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In silico analysis of the 16S rRNA gene of endophytic bacteria, isolated from the aerial parts and seeds of important agricultural crops

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ABSTRACT. Because of human population growth, increased food production and alternatives to conventional methods of biocontrol and development of plants such as the use of endophytic bacteria and fungi are required. One of the methods used to study microorganism diversity is sequencing of the 16S rRNA gene, which has several advantages, including universality, size, and availability of databases for comparison. The objective of this study was to analyze endophytic bacterial diversity in agricultural crops using published papers, sequence databases, and phylogenetic analysis. Fourteen papers were selected in which the ribosomal 16S rRNA gene was used to identify endophytic bacteria, in important agricultural crops, such as coffee, sugar cane, beans, corn, soybean, tomatoes, and grapes, located in different geographical regions (America, Europe, and Asia). The corresponding 16S rRNA

gene sequences were selected from the NCBI database, aligned using the Mega 5.2 program, and phylogenetic analysis was undertaken. The most common orders present in the analyzed cultures were Bacillales, Enterobacteriales, and Actinomycetales and the most frequently observed genera were *Bacillus*, *Pseudomonas*, and *Microbacterium*. Phylogenetic analysis showed that only approximately 1.56% of the total sequences were not properly grouped, demonstrating reliability in the identification of microorganisms. This study identified the main genera found in endophytic bacterial cultures from plants, providing data for future studies on improving plant agriculture, biotechnology, endophytic bacterium prospecting, and to help understand relationships between endophytic bacteria and their interactions with plants.

Key words: Endophytic bacteria; 16S rRNA gene; Agronomic crops; Phylogenetic analysis; Biotechnology; Bioprospection

INTRODUCTION

According to current estimates by the United Nations, the world population is expected to reach 9 billion by the year 2050. Accordingly, food production is expected to be more than double worldwide. In order to achieve this goal, agricultural production needs to be extended and intensified in a sustainable manner.

In addition, modernization of agricultural techniques, the use of fertilizers and pesticides, and mechanization should also be improved to increase production, reduce costs, and minimize environmental damage (Mazoyer and Roudart, 2009). In this view, the use of endophytic microorganisms or endophytes (mostly fungi and bacteria) that develop within host plants may be advantageous.

Endophytes spend at least one phase of their lifecycle within the host plant without causing damage, and they also interact in complex ways with their host. This group of microorganisms can be found in different parts of the plant, such as leaves (Garcia et al., 2012; Rhoden et al., 2012; Leme et al., 2013), flowers, seeds, stems, and roots (Vega et al., 2005; Azevedo and Araújo, 2007; Compant et al., 2011). Furthermore, endophytes may also be used as an alternative to fertilizers and pesticides because these microorganisms have been shown to be a sustainable alternative, enhancing the growth of plants and biologically controlling insects (Sessitsch et al., 2004; Compant et al., 2005).

The 16S rRNA gene has been used to evaluate the diversity of endophytic bacteria with outstanding success (Figure 1). Carl Woese pioneered the use of 16S rRNA more than 30 years ago (Woo et al., 2008). Among the advantages of using 16S rRNA is its universality, because it is present in almost all bacteria, allowing its use in taxonomic and phylogenetic identification (Kembel et al., 2012).

Figure 1. Representation of the 16S rRNA gene. Gray regions represent conserved sequences, while V1 toV9 represent variable sequences that are important regions for phylogenetic analyses. Adapted from Petrosino et al. (2009).

The high degree of conservation of the 16S rRNA sequence within species is an important factor for differentiating organisms, in addition to its size, because it is large enough to be amplified using PCR, and thus, can be used in phylogenetic analyses (Woo et al., 2008). Furthermore, the 16S rRNA gene has also become very important in the medical field because it enables identification of pathogens faster than using traditional biochemical tests, and it provides an alternative when these biochemical tests cannot be used (Woo et al., 2008).

A number of bioinformatic tools can be used to analyze the 16S rRNA gene when identifying organisms. One of the most prominent ones is BLAST (Basic Local Alignment Search Tool), which compares all the sequences deposited in public domain databases, such as the National Center for Biotechnology Information (NCBI). Another tool used in bioinformatics is the MEGA (Molecular Evolution Genetics Analysis) software, which, in addition to sequence alignment and phylogenetic inference, has many parameters available to the user for analysis (Tamura et al., 2011).

Due to the importance of endophytic bacteria as biological controls, in promoting the growth of plants, and their diversity, the present study aimed to analyze the diversity of endophytic bacteria in the aerial parts of plants and seeds using *in silico* analysis of sequences of the 16S rRNA gene obtained from databases. Particularly for crops of sugar cane, beans, corn, soybeans, tomatoes, coffee, and grapes, it is important to verify information on the prevalence of a certain genus in these cultures and the robustness of this tool for the analysis of diversity. The data generated from this study can be used in future studies of diversity to help understand the relationships between endophytic bacteria and their interactions with plants, as well as in programs related to prospecting and biotechnology of this group of microorganisms.

MATERIAL AND METHODS

To study the genetic diversity of endophytic bacteria, 14 papers (published between 2007 and 2013) deposited in Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Periodics database - CAPES were used (Table 1).

Table 1. Summary of articles evaluated in a comparison of the genetic diversity of endophytic bacterial cultures, including organ of plants sampled, geographical origin of the plant in isolation, author, and year of publication.

Crop	Organ of origin in plant	Geographic origin	Publication
Coffee (*Coffea canephora*)	Fruit	Brazil	Miguel et al., 2013
(*Coffea arabica*)	Fruit	Not determined	Oliveira et al., 2013
Sugar cane (*Saccharum officinarum*)	Stem	Brazil	Mendes et al., 2007
	Stem	Cuba	Velázquez et al., 2008
Bean (*Phaseolus vulgaris*)	Leaf	Brazil	de Oliveira Costa et al., 2012
	Seed	Colombia	López-López et al., 2010
Corn (*Zea mays*)	Flower	Brazil	Figueiredo et al., 2009
	Stem, seed, Flower	Not determined	Montañez et al., 2012
	Seed	China	Liu et al., 2012
Soybean (*Glycine max*)	Stem	Japan	Okubo et al., 2009
Tomato (*Lycopersicon esculentum*)	Stem and Leaf	Brazil	Barretti et al., 2009
Grape (*Vitis vinifera*)	Leaf	Italy	Bulgari et al., 2009
	Leaf		Piccolo et al., 2010
	Flower, fruit, seed	Austria	Compant et al., 2011

Of these articles, seven investigated crops of agricultural importance (coffee, sugar cane, beans, corn, soybeans, tomatoes, and grapes) using the 16S rRNA gene for identification,

which was also present in GenBank. After selecting the papers, sequences of the 16S rRNA gene were selected from the NCBI database and organized based on the tissue of origin of the microorganisms in the plant, as well the geographical location of the plant (crop type and geographical location can be visualized in Figure 2).

