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Fungal Endophyte Communities in *Begonia* Species from the Brazilian Atlantic Rainforest

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Abstract
Tropical plants represent hotspots of endophytic fungal species diversity. Based on culture-dependent methods, we evaluated the endophytic fungal communities in leaves of three plant species found in the Brazilian Atlantic Rainforest: *Begonia fischeri, Begonia olsoniae*, and *Begonia venosa*. These species are found in two distant sites: a continental region and an insular area. A total of 426 fungal endophytes in 19 genera were isolated in pure culture including *Colletotrichum* (51.6% of isolates) and *Diaporthe* (22.5%) as the most abundant, followed by *Phyllosticta* (3.5%), *Neopestalotiopsis* (1.8%), *Stagonospora* (1.8%), and *Nigrospora* (1.6%) among the genera found in minor abundance. The diversity and composition of fungal taxa differed across plant hosts. Richness and diversity of fungi were higher in *B. fischeri* in comparison to *B. olsoniae* and *B. venosa*. Discriminatory analysis revealed that fungal communities are structured according to hosts, which means that each plant species had its distinct endophytic communities, but dominated by common fungal taxa. This is the first study to report fungal endophytes in begonia leaves and characterize their communities.

Keywords Fungus–plant interaction · Diversity · Begoniaceae · Endophytic

Introduction

Endophytic microorganisms live within plant tissues without causing any disease symptoms [1, 59]. These microbes may contribute to the nutrition, growth, and resistance of hosts against pathogens [2]. Considered a hyper-diverse group, endophytes also represent an important source of new bioactive metabolites [47, 58].

Endophytic microorganisms are widely distributed among terrestrial plants. It is thought that each plant species harbors at least one endophytic fungus species, however, this number can vary from tens to hundreds, depending on the host [2]. Several studies covering the geographical distribution of plant hosts and their associated endophytic community unraveled distribution patterns of these interactions [3, 9, 51, 60]. These studies clearly showed that a myriad of interactions occur between hosts and endophytes (ranging from specific to generalist), as well as among endophytes within the same host [42].

Begonia is one of the largest genus of vascular plants, comprehending about 1500 species [23], distributed throughout the tropical zones [18]. Of the 213 species that occur in Brazil, 186 are endemic of the Atlantic Rainforest [25]. *Begonia* species were target of several studies as indicators of biogeographic variation, population analysis of endemic species, hybridization, and genomics, among others [10, 11]. Some studies point to the biotechnological potential of *Begonia malabarica* Lam. in the treatment of diabetes [36]. In addition, leaf extracts of the same species presented antimicrobial activity against the bacteria *Pseudomonas aeruginosa, Staphylococcus aureus*, and *Klebsiella pneumoniae* [41]. However, no study examined the association of endophytic microorganisms with *Begonia* species.

It is known that tropical plants represent hotspots of fungal species diversity [3] because such plants harbor

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a great diversity of endophytes that are promising for applied studies [46]. For this reason, we focused on three begonia species: *Begonia venosa* Skan ex Hook. f. found only on the north coast islands of São Paulo State. On the other hand, *Begonia olsoniae* L.B. Sm. & B.G. Schub. is only found in the continental region of southeastern Brazil. Yet, *Begonia fischeri* Schrank is found in all Brazilian biomes and also occurring from the Antilles to Argentina [26]. These species occur in different habitats, but were found in the same biome, which is interesting for endophyte diversity studies. *B. venosa* occurs in low slope areas with high accumulation of organic matter, such as hilltops and rock crevices. On the other hand, *B. olsoniae* is rupicolous and preferably occurs in shaded areas, while *B. fischeri* has a terrestrial habitat and occurs mainly in sites with high incidence of light and moist soils, usually in disturbed or in regeneration areas [26, 56].

The distribution of endophytic fungi across terrestrial plants is an intriguing question. In an attempt to expand the knowledge about this group of fungi, we profiled the endophytic fungal communities associated with: *B. fischeri, B. olsoniae*, and *B. venosa* founded in the same biome but in different areas (island and continent) and habitats to understand their composition, structure, and diversity.

