GMR Genetics and Molecular Research
Online Journal
ISSN:1676-5680

# Phylogenetic analysis of endophytic bacterial isolates from leaves of the medicinal plant *Trichilia elegans* A. Juss. (Meliaceae)

**S.A. Rhoden[1], A. Garcia[1], M.C. Santos e Silva[2], J.L. Azevedo[2] and J.A. Pamphile[2]**

[1]Laboratório de Biotecnologia Microbiana, Departamento de Biotecnologia, Genética e Biologia Celular, Universidade Estadual de Maringá, Maringá, PR, Brasil
[2]Laboratório de Genética de Microrganismos "Prof. Dr. João Lúcio de Azevedo", Departamento de Genética,
Escola Superior Agrícola "Luiz de Queiroz", Piracicaba,
SP, Brasil

Corresponding author: S.A. Rhoden
E-mail: sandro_ar@hotmail.com

Genet. Mol. Res. 14 (1): 1515-1525 (2015)
Received October 21, 2014
Accepted November 11, 2014
Published February 20, 2015
DOI http://dx.doi.org/10.4238/2015.February.20.7

**ABSTRACT.** Various organisms such as fungi and bacteria can live inside plants, inhabiting the aerial parts (primarily the leaves) without causing damage. These microorganisms, called endophytes, produce an extensive variety of compounds that can be useful for medical and agronomic purposes. *Trichilia elegans* A. Juss., belonging to the Meliaceae family, shows wide dispersion in South America, and phytochemical analyses from these plants and endophyte isolates have shown biological activity. Accordingly, the aim of this study was to verify the diversity of bacterial endophytes from *T. elegans* using partial sequencing of 16S rRNA, followed by phylogenetic analysis. Isolation was performed by cutting the leaves, after disinfection with 5% sodium hypochlorite (NaOCl), in 1-2-mm$^2$ fragments, which were

 

equally placed on dishes containing TSA and fungicide BENLATE at 75 µg/mL. All dishes were incubated at 28°C in the biochemical oxygen demand system for 5 days and periodically checked. Afterwards, the colonization frequency (%) was determined: (number of fragments colonized by bacteria/total number of fragments) x 100. Three isolations between September 2011 and March 2012 were performed; the growth frequency ranged between 1.6 and 13.6%. Following sequencing of 16S rRNA and phylogenetic analysis, the genera identified were: *Staphylococcus*, *Bacillus*, *Microbacterium*, *Pseudomonas*, and *Pantoea*. These results will provide important knowledge on the diversity of endophytic bacteria inhabiting medicinal plants, and a better understanding of the microbiome of *T. elegans* would reinforce the necessity of endophyte studies with a focus on their future applications in biotechnological areas of agriculture, medicine, and the environment.

**Key words:** Biotechnology; 16S rRNA; Meliaceae; DNA barcode; Microbiome

## INTRODUCTION

Endophytic microorganisms live inside plants without causing them any damage (Bernardi-Wenzel et al., 2010; Garcia et al., 2012; Orlandelli et al., 2012; Rhoden et al., 2012a; Leme et al., 2013). They can be found in the aerial parts of the plant, primarily in the leaves, and occupy intra- and intercellular spaces (Bernardi-Wenzel et al., 2010; Garcia et al., 2012), characterizing a symbiotic relationship. Most studies about endophytes are associated with fungal and bacterial groups, although algae, when inside plants, are also considered endophytes. The close relationship between endophytes and plant species and the degree of specialization in the interactions can be an indication that the species have evolved together (Saikkonen et al., 2004).

Numerous studies have shown that certain medicinal properties of plants may be related to the endophytic fungi hosted by these plants (Azevedo et al., 2002); this has also been shown to be valid in the case of endophytic bacteria. Endophytes have been intensively studied because they have properties that can be applied across multiple areas, and have been shown to be potentially useful in agriculture, biological control, and the development of bioactive compounds. For example, some studies have indicated that the presence of endophytes in large quantity can reduce attacks by insects (Kogel et al., 2006; Koulman et al., 2007).