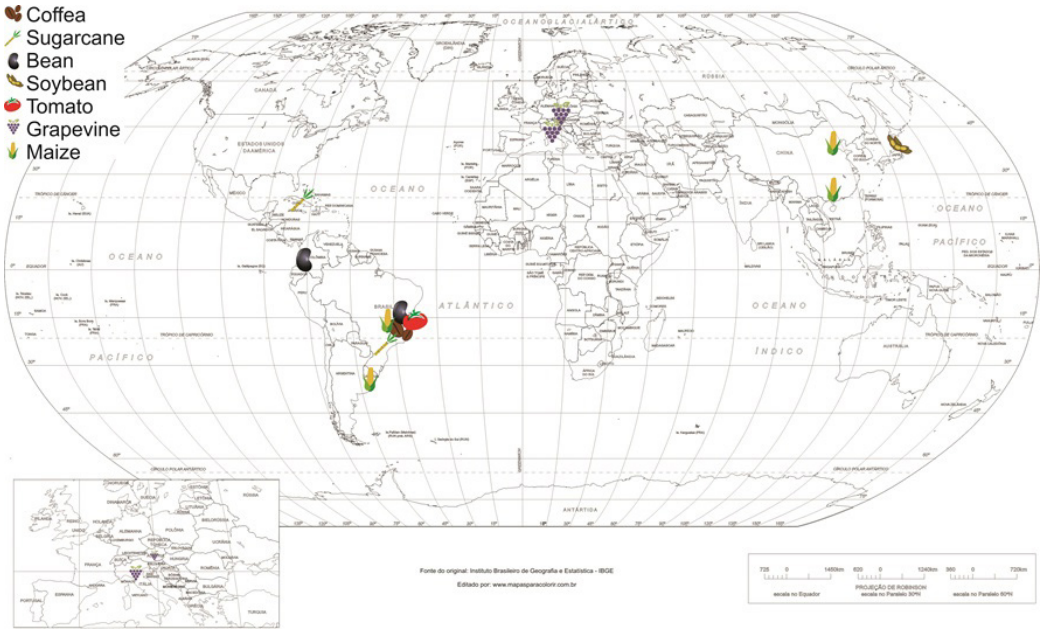

Figure 2. Geographical distribution of different crops used in the present analyses. Original image of the map belongs to Instituto Brasileiro de Geografia e Estatística (IBGE) and was edited by the authors.

Because of the great diversity of microorganisms in the roots of plants, including nitrogen-fixing species, the current study only considered endophytes isolated from the aerial parts and seeds of the plants.

Sequences were aligned using the MEGA 5.2 software with the following parameters: neighbor-joining (Saitou and Nei, 1987) using the p-distance matrix for nucleotides with pair-wise gap deletion and bootstrap with 1000 replications. When analyzing the bacterial diversity, agronomic crops and geographical origin were also taken into account (Figure 3). Partial sequences (less than 1000 bases) were not used because the sequences do not have sufficient coverage for alignment.

The numerical diversity of endophytic bacteria isolated from each plant species was also investigated by counting the number of each genus of bacteria isolated from each crop (Figure 4) and the frequency of each order of endophytic bacteria isolated from all papers (Figure 5).

RESULTS

The result of phylogenetic analysis of the isolated endophytic bacteria as well as the host plant can be seen in Figure 3.

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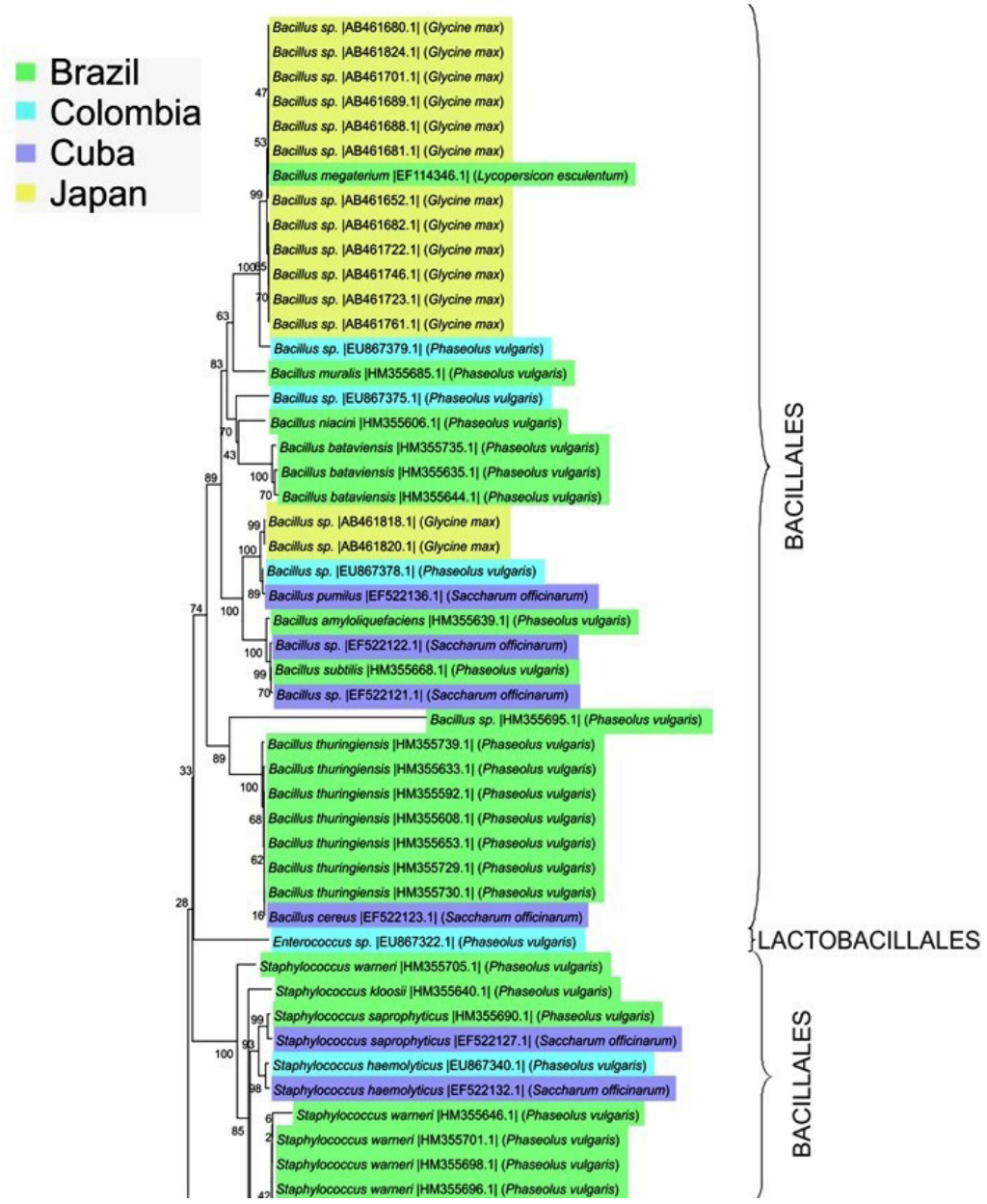

Figure 3. Phylogenetic analysis. The sequences collected were aligned using the MEGA program (version 5.0; Tamura et al., 2011), with grouping using the NJ method (Saitou and Nei, 1987) with a p-distance matrix for nucleotides and the pairwise gap deletion option adopted and 1000 bootstrap repetitions.