Materials and Methods

Study Areas and Sampling

The present study was conducted at two sites: (i) on the continent, in the municipality of Ubatuba and (ii) on the Alcatrazes Island, in the municipality of São Sebastião, both located on the northern coast of São Paulo State. The Alcatrazes Island is located near four other islands, making up the Alcatrazes Archipelago, located 43 km off the Brazilian coast.

We carried out two expeditions: the first was held on the continental area in June 2014, in Parque Estadual da Serra do Mar, Ubatuba, SP, Brazil. A total of five specimens of *B. fischeri* and five of *B. olsoniae* were collected (Table S1). The specimens were placed in plastic bags and stored under refrigeration until processing, which was carried out on the same day of collection. The second expedition was held in February 2015 on Alcatrazes Island. Five specimens of *B. venosa* were found, collected, and transported as described (Table S1).

Mature individuals of all species were collected as vouchers for identification. The vouchers were taken to the Herbário Rioclarense (HRCB) of UNESP, Rio Claro, SP, as HRCB 64226 (*B. fischeri*), HRCB 64227 (*B. venosa*), and HRCB 64229 (*B. olsoniae*).

Isolation of Endophytic Fungi

Four healthy leaves were selected from each plant specimen; then a total of 60 leaves were examined considering all specimens of the three *Begonia* species. The leaves were first washed with tap water to remove soil particles and other detritus. Subsequently, the leaves were submerged for 1 min in sterile distilled water, followed by 1 min in 70% ethanol, 2 min in 2.5% NaOCl, 1 s in 70% ethanol, and 2 min in sterile distilled water [39]. To check the efficiency of the disinfection process, 100 µL of the final washing batch was plated on malt agar 2% (MA2%) and incubated at 25 °C for 15 days. After disinfection, each leaf was cut into fragments (0.8–1.0 cm) with a flame-sterilized scalpel. Five fragments of each leaf were inoculated on the surface of potato dextrose agar plates (PDA—Acumedia, nutrient-rich medium). Thus, 20 leaf fragments were analyzed for each of the 15 *Begonia* specimens in PDA. The remaining fragments were cut into several smaller pieces, placed in sterilized empty Petri dishes, and then malt agar 2% was poured on the leaves (MA2%—Acumedia, intermediate-nutrient medium). Both media were supplemented with tetracycline (100 mg L^{-1}) and streptomycin sulfate (20 mg L^{-1}) to prevent bacterial growth.

Isolation plates were incubated at 25 °C for 10 days in darkness and observed daily. Fungal colonies were transferred to MA2% plates and incubated under the same conditions to obtain pure cultures. Monosporic cultures were prepared from fungi that presented sporulation. For those fungi that did not sporulate, axenic cultures were prepared by successive transfers in MA2%. Cultures are maintained in MA2% agar slants at 10 °C and in 10% glycerol at −80 °C. Isolates are deposited in the fungal collection of UNESP-Microbial Resources Center.

Fungal Identification

Fungal isolates were grouped into morphospecies and characterized based on general aspects of the colony and microscopic observations of the reproductive structures. Microscopic preparations were made from fresh mycelium using water or cotton blue as mounting fluid. The observed structures were compared with those found in taxonomic keys [14, 15]. Genomic DNA from representative strains of all morphospecies was extracted and submitted to ITS sequencing with primers ITS4 and ITS5 [7, 44]. However, for some taxa we amplified and sequenced additional DNA regions to obtain better taxonomic resolution as follows: for *Aspergillus* and *Penicillium*, we amplified the beta tubulin gene (with primers ßT2a and ßT2b); for

Trichoderma we amplified and sequenced the elongation factor alpha gene (with primers tef1r and 728F). Amplification reactions and conditions were those described in Montoya et al. [32].