*Trichilia elegans* A. Juss. belongs to the Meliaceae family and shows wide dispersion, being found from Venezuela to Uruguay with approximately 70 species of this genus occurring in the American tropical region. However, in the remnant forests and in the regions near Maringá, Paraná, Brazil, there are only three species of *Trichilia*: *T. catigua* A. Juss. (catiguá), *T. elegans* A. Juss. (pau-de-ervilha) and *T. pallida* Sw. (baga-de-morcego) (Souza et al., 2001).

*T. elegans* is a medicinal plant and its importance was made evident in previous papers such as on phytochemical studies (Garcez et al., 1996), the diversity of endophytic fungi (Rhoden et al., 2012a), the efficiency of endophytic fungi in control of human pathogenic bacteria (Rhoden et al., 2012b), and the production of 3-nitropropionic acid by the endophytic fungi *Phomopsis longicolla*, isolated from the same species plant (Flores et al., 2013). Another

specimen of the same genus (*Trichilia*) in phytochemical studies showed anti-inflammatory activity (Benencia et al., 2000) and a change in the eating behavior of *Diabrotica speciosa* (Seffrin et al., 2008).

Analysis of the 16S rRNA gene sequence is important for the study of phylogeny and taxonomy for several reasons: it is present in almost all bacteria; the function of the 16S rRNA gene has not changed over time, suggesting that random sequence changes are a relatively accurate measure of time (evolution); and the 16S rRNA genes are large enough for informatic purposes (Patel, 2001).

In fact, it is of importance to realize that the diversity of endophytic bacteria can bring a new perspective to the research of: a) new compounds, which in the future could be used as biological controls; b) preservation, through reduction of the use of pesticides; c) growth promoters in plants; and d) new pharmaceutical drugs. Interactions between endophytic fungi, bacteria, and their plant host might reveal evolutionary aspects of the relationships, showing how these organisms can assist plant growth. Therefore, the aims of the present study were to perform phylogenetic analysis and 16S rRNA sequence analysis of endophytic bacteria, and to determine the endophytic diversity in *T. elegans*.

## MATERIAL AND METHODS

### Biological materials and collection site

Leaves from *T. elegans* trees with no evident blotches or herbivore damage were used for these analyses. Trees were in "Horto Florestal Dr. Luiz Teixeira Mendes", in Brazil, Maringá, located at 51°57'W, 23°26'S, at 556 m in altitude. The collections occurred during different dates: the first collection was in September 2011 (total precipitation 29.7 mm and monthly average temperature 22.22°C), the second in October 2011 (total precipitation 210.4 mm and monthly average temperature 22.95°C), and the third in March 2012 (total precipitation 62.8 mm and monthly average temperature 24.81°C). The temperature and rainfall data were recovered from http://www.inmet.gov.br (National Institute of Meteorology), station 83767.

### Isolation of endophytic bacteria

For surface disinfection of the tree samples, a modified methodology incorporating sodium hypochlorite (NaOCl) was used according to Rhoden et al. (2012a). For each collection, three washes were performed: 1 min in 70% EtOH, 5 min in 5% hypochlorite solution, 30 s in 70% alcohol, and twice in sterile distilled water. The disinfection process was checked by spreading 100 µL final water used on Petri dishes containing triptone soy agar (TSA) (Hi-Media Laboratories Pvt. Ltd.; Mumbai, India).

Disinfected leaves were cut into 1-2-mm$^2$ fragments and equally placed on TSA dishes containing the fungicide BENLATE® at 75 µg/mL. All dishes were incubated at 28°C in a biochemical oxygen demand (BOD) system (Eletrolab model EL 202), for 5 days and periodically checked. Afterwards, the colonization frequency (%) was determined using the formula: (number of fragments colonized by bacteria/total number of fragments) x 100. Bacteria grown in striation were transferred to new plates containing Luria broth medium (LB) and agar for starting the purification process. For the stock, bacteria remained in LB at 4°C until the time of DNA extraction.

