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Short Communication

Operational taxonomic units (OTUs) of endophytic bacteria isolated from banana cultivars in the Amazon

Á. Souza[1], V.B. Nogueira[2], J.C. Cruz[3], N.R. Sousa[3], A.R.L. Procópio[1] and G.F. Silva[3]

[1]Universidade do Estado do Amazonas, Manaus, AM, Brasil
[2]Universidade Federal do Amazonas, Manaus, AM, Brasil
[3]Laboratório de Biologia Molecular, Embrapa Amazônia Ocidental/CPAA, Manaus, AM, Brasil

Corresponding author: G.F. Silva
E-mail: gilvan.silva@embrapa.br

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ABSTRACT. Endophytic microorganisms colonize plants, inhibit the growth of pathogens (by competing for nutrients and/or space), or produce antagonistic substances. Fifty-five endophytic bacteria were isolated from the leaf tissue of the FHIA 18 banana cultivar. Genetic diversity analyses were performed using the enterobacterial repetitive intergenic consensus sequence polymerase chain reaction method and BOX molecular markers. These analyses resulted in 33 and 21 polymorphic bands, respectively. The similarity data, obtained using the Dice coefficient based on the polyphasic analysis method, ranged from 22 to 100%. This indicated a high genetic diversity among the analyzed isolates. Sixty percent similarity was utilized as the cut-off criterion for the formation of operational taxonomic units (OTUs); this resulted in the identification of 32 possible OTUs, indicating

a high number of potential species.

Key words: BOX-PCR; Endophytic bacteria; FHIA 18 banana cultivar; Operational taxonomic units; Enterobacterial repetitive intergenic consensus sequence polymerase chain reaction

INTRODUCTION

Endophytic microorganisms are organisms present in the plant tissue that do not damage the plant or facilitate the development of any external structures from the plant tissues (Azevedo and Araujo, 2007). Endophytic bacteria play an important role in the phytoremediation of contaminated soil, improvement of soil fertility through phosphate solubilization, and nitrogen fixation (Ryan et al., 2008). Interactions between the plants and endophytic bacteria could promote plant health; therefore, this method is an environmentally sustainable agricultural model for food and non-food crops. Endophytes have been used as alternatives to fertilizers and chemical pesticides, because of their lower cost and contribution to a sustainable agricultural system (Zinniel et al., 2002).

Various methods have been used to identify and characterize endophytic bacteria. Taxonomical studies have provided a way to measure endophytic biodiversity, enabling communication among different organisms in the scientific community. Recent studies have shown the applicability of the latest molecular methods for the determination of species-level bacterial taxonomy. One of the most relevant molecular approaches currently available is the identification of the operational taxonomic unit (OTU), which was previously described by Sokal and Sneath (1963) in a non-molecular context. The original application of OTUs involved the identification of as many characteristics of the organism as possible. Recent research has described a subtype of OTU, the environmental molecular operational taxonomic unit (eMOTU), for the identification of certain characteristics in a molecular context. These units have been defined as "clusters of sequences (that act as representatives of the genomes from which they are derived) generated by an explicit algorithm" (Floyd et al., 2002; Galimberti et al., 2012).

Enterobacterial repetitive intergenic consensus sequence polymerase chain reaction (ERIC-PCR) using BOX molecular markers is a simple, rapid, and inexpensive tool with sufficient discriminatory power that can be applied to molecular characterization. Therefore, this technique has been widely used in the molecular analysis of microorganisms, especially bacteria (Becerra-Castro et al., 2011; Macedo et al., 2011). This method is also capable of identifying a sufficient number of characteristics to facilitate OTU analysis. The diversity analysis of endophytic bacteria using BOX-PCR has previously shown a high rate of success in the discrimination of closely related bacteria from the Enterobacteriaceae family (Torres et al., 2008). Similarly, ERIC-PCR has shown great applicability in the diversity analysis of microorganisms. For example, Katara et al. (2012) conducted a molecular typing analysis of *Bacillus thuringiensis* isolates obtained from diverse habitats in India using repetitive extragenic palindromic PCR (REP-PCR) and ERIC-PCR, and identified a sufficient number of patterns to discriminate among the various isolated *B. thuringiensis* strains.