The amplicons were visualized in 1% agarose gel stained with GelRed® (Biotium) after electrophoresis. Then, amplicon purification was performed with Wizard® Genomic DNA Purification Kit (Promega) and quantified in NanoDrop (Thermo Scientific). An amount of 20 ng of DNA was used as template in cycle sequencing reactions using BigDye® Terminator Cycle Sequencing Kit v. 3.1 (Life Technologies), following the manufacturer's protocol. After bidirectional sequencing in ABI 3500 (Life Technologies), contigs were assembled in BioEdit v. 7.0.5.3 [19] and compared with homologous sequences deposited in the NCBI-GenBank. Sequences belonging to the genus *Trichoderma* were compared with those deposited in the International Subcommission database on *Trichoderma* and *Hypocrea* Taxonomy (ISTH) using *TrichOKEY* [13].

Sequences were grouped into molecular operational taxonomic units (MOTUs) in MOTHUR, considering a cutoff of 97% similarity [43]. Phylogenetic trees were inferred to assist in the fungal taxonomic affiliation of some groups namely, *Aspergillus, Colletotrichum, Diaporthe, Epicoccum, Mucor, Penicillium, Stemphylium*, and *Trichoderma* (Figures S1–S8). These analyses were conducted with sequences obtained from the NCBI-GenBank as well as sequences obtained from several studies [5, 12, 16, 24, 50, 55, 57]. Sequences were aligned in MAFFT [27] and the phylogeny was inferred using the neighbor-joining method under the Kimura 2-parameter model [28]. Branch support values were calculated with 1000 bootstrap replicates using MEGA v. 6.0 [49]. Sequences of accession numbers used in all phylogenetic analyses are listed in Table S2.

Analysis of Endophyte Communities

We estimated the fungal richness for each community using the Chao1 estimator [31]. Rarefaction curves were generated to compare the observed richness of fungal taxa between plant species. Moreover, the Simpson (1/D) and Shannon diversity indices were calculated. To determine the number of shared species we used the Jaccard, Sorensen, and Bray–Curtis indices [31]. To check which taxa accounted for the observed differences among samples, we carried out a percentage of similarity analysis (SIMPER) in Past v. 2.17c using the Bray–Curtis distance [6]. Rarefaction curves and all the indices were calculated in EstimateS v.9.1.0 [8]. To verify significant differences between the indices, we used Kruskal–Wallis test in R v. 3.0.1. In addition, the number of fungal taxa and the unique species found in each plant species were represented in a Venn diagram.

To investigate the relationship between fungal communities and plant hosts, a correspondence analysis (CA) was performed in Past v. 2.17c [20]. In this analysis, we used the Bray–Curtis distance considering the abundance of fungi found in each plant species. This dissimilarity index was calculated from each pair of host plants. A dissimilarity matrix was generated which was used for mapping the interrelation between plants and fungal abundance [40].

Results

Diversity of Endophytic Fungi in *Begonia* Species

We evaluated 60 leaves (20 of each of the three *Begonia* species), all of which were positive for endophytes. No fungal growth was observed in the control plates inoculated with leaf washings, indicating the disinfection process was effective in eliminating epiphytic microorganisms. A total of 426 fungal isolates were obtained: 120 from *B. fischeri*, 151 from *B. olsoniae*, and 155 from *B. venosa* in both culture media. Based on morphological characters, the 426 isolates were grouped into 71 morphospecies. Representative isolates ($n = 302$ out of 426 isolates) of each morphospecies were sequenced. Then, the sequences were grouped into 46 MOTUs, comprising the phyla Ascomycota (93.5% of the 426 isolates), Basidiomycota (4.3%), and Mucoromycotina (2.2%). For representatives of the phylum Ascomycota, nine orders were found: Botryosphaeriales, Capnodiales, Diaporthales, Eurotiales, Hypocreales, Pleosporales, Sordariales, Trichosphaeriales, and Xylariales. The phylum Basidiomycota was represented by two isolates: one belonging to the Cantharellales order and one unidentified isolate. Only one isolate of the Mucoromycotina was recovered, which belongs to the Mucorales order (Figures S1–S9 and Table 1).