## DNA extraction

DNA bacterial extraction was conducted according to Nogueira et al. (2004), with modifications. Endophytic bacterium isolates were grown in 5 mL LB for 24 h at 28°C. A 400-µL aliquot of the solution was transferred to a microtube and 400 µL saturated phenol solution was added. The mixture was shaken in a vortex apparatus and subjected to centrifugation at 16,000 *g* for 5 min.

The supernatant (aqueous layer) was transferred to a new microtube and the phenolic step was repeated. After centrifugation the supernatant was again transferred to a new microtube and 400 µL chloroform was added. The microtube was shaken in a vortex and centrifuged for 5 min at 16,000 *g*. The aqueous layer was transferred to another microtube, to which 1 mL cold EtOH was added.

To complete the process of extracting the DNA, the microtube was centrifuged for 3 min at 16,000 *g*, the EtOH discarded, and the tubes incubated at 37°C for 30 min to evaporate residual EtOH. The extracted material was resuspended in 15 µL Mili-Q sterile water.

## DNA amplification by polymerase chain reaction (PCR)

Amplification of the 16S rRNA gene was carried out according to Procópio et al. (2009), with modifications. PCR was performed in a solution containing 5 µL buffer (200 mM Tris-HCl, pH 8.4, 500 mM KCl), 5µL 2.5 mM dNTPs, 3.0 µL 10 pM of each primer (Invitrogen; Carlsbad, CA, USA): (R1378: 5'-CGG TGT GTA CAA GGC CCG GGA ACG-3' and PO27F:5'-GAG AGT TTG ATC CTG GCT CAG-3'), 0.4 µL 5 U/µL Taq DNA polymerase, 3.75 µL 50 mM $MgCl_2$, 27.85 µL Mili-Q sterile water, and 2 µL 10-20 ng/µL previously extracted sample DNA. PCR conditions were: initial denaturation 94°C for 4 min, followed by 25 cycles of 30 s denaturation at 94°C, 1 min annealing at 63°C, 1 min extension at 72°C, and a 7-min final extension at 72°C. PCR was performed in a Peltier Based model MG96G thermocycler (BioCycler).

## Purification of the amplification products

The amplified 16S genes were purified using the GFX PCR DNA kit and Gel Band Purification (Amersham Biosciences; Munich, Germany), according to manufacturer instructions. DNA PCR products were quantified by using 1% agarose gel analysis, photographed, and documented.

## Partial sequencing of 16S rRNA

Samples corresponding to 16S rRNA were partially sequenced. Reaction to sequencing was performed by PCR to a final volume of 20 µL, which included 8 µL sequencing solution (DYEnamic ET dye Terminator Cycle kit, MegaBACETM, GE Healthcare; Munich, Germany), 2.5 µL 10 µM primer PO27F (Invitrogen), 2 µL 100-200 ng/µL previously purified DNA, and 7.5 µL Mili-Q sterile water. PCR was performed in a thermocycler, programmed to carry out 25 cycles of 20 s denaturation at 95°C, 1 min annealing at 50°C, and 1 min extension at 60°C. The sequencing was performed in a MegaBACE TM 1000 sequencer (Amersham Biosciences), with injection and electrophoresis conditions of 1 Kv/90 s and 7 Kv/240 min, respectively.

The sequences were analyzed and edited. The nucleotide sequences were compared to those deposited in the National Center for Biotechnology Information (NCBI) database. For the genera or species research, the BLASTn program was used. Determination was based on the best result obtained for identity.

## Estimation of genetic distance of the isolates

Sequences corresponding to the partial 16S rRNA gene were aligned using the MEGA program (version 5.0) (Tamura et al., 2011) with grouping by the neighbor-joining method (Saitou and Nei, 1987), using a p-distance matrix for nucleotides with the pair-wise gap deletion option adopted and with 10,000 bootstrap repetitions. Similar sequences from NCBI were also used for the phylogenetic analysis, which were redeemed from the environment where the source material was collected to check the relationship between the isolates from *T. elegans* and the bacteria of another environment. For generation of the phylogenetic tree, EvolView (Zhang et al., 2012) was used.