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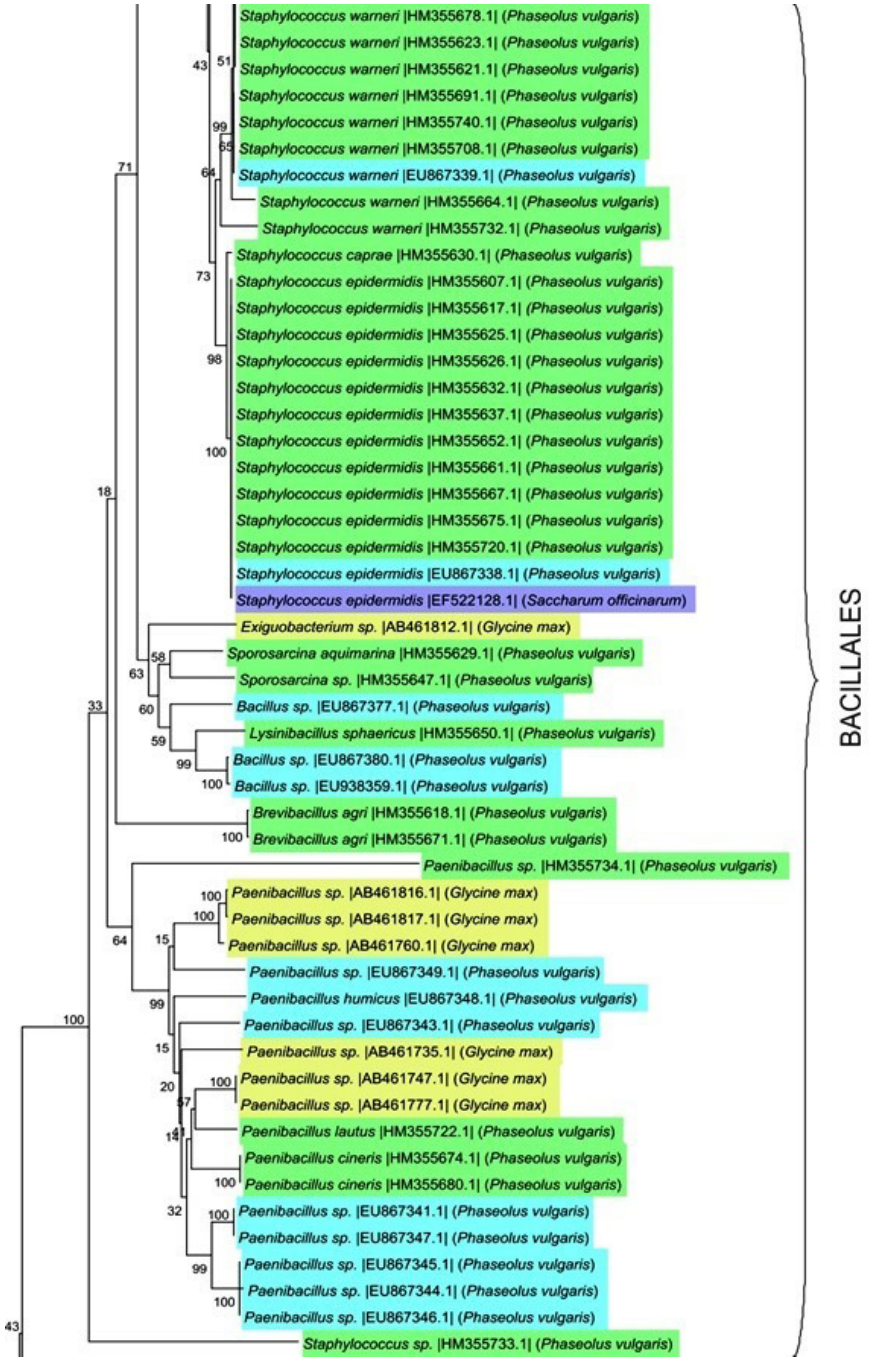

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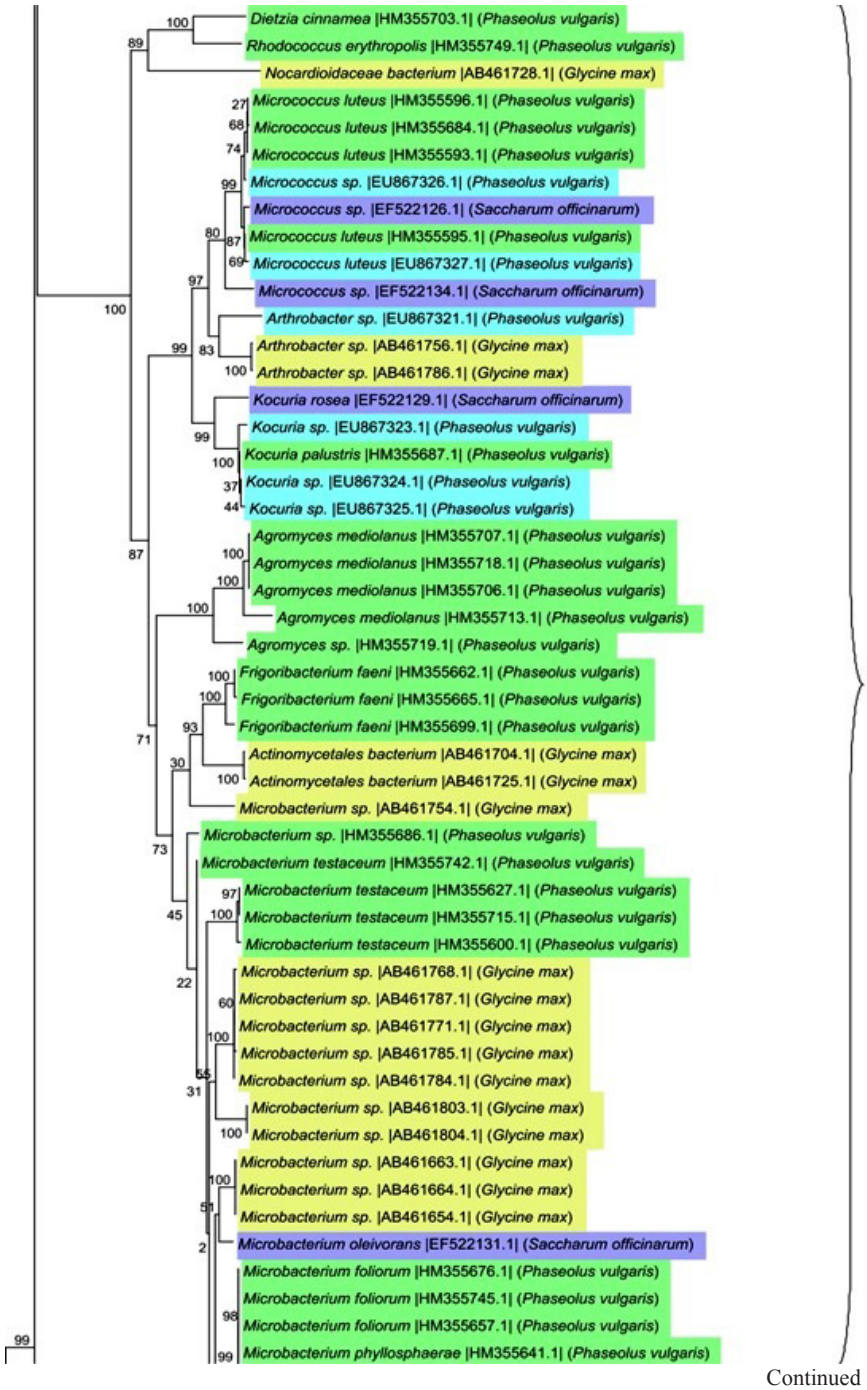