Among the 46 MOTUs, 19 genera were identified including *Colletotrichum* (51.6% of the 426 isolates) and *Diaporthe* (22.5%) as the most abundant (Table 1). All plants exhibited as dominant genera *Colletotrichum* and *Diaporthe*. Specifically, *B. fischeri* had *Colletotrichum* sp3 (20%) and *Diaporthe* sp1 (11.6%) as the most abundant MOTUs. In *B. olsoniae* leaves we found *Colletotrichum* sp1 (53.6%) and *Diaporthe* sp1 (12.5%), whereas *B. venosa* had *Colletotrichum* sp2 (34%) and *Diaporthe* sp3 (14%). The latter fungus was found only in *B. venosa*. Sequences that exhibited low similarity (< 97%) with those available in the databases belonging to isolates that could not be identified by morphology are presented as unidentified Ascomycota or Basidiomycota (Table 1 and Table S2).

None of the rarefaction curves reached an asymptote, suggesting that richness of fungal taxa would continue to increase with the sampling effort (Figure S10). In addition, the estimated richness (Chao1) of fungal taxa was similar

Table 1 Distribution of 46 MOTUs of endophytic fungi obtained from *Begonia fischeri, Begonia olsoniae*, and *Begonia venosa* collected in the Atlantic Rainforest

Taxa	*B. fischeri*	*B. olsoniae*	*B. venosa*	Total
Aspergillus niger Tiegh.	3			3
Aspergillus sp.	1			1
Bartalinia sp.			1	1
Cantharellales	1			1
Cladosporium sp.	3			3
Colletotrichum sp1	13	81	2	96
Colletotrichum sp2	12	6	53	71
Colletotrichum sp3	24	7	14	45
Colletotrichum sp4		7		7
Colletotrichum sp5		1		1
Curvularia sp1	3			3
Curvularia sp2	1			1
Diaporthe sp1	14	19	21	54
Diaporthe sp2			11	11
Diaporthe sp3			22	22
Diaporthe sp4		5		5
Diaporthe sp5	1			1
Diaporthe sp6		1		1
Diaporthe sp7	1			1
Diaporthe sp8		1		1
Endomelanconiopsis sp.		3		3
Epicoccum nigrum Link	2	1		3
Mucor bainieri B.S. Mehrotra & Baijal		1		1
Neofusicoccum sp.			5	5
Neopestalotiopsis sp.	8			8
Neurospora sp.		2		2
Nigrospora sp.	6	1		7
Penicillium thomii var. *flavescens* S. Abe	4			4
Phoma sp.			2	2
Phyllosticta sp.			15	15
Pleosporales	6	2		8
Stagonospora sp.	8			8
Stemphylium sp.			1	1
Trichoderma atroviride P. Karst			2	2
Trichoderma sp.		1		1
Xylariales 1	1	1	2	4
Xylariales 2	2	1		3
Xylariales 3	2			2
Xylariales 4	1		1	2
Xylariales 5	1			1
Xylariales 6	1			1
Unidentified ascomycota 1		10		10
Unidentified ascomycota 2			1	1
Unidentified ascomycota 3			1	1
Unidentified ascomycota 4	1			1
Unidentified basidiomycota			1	1
Total	120	151	155	426

Figures represent the number of isolates recovered using culture-dependent methods