# RESULTS

## Isolation of endophytic bacteria from *T. elegans*

The collection periods and frequency of colonization of plants by endophytic bacteria are presented in Table 1.

**Table 1.** Collection dates and frequency of endophyte isolation.

| Isolation | Date of collection | No. of *Trichilia elegans* leaf fragments | No. of fragments that showed growth | Frequency of growth (%) | Numeration |
|---|---|---|---|---|---|
| 1 | September 2011 | 480 | 8 | 1.6 | 1-8 |
| 2 | October 2011 | 530 | 37 | 6.9 | 9-45 |
| 3 | March 2012 | 360 | 49 | 13.6 | 46-95 |

## Molecular identification of isolates based on 16S rRNA sequencing

Among the endophytic bacterial isolates, it was possible to identify the genera and the species of 16 isolates, through sequencing analysis of the partial 16S rRNA region and through BLAST analysis (http://www.ncbi.nlm.nih.gov/blast/) using the NCBI database. After sequencing the bacteria, five endophytic bacterial genera were identified: *Staphylococcus* (Bacillales), *Bacillus* (Bacillales), *Microbacterium* (Actinomycetales), *Pseudomonas* (Pseudomonadales), and *Pantoea* (Enterobacteriales). The identity between the isolated and NCBI sequences is presented in Table 2 and the abundance of each genus is identified in Figure 1. The nucleotide sequences obtained in this study have been submitted to GenBank and were assigned accession Nos. KC700345 through KC700360.

## Phylogenetic analysis of *T. elegans* endophytes based on data of 16S rDNA sequencing

The phylogenetic analysis was divided into 5 groups (Figure 2) according to genus:

*Staphylococcus*, *Bacillus*, *Microbacterium*, *Pseudomonas*, or *Pantoea*, as follows: four bacteria belonged to the *Staphylococcus* group (isolates 6, 15, 49, and 67), six belonged to *Bacillus* (isolates 5, 10, 21, 38, 50, and 62), three belonged to *Microbacterium* (isolates 65, 46, and 75), one belonged to *Pseudomonas* (isolate 41), and two belonged to *Pantoea* (isolates 7 and 3). The analysis is represented by the orders Bacillales (*Staphylococcus* and *Bacillus*), Actinomycetales (*Microbacterium*), Pseudomonadales (*Pseudomonas*), and Enterobacteriales (*Pantoea*). The phylogenetic results are consistent with the initial grouping.

**Table 2.** Isolated bacterial endophytes identified with relationship to genus or species and the identity percentage found in the National Center for Biotechnology Information database.

| Endophytic isolates (Bacteria) | Bacterial genus/species with greatest similarity | Accession number with greatest similarity | Identity (%) |
|---|---|---|---|
| 3 | *Pantoea agglomerans* | FJ546348 | 98 |
| 5 | *Bacillus cereus* | JX534506 | 97 |
| 6 | *Staphylococcus* sp | AJ936959 | 98 |
| 7 | *Pantoea conspicua* | JF505939 | 98 |
| 10 | *Bacillus* sp | KC256826 | 99 |
| 15 | *Staphylococcus* sp | CP003668 | 93 |
| 21 | *Bacillus cereus* | JQ834164 | 96 |
| 38 | *Bacillus* sp | JN944558 | 98 |
| 41 | *Pseudomonas aeruginosa* | JQ337950 | 97 |
| 46 | *Microbacterium* sp | KC160929 | 98 |
| 49 | *Staphylococcus* sp | KC119123 | 99 |
| 50 | *Bacillus cereus* | HQ457016 | 99 |
| 62 | *Bacillus cereus* | HM451440 | 96 |
| 65 | *Microbacterium oxydans* | EU239169 | 99 |
| 67 | *Staphylococcus epidermidis* | AJ508368 | 99 |
| 75 | *Microbacterium foliorum* | KC139419 | 98 |