Therefore, this study aimed to isolate and characterize (the genetic diversity of) endophytic bacteria isolated from the FHIA 18 banana cultivar (grown in the Amazon Basin).

MATERIAL AND METHODS

Isolation of samples

The samples were collected from the leaf tissues of a single FHIA 18 banana cultivar in the Embrapa Amazônia Ocidental experimental area. The collected samples were stored at room temperature and processed within 24 hours after collection. The protocols used for disinfection and isolation were adopted from those detailed by Procópio et al. (2009).

DNA extraction and amplification

Bacterial DNA was extracted using the protocol described by Sun et al. (2008). The PCR was conducted in a Veriti® thermal cycler (Applied Biosystems; Life Technologies, Carlsbad, CA, USA).

The following primers [designed and described by Hulton et al. (1991)] were used for ERIC-PCR: ERIC1R 5'-ATGTAAGCTCCTGGGGATTCAC-3' and ERIC2 5'-AAGT AAGTGA CTGGGGTGAGCG-3'. The amplification was performed in a 15-µL reaction mixture containing 1X buffer (500 mM KCl, 100 mM Tris-HCl, pH 8.4, 1% de Triton X-100), 2 mM $MgCl_2$, 0.2 mM dNTP mix, 0.5 mM primers, 1 U Taq polymerase (Phoneutria), and 50 ng bacterial genomic DNA. The thermal cycling program was set as follows: denaturation for 3 min at 94°C, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 45.5°C for 1.5 min, and extension at 72°C for 2 min, and a final extension at 72°C for 8 min.

The BOX-PCR primers were previously detailed by Versalovic et al. (1994), and the primer sequence was as follows: BOX 1R (5'-CTCCGGCAAGGCGACGCTGAC-3'). The amplification mixture (20 µL) was comprised of 1X buffer (500 mM KCl, 100 mM Tris-HCl, pH 8.4, 1% de Triton X-100), 3 mM $MgCl_2$, 0.25 mM dNTP mix, 2 mM primers, 1 U Taq polymerase (Phoneutria) and 50 ng fungal genomic DNA. The thermal cycling program was set as follows: an initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1.5 min, and extension at 68°C for 8 min, and a final extension at 68°C for 8 min.

Genetic diversity analysis

The genetic diversity was evaluated via a comparative analysis of the patterns generated by the amplification of conserved and repetitive DNA genome sequences. The binary matrix constructed from the previously obtained band profile was analyzed based on the Dice coefficient; the dendrogram was generated using the UPGMA method, using the NTSYS v.2.1 software (Rohlf, 2000). The electrophoretic patterns were also analyzed, using both molecular markers to obtain the operational taxonomic units (OTUs).

RESULTS

In total, 55 endophytic bacteria were isolated (Table 1). The results of BOX molecular marker and ERIC-PCR analyses indicated a high genetic diversity among the members of the endophytic bacterial community. The analysis of the group structure created by the Dice coefficient using the data from both the BOX and ERIC markers revealed the formation of six groups. The groups 1, 2, and 4 were comprised of two subgroups, while the remaining groups (3, 5, and 6) did not contain any subgroups. Two isolates, FH14 and FH10C, displayed unique (prominent) profiles; these isolates showed 24% similarity with other isolates. Of the 55 isolates, only 2 showed 100% similarity (FH12D and FH6D; Figure 1).

Table 1. Isolates obtained from the FH18 banana cultivar and the access numbers obtained from the Amazon Biotechnology Center (CBA, Manaus, AM, Brazil) collection.