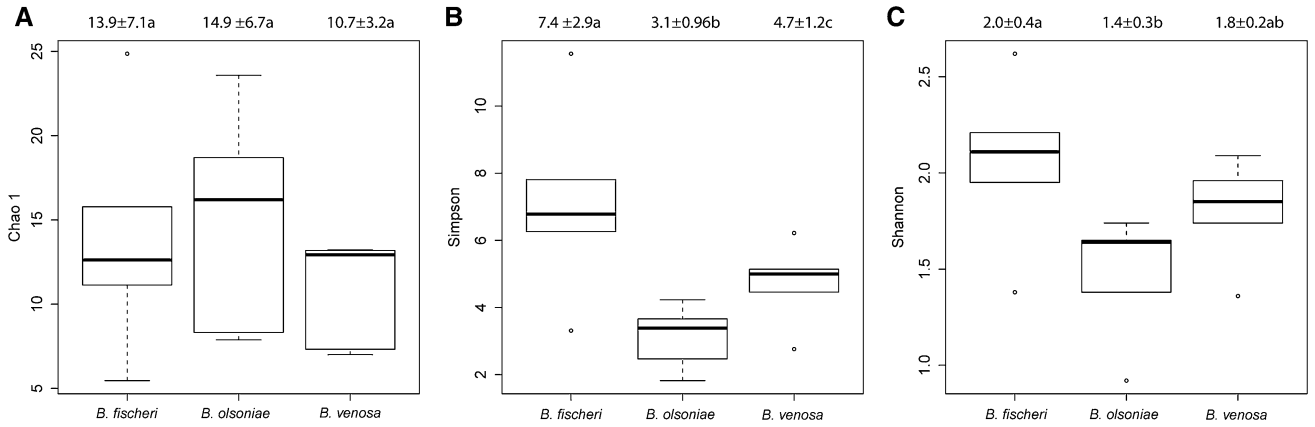

Fig. 1 Estimated species richness (Chao1) and diversity indices (Simpson and Shannon) of endophytic fungi found in *Begonia fischeri, Begonia olsoniae*, and *Begonia venosa*. The mean and standard deviation of the indices are provided above each boxplot. Similar letters indicate no significant differences (Kruskal–Wallis, $P > 0.1$). Circles indicate outliers. **a** Chao1, **b** Simpson, **c** Shannon

Table 2 Indices of shared taxa between fungal communities of three *Begonia* species found in the Atlantic Rainforest

Plants		Jaccard	Sorensen	Bray–Curtis
B. fischeri	*B. olsoniae*	0.257	0.409	0.339
B. fischeri	*B. venosa*	0.167	0.286	0.32
B. olsoniae	*B. venosa*	0.161	0.278	0.229

between plant species (Kruskal–Wallis, $P > 0.1$, Fig. 1a), indicating that it is expected to find more rare species in similar proportions in all three plant species.

According to the Simpson diversity index, communities of endophytes in *B. fischeri, B. olsoniae*, and *B. venosa* significantly differed (Kruskal–Wallis, $P > 0.1$, Fig. 1b). In addition, differences in diversity were found between fungal communities of *B. fischeri* and *B. olsoniae* that exhibited the highest and the lowest diversity of fungi, respectively (Fig. 1b). These differences accounted for *Colletotrichum* sp1 (81 isolates), which was the dominant fungus found in *B. olsoniae* and that influenced the results of the Simpson index. On the other hand, *B. venosa*, the endemic species, showed no significant differences in diversity in comparison to the non-endemic *Begonia* species (Fig. 1c).

Structure and Composition of Endophyte Communities

Plant hosts shared few fungal taxa as indicated by indices below 0.5 (Table 2). These results corroborate with those obtained in the SIMPER analysis, which demonstrated that endophyte communities have an overall difference of 77.16%. From this dissimilarity, *Colletotrichum* sp1 accounted for 24.58% of the difference, which shows the importance of this taxon for all communities. In addition,