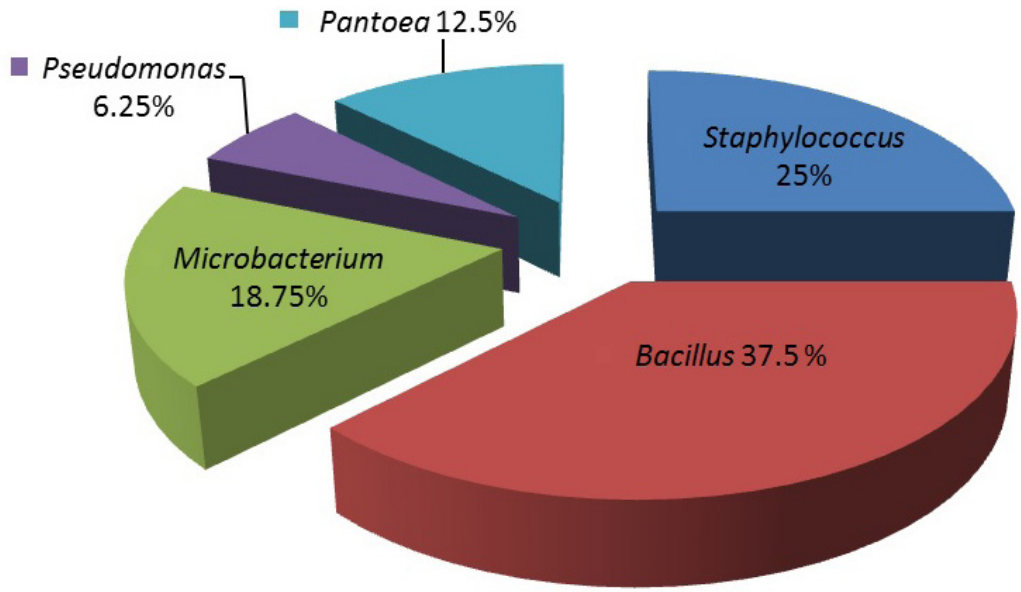


**Figure 1.** Abundance of each genus identified among endophytic bacterial isolates from *Trichilia elegans* leaves.

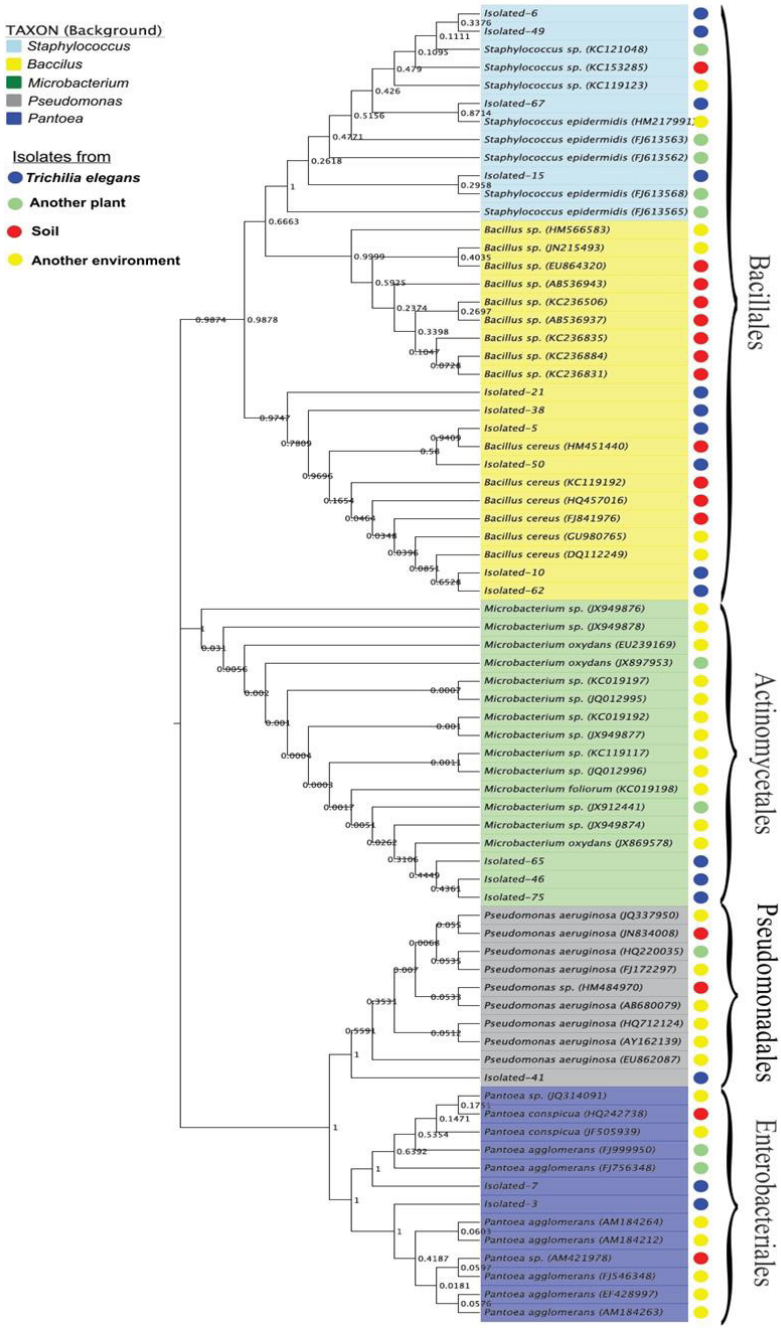


**Figure 2.** Phylogenetic tree constructed with sequences of the 16S rRNA regions of endophytic bacteria isolated from *Trichilia elegans* and sequences from GenBank (indicated by accession number), using the neighbor-joining method and utilizing p-distance for nucleotides, with the pairwise gap deletion option. Numbers indicate frequency of each branch from bootstrap analyses of 10,000 replicates.

## DISCUSSION

### Isolation of endophytic bacteria from *T. elegans*

Rhoden et al. (2012a) isolated endophytic fungi using the same plant species (*T. elegans*) as utilized here and obtained 100% colonization of leaf fragments. However, the frequency of endophytic bacteria has been shown to be lower than isolation of fungi, which could potentially be explained because the conditions of rainfall and temperature were not favorable to obtain maximal bacterial colonization. Similarly, Vega et al. (2005) reported that no difference in bacterial colonization was observed between the different parts of plants, which was confirmed by Arnold and Herre (2003) who discussed that rain, humidity, and temperature can influence the occurrence of endophytes.

### Molecular identification of isolates based on partial 16S rDNA sequencing and phylogenetic analysis

According to Janda and Abbot (2007), the use of 16S rRNA gene sequence for identification is attractive, because it is an important