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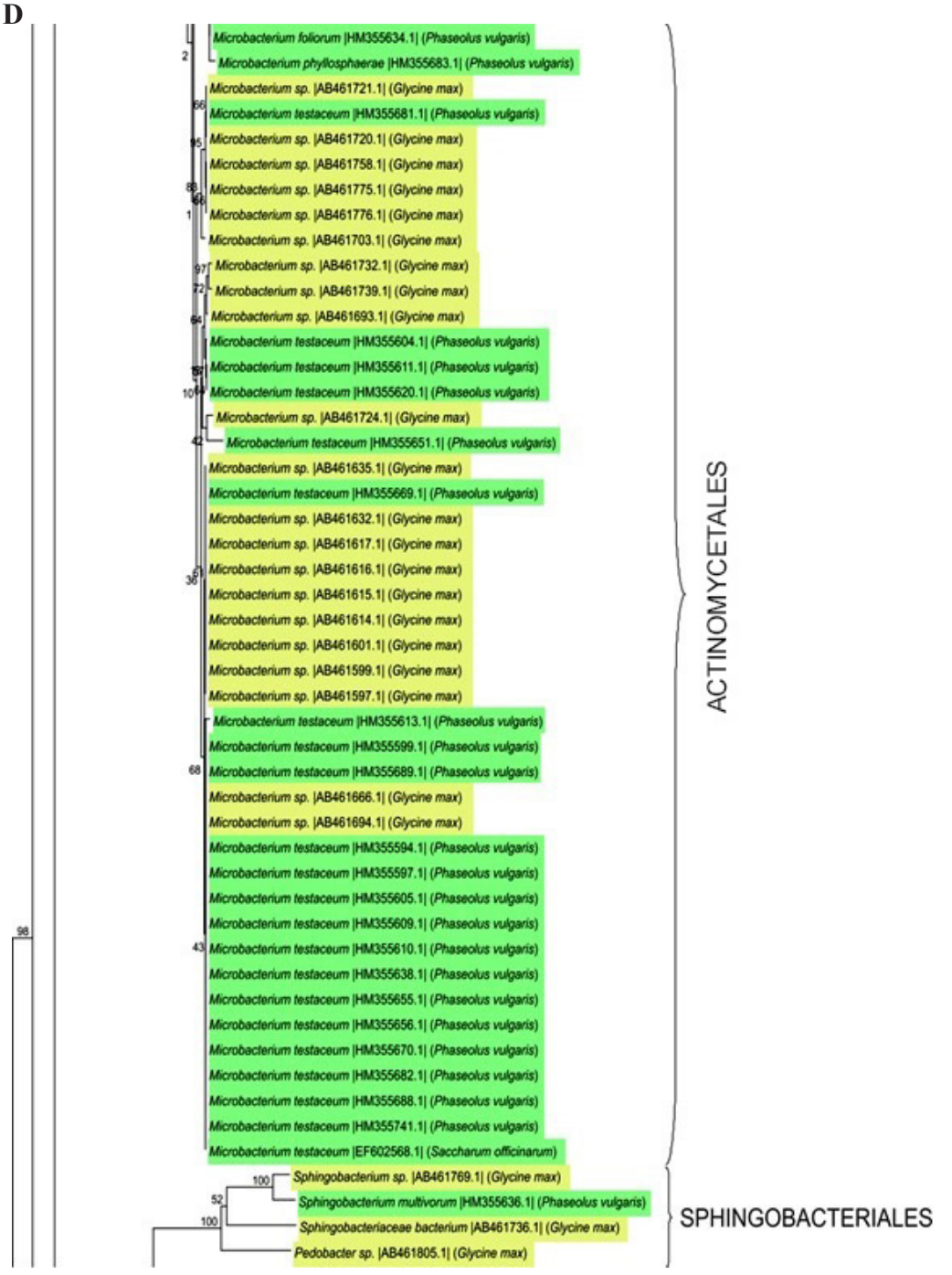

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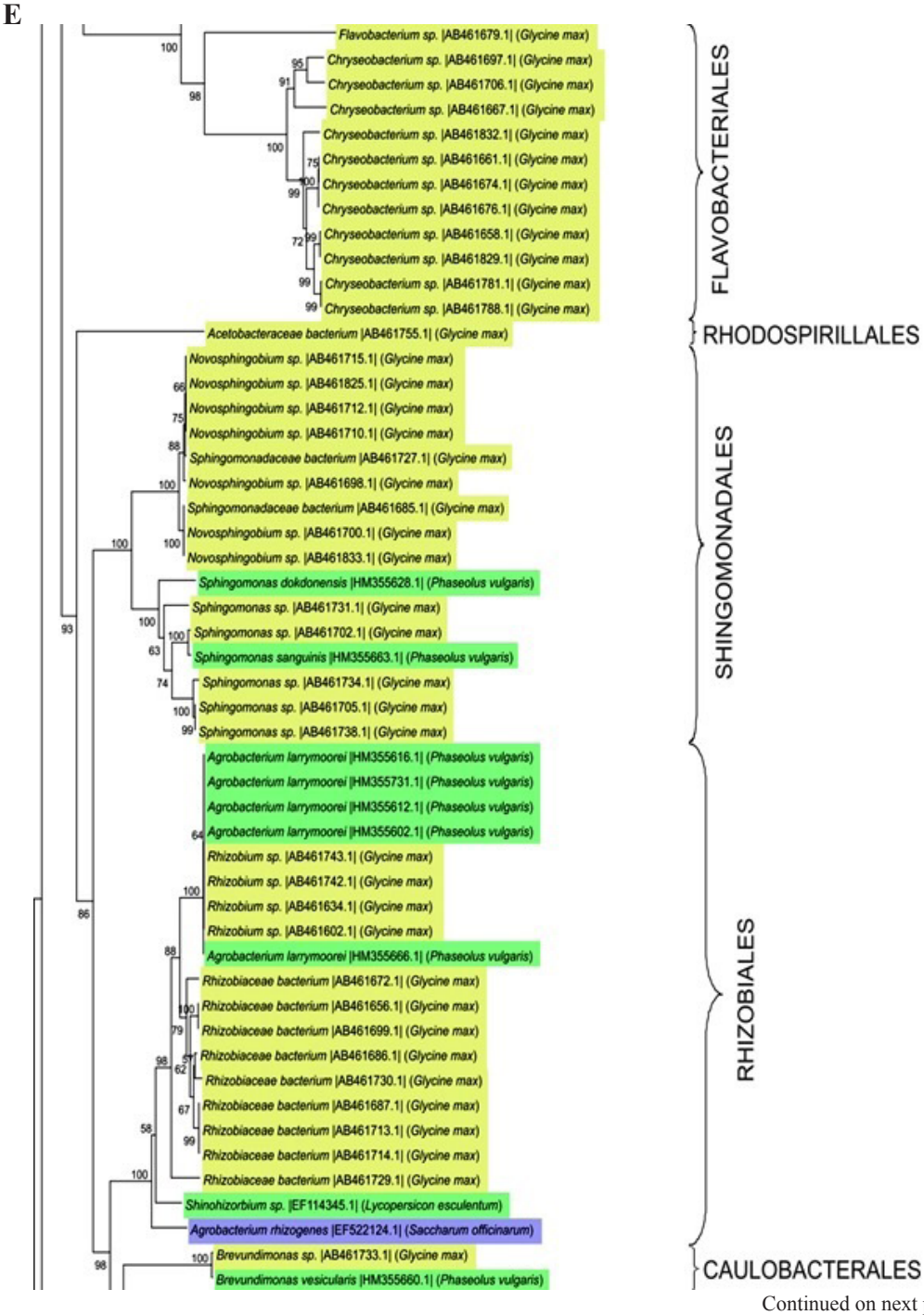