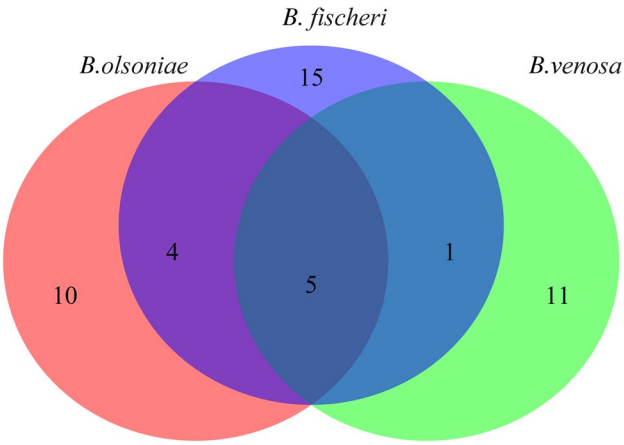

Fig. 2 Venn diagram indicating the number of shared and rare fungal taxa among *Begonia fischeri, Begonia olsoniae,* and *Begonia venosa*

the pairwise analysis showed that *B. fischeri* and *B. venosa* exhibited 74% dissimilarity. From this total, *Colletotrichum* sp2 and *Diaporthe* sp3 accounted for 15 and 8% of the overall difference, respectively. Moreover, *B. venosa* and *B. olsoniae* differed by 83.6%, and the taxa *Colletotrichum* sp1 and *Colletotrichum* sp2 accounted for 25.9 and 15% of this difference, respectively. Finally, *B. fischeri* and *B. olsoniae* differed by 73.8%, with the expressive collaboration of *Colletotrichum* sp1 (26.8%) and *Colletotrichum* sp3 (6.9%) for the dissimilarity.

A total of five taxa (*Colletotrichum* sp1, *Colletotrichum* sp2, *Colletotrichum* sp3, *Diaporthe* sp1, and Xylariales 1) were found in all three *Begonia* species, representing 270 isolates from the total of 426. Furthermore, *B. fischeri* and *B. olsoniae* shared four taxa, which were not shared by *B. venosa* (*Epicoccum nigrum* Link, *Nigrospora* sp., Pleosporales, and Xylariales 2; $n = 21$ isolates). However, only

one taxa (Xylariales 4; $n=2$ isolates) was shared between *B. venosa* and *B. fischeri*. No taxa were shared between *B. venosa* and *B. olsoniae* (Fig. 2). Among the taxa found in *B. fischeri*, eight of them were singletons. *B. olsoniae* and *B. venosa* present five and six singletons, respectively. These results show that each plant host harbors unique endophytes considered rare taxa. In addition, at least one individual of each plant species showed one isolate which was not possible to identify using our polyphasic approach. Such isolates showed low similarity to sequences found in the database. This finding suggests that begonias may harbor new fungal species for science.

The CA was markedly different among all plants species (Fig. 3). Both axes 1 and 2 accounted for 24 and 17% of the observed variation in these communities, respectively; thus, the remaining 59% of the difference may be explained by other factors in addition to the plant host. This result may suggest homogeneity between the samples from the same host species.

Discussion

Here we analyzed the endophytic fungal communities in three *Begonia* species in the Atlantic Rainforest. Based on culture-dependent methods, we studied the diversity, composition, and structure of fungal communities. Endophyte composition differed among *Begonia* species, but all presented *Colletotrichum* as dominant genus. In addition, the structure of endophytes communities was different for each plant species.

In this study, a few genera were abundant in each *Begonia* species (Table 1). We observed that the *B. fischeri* endophytic community was more homogenous and with less number of dominant genera than *B. venosa* and *B. olsoniae*, as well as *B. venosa* showed less dominant genera than *B. olsoniae*. Variation in endophyte composition among hosts is influenced by quantitative differences in the relative abundance of generalists and the presence or absence of rare taxa [54]. Thus, the patterns of most abundant and rare taxa found