informatics tool that provides genera and species identification for isolates that do not fit any recognized biochemical profiles. In this way, 16S rRNA sequencing in the present study showed that the most representative genus in our isolates was *Bacillus*, comprising 37.5% of identified bacteria. *Bacillus* is a Gram-positive, spore-forming, rod-shaped genus with high tolerance to adverse ecological conditions. Previous studies have shown that endophytic bacteria of the genus *Bacillus* are present in: *Coffea arabica* L. (Vega et al., 2005), sunflower (Forchetti et al., 2007), cotton (Misaghi and Donndelinger, 1990), potato (Sessitsch et al., 2004), strawberry (Dias et al., 2008), *Panaxnoto ginseng* (Ma et al., 2013), and citrus plants (Araujo et al., 2001). Among the bioprospection factors, the endophyte *Bacillus* stands out for phosphate solubilization and auxin production (Dias et al., 2008) and activity against *Fusarium oxysporum*, *Ralstonia* sp, and *Meloidogyne hapla* (Ma et al., 2013).

Forchetti et al. (2007) reported that the use of biopesticides based on endophytic bacteria to biologically control diseases is a promising alternative to avoid chemical treatments. In this way the genus *Bacillus* has advantages over other biocontrol bacteria, because it is easy to cultivate and store, and it can be used as spores on plant seeds or in inoculants; it also displays protective effects against various microbial pathogens, thus being able to promote plant growth. Therefore, these *Bacillus* isolates may present several properties that can be useful in agricultural aspects.