FHIA18	Collection No.	FHIA18	Collection No.
FH6B	CBA-1716	FH14A	CBA-1702
FH7C	CBA-1722	FH9B	CBA-1730
FH14	CBA-1701	FH1A	CBA-1706
FH5A1	CBA-1711	FH5A	CBA-1712
FH12C	CBA-1691	FH6C	CBA-1717
FH11E1	CBA-1688	FH9	CBA-1728
FH9A1	CBA-1693	FH11C	CBA-1762
FH14C	CBA-1705	FH11D	CBA-1764
FH12B	CBA-1690	FH10C	CBA-1685
FH10B1A	CBA-1683	FH8	CBA-1724
FH5	CBA-1710	FH12D	CBA-1694
FH11	CBA-1686	FH8B	CBA-1726
FH10B1	CBA-1681	FH6D	CBA-1719
FH6C1	CBA-1718	FH11E	CBA-1668
FH11E1A	CBA-1689	FH14A1	CBA-1703
FH7B	CBA-1721	FH1D	CBA-1709
FH13	CBA-1695	FH7A	CBA-1720
FH10B	CBA-1680	FH8A	CBA-1725
FH13A	CBA-1696	FH13D1	CBA-1700
FH10B1	CBA-1681	FH11	CBA-1686
FH13B	CBA-1697	FH10	CBA-1677
FH13D	CBA-1699	FH5A1A	CBA-1713
FH18C	CBA-1746	FH16E	CBA-1749
FH11B	CBA-1727	FH1B	CBA-1707
FH14B	CBA-1704	FH1C	CBA-1708
FH9A	CBA-1729		
FH7D	CBA-1723		
FH9C	CBA-1682		
FH10F	CBA-1714		

The cut-off criterion for the preparation of OTUs is a widely debated topic (no consensus value, has some discrepancy) among different authors. Some of the observed values include 60% sequence similarity (Yang et al., 2004; Torres et al., 2008) and 70% (Grange and Hungary, 2004; Alberton et al., 2006; Schloss and Handelsman, 2006). In this study, the selected cut-off took into account the 60% sequence similarity; this allowed for the identification of 32 OTUs (Figure 1).