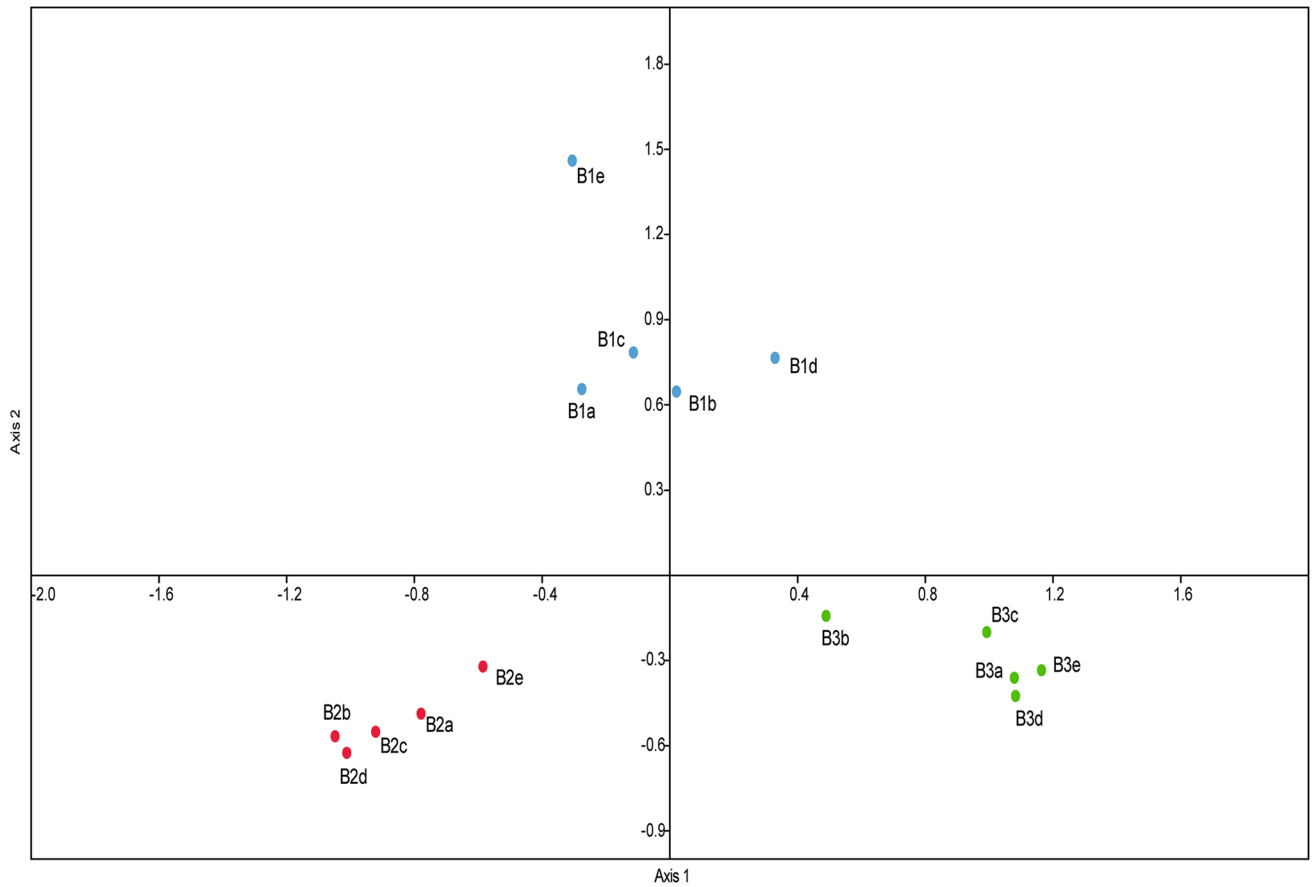

Fig. 3 Structuring of fungal communities in three begonia species (*Begonia fischeri, Begonia olsoniae*, and *Begonia venosa*) revealed by correspondence analysis. Figures in axis 1 (24%) and axis 2 (17%) correspond to inertia values. Plant species IDs: B1: *B. fischeri*, B2: *B. olsoniae*, B3: *B. venosa*. Letters that follow each plant species represent each individual plants

in the present study may explain differences in the endophyte community in each *Begonia* species.

The genus *Colletotrichum* was the dominant in all begonias. This genus has been isolated with a relatively high frequency from various tropical and temperate hosts including fruit trees and medicinal plants [5, 53]. In addition, *Colletotrichum* are commonly found in a range of host plants in Brazil as the medicinal *Carapa guianensis* Aubl. [17], mangrove plants [45], *Mangifera indica* L. [53], and *Eugenia neomyrtifolia* Sobral [52], which shows the remarkable flexibility of this fungal genus in host preference.