The second most representative group in our isolates was the genus *Staphylococcu*s, with 25% of identified bacteria. The genus *Staphylococcus* has been previously isolated from other plants such as potatoes (Reiter et al., 2002; Sessitsch et al., 2004) and maize (Rijavec et al., 2007). Sessitsch et al. (2004) reported that the endophyte *Staphylococcus* showed potato plant growth-promoting activity. The most representative genera were grouped in the phylogenetic analysis in the order Bacillales with 98.78% of Bootstrap (BP).

The third most representative group, with 18.75% of identified bacteria, was represented by the order Actinomycetales (clade with 100% of BP) and by genus *Microbacterium*. The genus *Microbacterium* was previously isolated from other plants such as maize

(Rijavec et al., 2007) and rice (Elbeltagy et al., 2000). The *Microbacterium* isolates from *T. elegans* were grouped with more similarity in the clade isolates of Actinomycetales.

The fourth group was represented by the genus *Pantoea*, which was previously isolated from plants such as *Trifolium pratense* L. and red clover (Sturz et al., 1997), *C. arabica* L. (Vega et al., 2005), potato (Sessitsch et al., 2004), *Calystegia soldanella* (Park et al., 2005), maize (Rijavec et al., 2007), citrus (Sturz et al., 1997; Araújo et al., 2001), and peas (Elvira-Recuenco and van Vuurde, 2000). Sessitsch et al. (2004) showed that the genus *Pantoea* produced siderophore and indole-3-acetic acid. Upon phylogenetic analysis, the clade Enterobacteriales, with 100% of BP, was divided in two other clades, where isolates 7 (98% of Blast identity to *Pantoea conspicua*) and 3 (98% Blast identity to *Pantoea agglomerans*) were grouped.

Bacon and Hinton (1996) described that the fifth genus *Pseudomonas* with 6.25% of total isolates, is a widely distributed plant-associated bacterium, found in the majority of plant species and tissues, often with positive effects on plant growth, such as in Norway spruce (Cankar et al., 2005), *C. arabica* L. (Vega et al., 2005), potato (Reiter et al., 2002; Sessitsch et al., 2004), *C. soldanella* and *Elymus mollis* (Park et al., 2005), *Glycine max* (Kuklinsky-Sobral et al., 2005).

Phylogenetic analysis grouped *Pseudomonas* in the clade Pseudomonadales with 100% of BP.

Phylogenetic analysis using isolates from the NCBI database and in comparison with other bacteria of the same genus isolated from different environments, assisted in the correlation of the sequences of 16S endophytic bacteria, which had high similarity with bacteria of the same genus from other environments that were not plant-based.

Similar to the present study, Velázquez et al. (2008) obtained endophytes from sugar cane that were phylogenetically very close to the following genera: *Bacillus* and *Staphylococcus,* from the Firmicutes group; *Microbacterium*, *Micrococcus*, and *Kocuria* from the Actinobacteria group; *Rhizobium* and *Gluconacetobacter* from the α-Proteobacteria group; *Comamonas* and *Xanthomonas* from the β-Proteobacteria group; and *Acinetobacter* and *Pantoea* from the γ-Proteobacteria group.

Endophytic strains isolated from *T. elegans* using 16S rRNA gene sequences proved to be diverse and belonged to divergent phylogenetic groups of bacteria: phylum Firmicutes of the order Bacillales (genera *Staphylococcus* and *Bacillus*), phylum Actinomycetales (genus *Microbacterium*) and phylum Proteobacteria of the order Pseudomonadales (genus *Pseudomonas*), and the order Enterobacteriales (genus *Pantoea*). These results are essential to provide the necessary knowledge of endophytic bacteria analysis for future applications of endophytes in areas related to agriculture, medicine, and biotechnology, and indicate the potential for very promising technological innovation in pathogenic microorganism control by endophytes.

## ACKNOWLEDGMENTS

Research supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) (project #480726/2011-6) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) to Doctorate Sanduíche scholarship (process #12331/12-0).

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