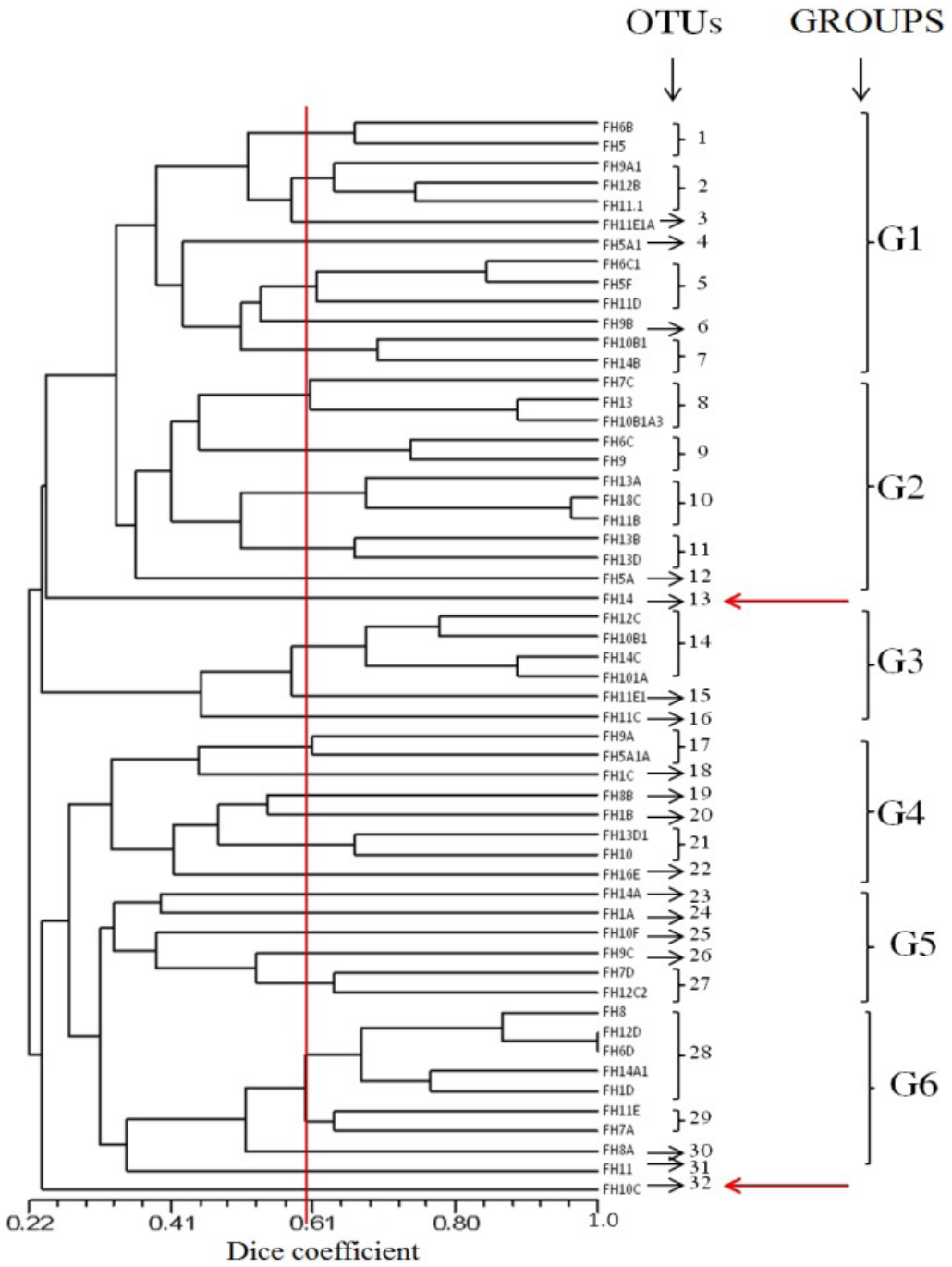

Figure 1. Similarity calculated using Dice coefficient and clustered using UPGMA algorithm based on ERIC-PCR and BOX-PCR data of 55 endophytic bacteria from FHIA 18 banana cultivar with similarity cut-off of 60% for OTU formation.

DISCUSSION

The results of the BOX and ERIC-PCR analyses revealed a high genetic diversity among the members of the endophytic bacterial community. A combination of the BOX- and ERIC-PCR techniques was used to obtain OTUs, as the use of both markers (together) resulted in the largest number of amplified bands. The community was composed of at least 32 possible OTUs; this indicated that the 55 analyzed isolates were composed of at least 32 different species, since individual OTUs can be defined as potential species (Yang et al., 2004). OTUs were also identified in endophytic bacteria isolated from different host plants.

Torres et al. (2008) identified 23 OTUs upon analyzing 53 bacterial endophytes isolated from different host plants, using BOX-PCR data, suggesting that this population was composed of 23 species.

The number of OTUs identified in this study shows the diversity of species colonizing the FHIA 18 cultivar. Grange and Hungria (2004) considered a similarity cut-off percentage of 70%, and observed a high genetic diversity using ERIC-PCR, with 81% of the isolates representing unique strains.

There is a general call for novel, effective, and sustainable antibiotics, chemotherapeutic agents, and agrochemicals. Endophytes, potential sources of natural products that could be applied to the medical, biotechnological, and agricultural fields, has not been extensively researched (relatively) in the past. The genetic diversity of these organisms is related to the diversity of their host plants (Strobel, 2012). For example, the Amazon rainforest has one of the highest levels of biodiversity in the world; therefore, it has been designated as a hot spot.

The number of different OTUs (32) identified from among the 55 isolates exemplifies the great genetic diversity. Finally, the results obtained could serve as a theoretical basis for the extension of research, in order to provide new and interesting insights into the endophytic community (which is known to inhabit banana plantations), including endophyte/plant interactions, and possible biotechnological applications.

Combined BOX- and ERIC-PCR analysis indicated the high genetic diversity of the endophytic bacteria community colonized in the FHIA 18 banana cultivar. Based on the OTU formation, it was concluded that the 55 isolates contained 32 different species; these results present a great potential for future biotechnological applications.

Conflicts of interest

The authors declare no conflict of interest.

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