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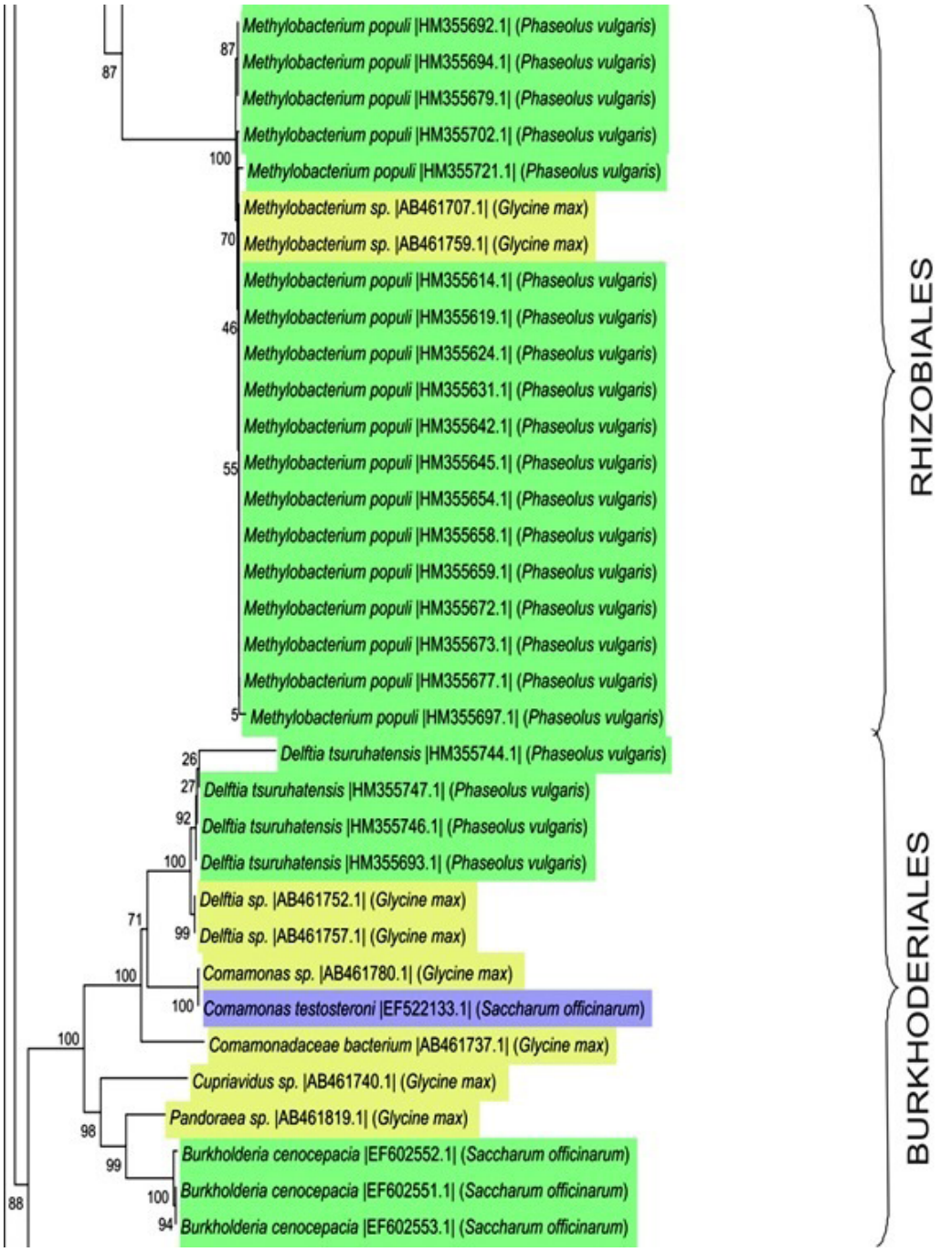

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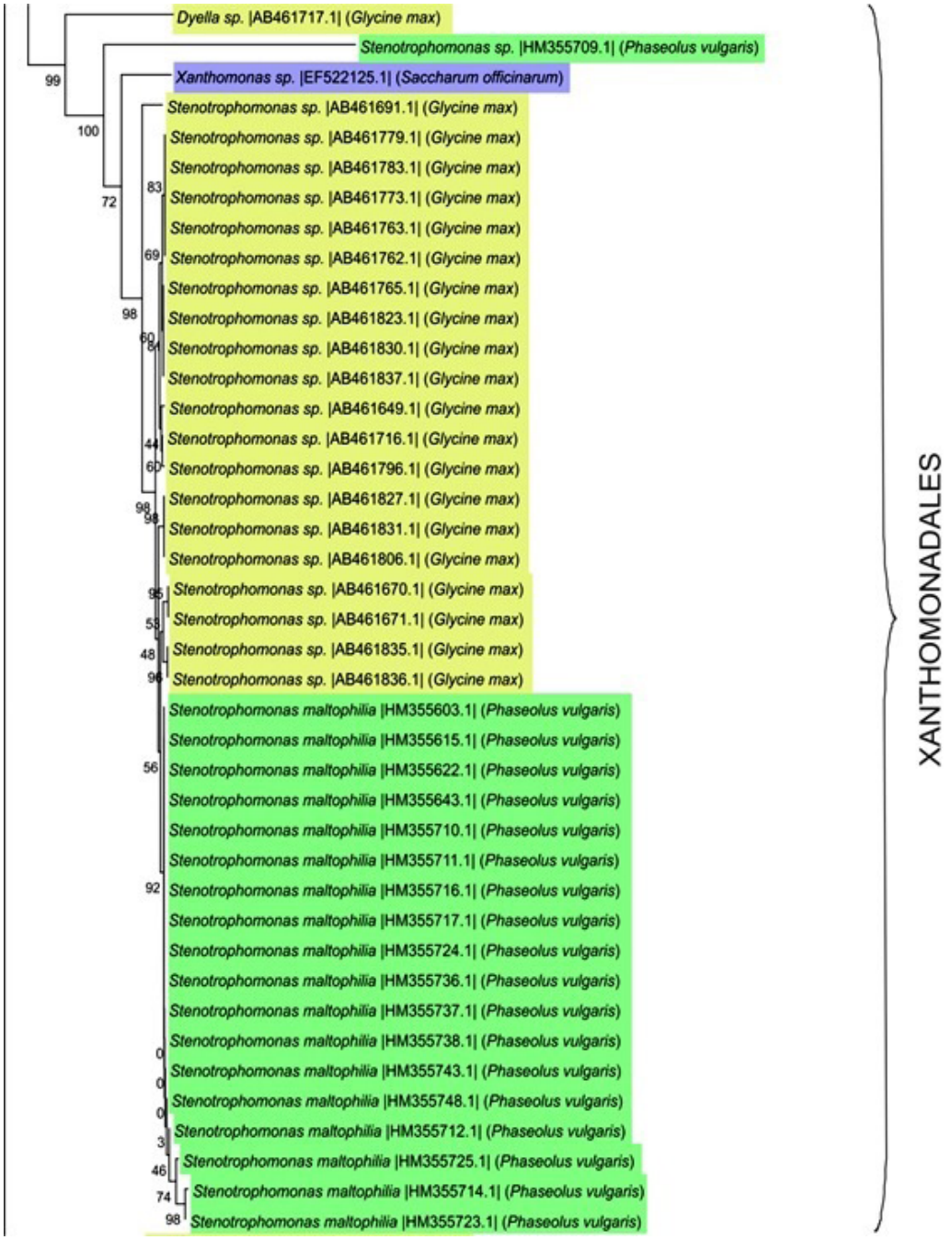