Diaporthe, the second most abundant genus found in our study may occur as latent pathogens as *Colletotrichum* [5, 21] but it is also described as a common endophytic fungus [45, 50]. In addition, in a survey of endophytic fungal communities on wide variety of tropical plant species, *Diaporthe* occurred in 20 out of 24 host species studied [48]. Like *Colletotrichum*, the genus *Diaporthe* is a common endophyte in begonias of this study.

Regarding the genera *Epicoccum* and *Xylaria*, these were also found in *Pinus halepensis* Mill. in Spain [4]. These fungi were reported as producers of active metabolites, for instance, *E. nigrum* has been reported as common sugarcane endophyte, which is capable of increasing the root system biomass and controlling pathogens in sugarcane from Brazil [16]. Furthermore a series of new compounds, which have antimicrobial or anticancer activities, has been isolated from *Xylaria* [22, 34].

Endophytes such as *Cladosporium, Nigrospora, Neopestalotiopsis, Penicillium, Phyllosticta, Stagonospora, Trichoderma*, and other species found in low frequency in this work are also reported as common endophytes [29, 35]. Our finds suggests that the endophytic fungal community is shaped by a few genera that are highly represented and by several rare genera represented by only a few members [45]. This result shows that *Begonia* species harbor a wide range of endophytes in their leaves.

In addition, using the ITS barcode marker for fungal identification some taxa found in the present study could not be identified to the genus level. These taxa are represented as unidentified Ascomycota and Basidiomycota. Also, we assigned one taxon to the Pleosporales order, one taxon to the Cantharellales order, and six taxa belonging to the Xylariales order. Considering that ITS does not resolve species identification in several groups of fungi, our findings reveal that underexplored hosts such as *Begonia* species are of great importance to discover putative new fungal taxa. These may have new metabolic mechanisms that can be explored for biotechnological purposes.

Although few fungal taxa have been shared among the three plant species, the begonias collected on the continent shared more taxa between them in comparison to *B. venosa*. This result might be related with differences in the collection sites; however, samplings on the continent took place eight months before the sampling on Alcatrazes Island. Furthermore, the expedition carried out in the continental area took place during the winter and the expedition on the island occurred during the summer. Collectively, these factors may also explain the observed differences in shared fungal taxa between begonias from the continent and the island.

Several studies suggest that the endophytic community differs according to the host [30, 37, 51, 60]. However, in these studies the majority of plant hosts were not phylogenetically closely related. Although in the present study the three plant species belong to the same genus, our results showed significant differences between the communities of each host as described by the CA. *B. olsoniae* and *B. venosa* belong to the same phylogenetic clade [33]. However, these species showed the highest dissimilarity between their communities (83.6%). That is, the genotypic and phylogenetic relationships of the hosts do not seem to be correlated with the composition of the endophytic community and may differ between hosts of the same species, but may be similar to hosts of other genera or even other families [38].

The observed differences in fungal diversity among plants can also be related to the intrinsic characteristics of each host such as the leaf thickness and abundance of trichomes, which may create a barrier for the entrance of endophytes. For example, *B. olsoniae* e *B. venosa* have thicker leaves and more trichomes than *B. fischeri*, but we did not find studies on endophytes of such plants that associate these plant features. Furthermore, secondary metabolites present in the leaves of each plant and the fungi interaction in the inside of the leaves can also play a role of selecting the endophytic community.

Understanding the structure of endophytic communities requires the study of a wide range of environmental factors, in addition to the plant host. Here, we show for the first time the community's diversity and structure of endophytic fungi in *Begonia* species from Atlantic Rainforest. Our results revealed that each *Begonia* species had its distinct endophytic communities composed of rare and putative undescribed species, and also dominated by common fungal taxa.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

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