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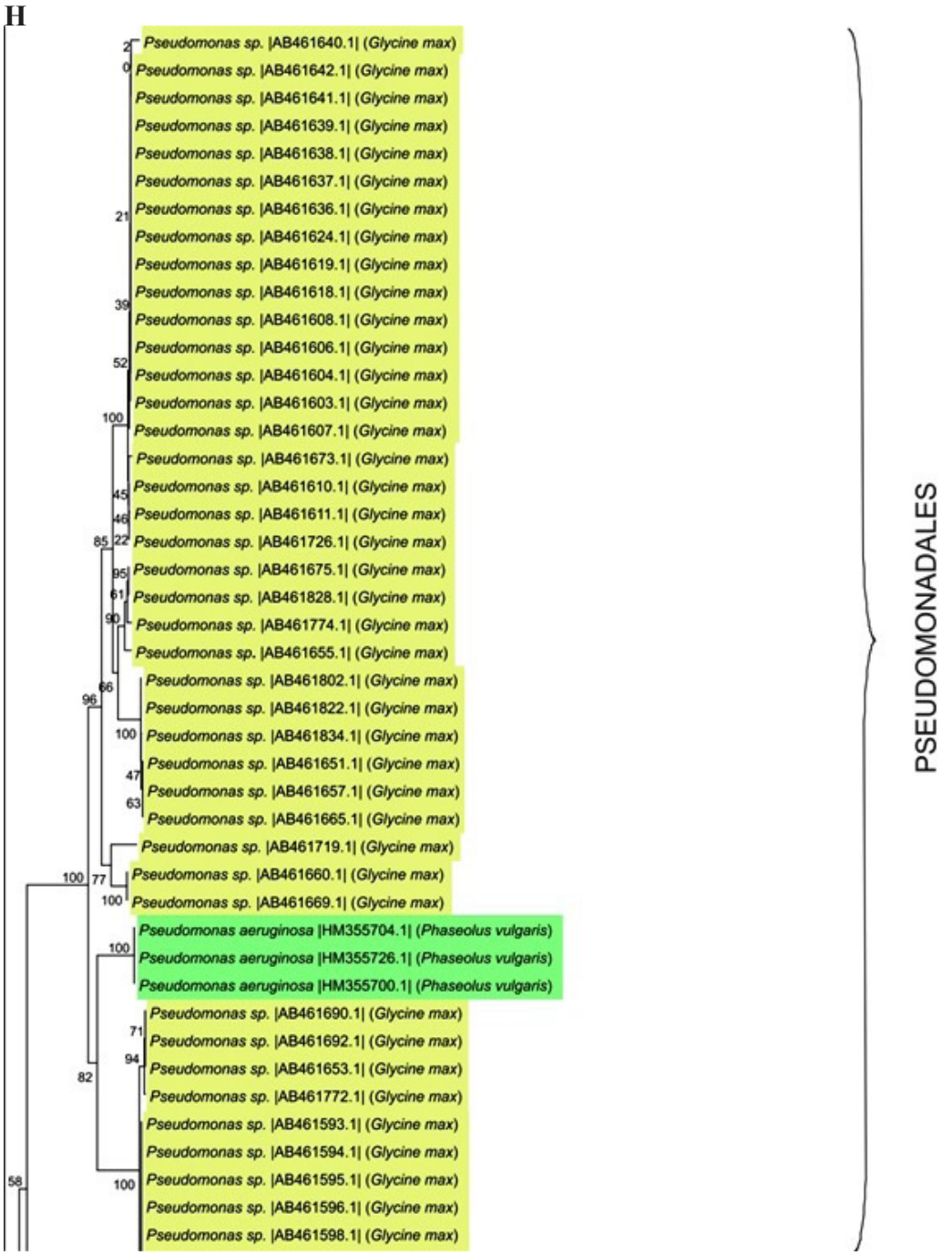

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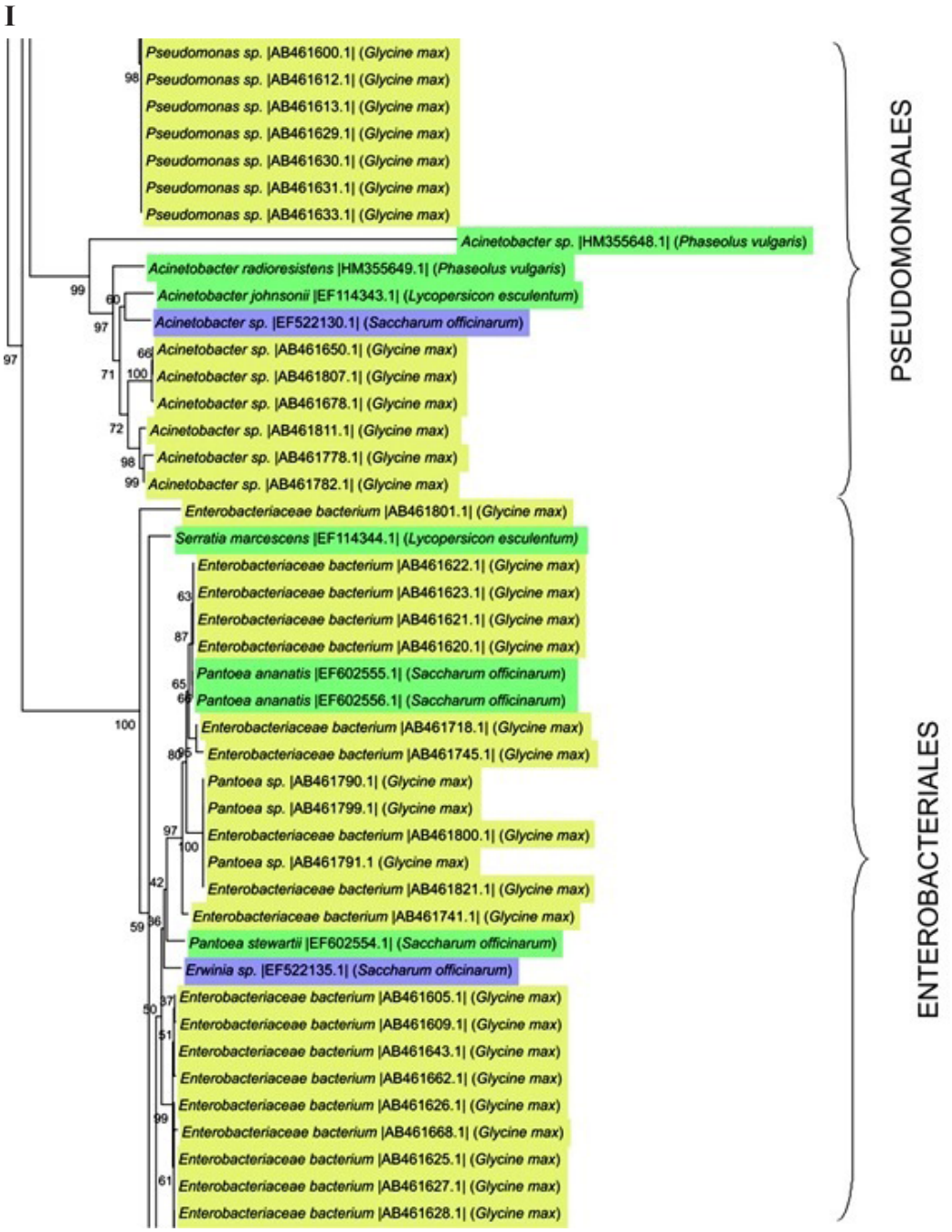

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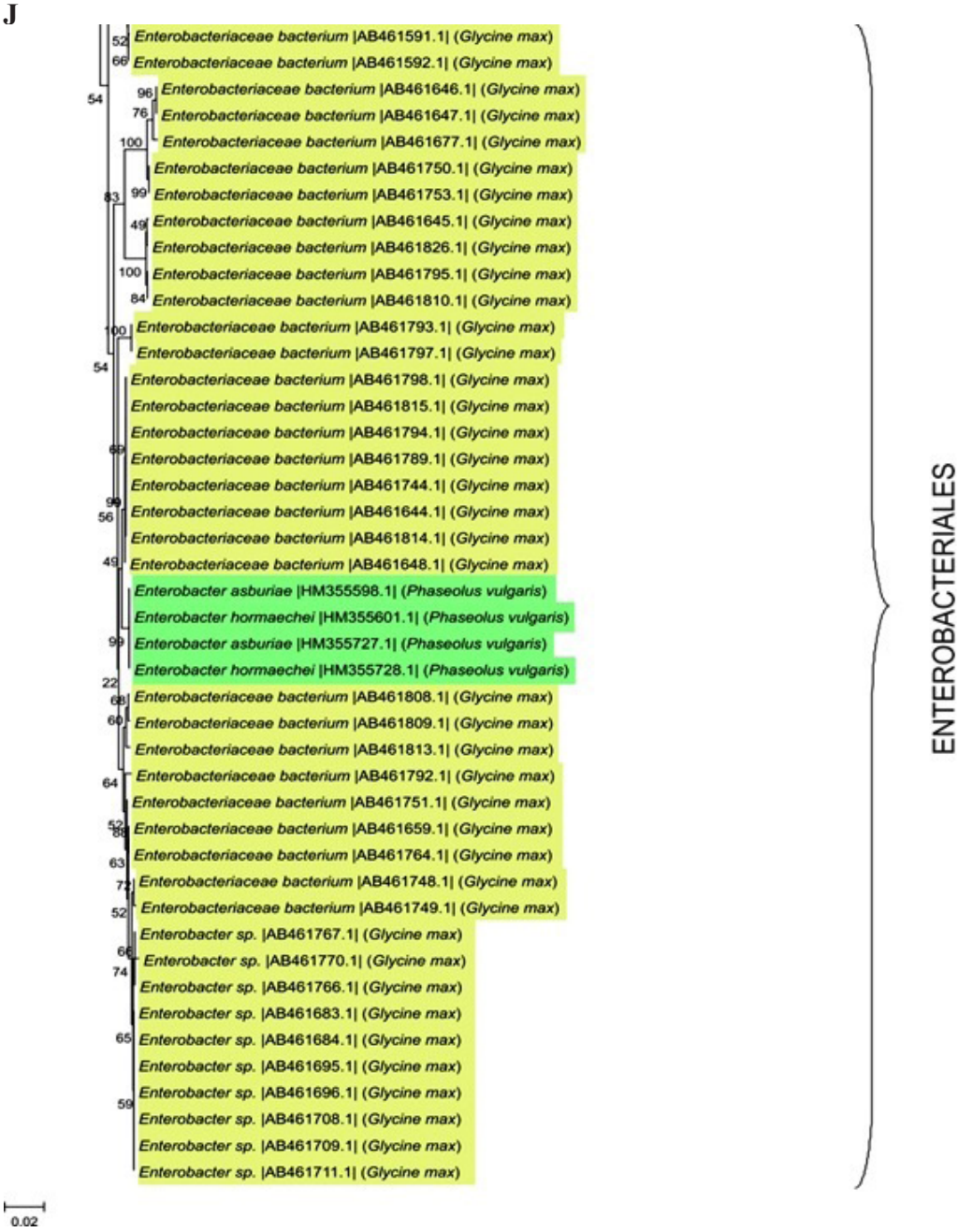

The result of numerical diversity can be seen in the graph below (Figure 4). The color is the host plant and the size of the bar represents numerical diversity.

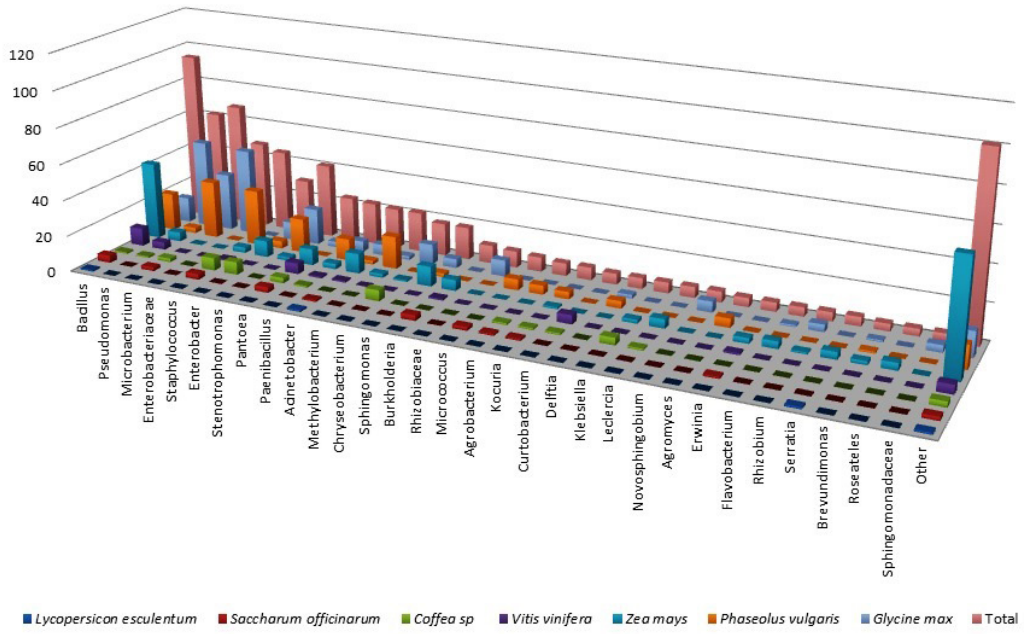

Figure 4. Numerical diversity of the genera of endophytic bacteria isolated from each plant species.

The frequency of each order can be seen in the graph below (Figure 5).

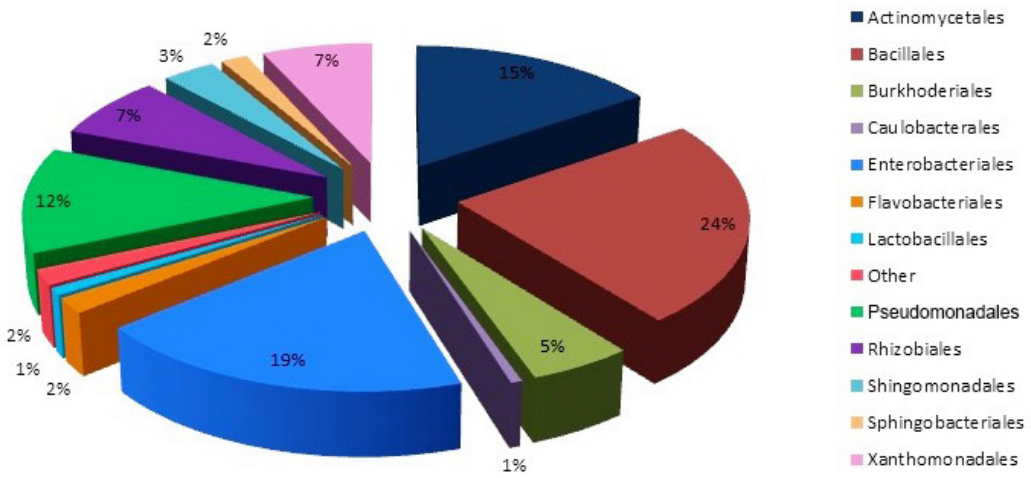

Figure 5. Frequency of each order of endophytic bacteria isolated from the plants.

DISCUSSION

The endophytic bacteria evaluated in the current study were isolated from various plant organs; however, only endophytic bacteria collected from tissues in the aerial parts of the plants were used because recent studies on endophytic microorganisms have focused on the aerial parts of plants, especially the leaves. The main objective of the study was to select bacteria from the plants following analysis of diversity, and then select endophytic strains with promising potential for biocontrol, and bacteria that stimulate the growth of the plant (Barretti et al., 2009; López-López et al., 2010; Montañez et al., 2012).

After analyzing the diversity of endophytic bacteria present in the articles (Figures 4 and 5), Bacillales was verified as the prevailing order, representing 24% of the total, with the genus *Bacillus* present in all cultures and more prevalent in corn than other crops. In the phylogenetic analysis, the following species belonging to the genus *Bacillus* were grouped together: *Bacillus* sp, *B. muralis*, *B. niacini*, *B. bataviensis*, *B. pumilus*, *B. cereus*, *B. subtilis*, *B. megaterium*, *B. amyloliquefaciens*, and *B. thuringiensis*.

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

Within the order Bacillales, the genus *Staphylococcus* was notable and was present in beans more frequently than other crops studied. In the phylogenetic analysis, *S. haemolyticus*, *S. warneri*, *S. kloosii*, *S. saprophyticus*, *S. caprae*, and *S. epidermidis* were clustered together. Some studies have reported that the genus *Staphylococcus* is important in plant growth-promoting activity (Sessitsch et al., 2004). The order Actinomycetales represented 15% of the total bacteria observed in the cultures, with the genera *Micrococcus* (beans) and *Microbacterium* (soy and beans) being observed most frequently. The order Flavobacteriales was represented by the genera *Flavobacterium* sp and *Chryseobacterium* sp and the order Sphingomonadales was represented by the genera *Novosphingobium* and *Sphingomonas* sp, representing 2 and 3% of the total endophytic bacteria, respectively. The order Rhizobiales (an important group of nitrogen-fixing bacteria) represented 7% of the total endophytic bacteria, of which the predominant genera were *Rhizobium* (soy), *Agrobacterium* (beans), and *Methylobacterium* (most frequent in beans). The order Burkholderiales represented 5% of the total endophytic bacteria, with the genera *Delftia* and *Burkholderia* occurring most frequently. The order Xanthomonadales represented 7% of the total endophytic bacteria analyzed, of which the predominant genus was *Stenotrophomonas* and was present in soy and bean. Similarly, the Pseudomonadales order represented 12% of total bacteria analyzed, with a notable presence of *Pseudomonas* (soybean and beans) and *Acinetobacter* (soybean). The second most dominant order of endophytic bacteria studied was that of Enterobacteriales, which represented 19% of the total, with the *Enterobacter* (bean) and *Pantoea* (sugar cane and soybeans) genera being most common.

Of the orders and genera of endophytic bacteria discussed above, previous studies have shown that *Staphylococcus*, *Rhizobium*, *Pseudomonas*, *Mycobacterium*, *Enterobacter*, *Methylobacterium*, *Micrococcus*, *Pantoea*, and *Bacillus* are capable of solubilizing phosphate,

making it more available to plants (Rodriguez and Fraga, 1999; Kuklinsky-Sobral et al., 2004; Sessitsch et al., 2004). Similarly, the genera *Sphingomonas*, *Pseudomonas*, *Microbacterium*, *Pantoea*, *Micrococcus*, *Agrobacterium*, *Rhizobium*, *Burkholderia*, *Enterobacter*, *Bacillus*, *Methylobacterium*, and *Acinetobacter* produce indole acetic acid (Costacurta and Vanderleyden, 1995; Kuklinsky-Sobral et al., 2004; Sessitsch et al., 2004; Mendes et al., 2007) and *Brevundimonas*, *Pseudomonas*, *Staphylococcus*, and *Microbacterium* stimulate plant growth (Sessitsch et al., 2004). These studies highlight the potential of endophytic bacteria in plants and their importance in improving agricultural crops.

The geographic distribution and the cultures of the organisms that were analyzed are poorly understood. Many studies have revealed that the diversity of endophytic microorganisms depends on several factors, including the host and the geographical origin (Li et al., 2012), abiotic factors, such as soil, pH, and the stage of plant development (Li et al., 2009), the tissue used for endophyte isolation (Vega et al., 2005), and also the variety used (Rodrigues et al., 2006). Thus, being poorly understood and in full development, additional studies are necessary in order to verify and satisfactorily explain the distribution of these organisms (Fierer and Jackson, 2006).

In the present phylogenetic study, it was possible to verify that the majority of bacterial species were grouped correctly and only approximately 1.56% of bacteria were grouped incorrectly in their genera, with the need for further assessment. Using *in silico* analysis of fungal endophytes from Brazilian plants, Rhoden et al., (2013) observed similar error rates using sequences from databases.

Endophytic bacteria, as discussed in the present study, are of great importance for plants, contributing to growth and protection, and assisting in the defense process. *Bacillus* was the most noteworthy genus in the cultures analyzed due to its high frequency. Further, in the cultures studied, the 16S rRNA gene was an important factor in identification of endophytic bacteria, allowing phylogenetic analyses to be undertaken.

The results of the current study may assist researchers in future studies, they may help to improve the crops being studied, and they may also contribute to the analysis and comparison of the diversity and biotechnological applications of endophytic bacteria, such as bioprospecting, plant growth promotion, nitrogen fixation, and biological control.

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