RESEARCH ARTICLE

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Euphorbia plant latex is inhabited by diverse microbial communities[1]

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PREMISE OF THE STUDY: The antimicrobial properties and toxicity of *Euphorbia* plant latex should make it a hostile environment to microbes. However, when specimens from *Euphorbia* spp. were propagated in tissue culture, microbial growth was observed routinely, raising the question whether the latex of this diverse plant genus can be a niche for polymicrobial communities.

METHODS: Latex from a phylogenetically diverse set of *Euphorbia* species was collected and genomic microbial DNA extracted. Deep sequencing of barcoded amplicons from taxonomically informative gene fragments was used to measure bacterial and fungal species richness, evenness, and composition.

KEY RESULTS: *Euphorbia* latex was found to contain unexpectedly complex bacterial (mean: 44.0 species per sample; 9 plants analyzed) and fungal (mean: 20.9 species per sample; 22 plants analyzed) communities using culture-independent methods. Many of the identified taxa are known plant endophytes, but have not been previously found in latex.

CONCLUSIONS: Our results suggest that *Euphorbia* plant latex, a putatively hostile antimicrobial environment, unexpectedly supports diverse bacterial and fungal communities. The ecological roles of these microorganisms and potential interactions with their host plants are unknown and warrant further research.

KEY WORDS culture-independent analysis; *Euphorbia*; Euphorbiaceae; latex; microbial communities; microbial ecology; sap

Some 20 000 species from over 40 angiosperm families exude the sticky, often white emulsion known as latex (Agrawal and Konno, 2009), which in *Hevea brasiliensis* (Euphorbiaceae) is famously useful to humans in the form of rubber. Latex is not known to contribute to primary functions of the plant, but rather has been shown to play a defensive role from microbial infection (reviewed in Agrawal and Konno, 2009). The genus *Euphorbia* (Euphorbiaceae) is a cosmopolitan clade of ca. 2000 species that comprises an impressive array of growth forms including herbs, shrubs, trees, geophytes, and multiple succulent forms. Despite this morphological diversity, the genus is defined by one primary characteristic: specialized, highly reduced, flowerlike inflorescences (pseudanthia) (Horn et al., 2012; Yang et al., 2012; Dorsey et al., 2013; Peirson et al., 2013; Riina et al., 2013). All members of this genus are lactiferous, producing a white latex that contains a number of secondary metabolites (Jassbi, 2006; Pintus et al., 2010) and can cause dermatitis and severe irritation to eyes (Evans and Schmidt, 1980; Lin, Marshall, and Kinghorn, 1983; Seigler, 1994; Basak et al., 2009; Shlamovitz et al., 2009). *Euphorbia* latex has also been shown to possess moderate antimicrobial properties (Sumathi et al., 2011; van Deenen, Prufer, and Gronover, 2011) and, as for other latex producers, is thought to provide protection from herbivores (Bernays, Singer, and Rodrigues, 2004). While there is evidence of microorganisms infecting *Euphorbia* laticifers (da Cunha et al., 2000), the composition and function of latex suggests that it would constitute an inhospitable environment for microorganisms (Salomez et al., 2014).

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Endophytes, microorganisms living in plant tissue without causing disease, occur in nearly all species of plants investigated and can play a number of functional and ecological roles (Rodriguez et al., 2009). In a recent report, the fungal endophytic communities associated with *Hevea brasiliensis* (rubber tree, Euphorbiaceae) were characterized in leaf and sapwood fragments (Gazis and Chaverri, 2010; Chaverri and Gazis, 2011; Chaverri, Gazis, and Samuels, 2011; Gazis, 2012; Unterseher et al., 2013; Martin et al., 2015), but not directly in latex. Among more than 2500 microbial isolates obtained from 190 individual trees, more than 700 operational taxonomic units (OTUs) were found, suggesting a high diversity of species (Gazis, 2012). Endophytic bacteria also are ubiquitous in plants and can form a range of different relationships with their hosts (Ryan et al., 2008). However, accounts of microbial communities in plant latex are scarce. Over a century ago, flagellate protozoa were found to inhabit *Euphorbia* latex and act as endobiotic pathogens (Lafont, 1909; Franchini, 1922a–c). Early studies on the latex of *Pedilanthus tithymaloides* Poit. (Euphorbiaceae) revealed abundant bacteria, although the communities were not characterized and whether the bacteria were pathogenic or beneficial was unclear (Picado, 1921). No recent studies have characterized the bacterial and fungal communities in *Euphorbia* latex.

Two separate observations motivated the present investigation of the possibility that *Euphorbia* latex could contain microbial communities. First, a rare specimen of *E. mandravioky* Leandri at the Fullerton Arboretum suddenly became diseased and eventually died, prompting a search for the (potentially microbial) cause. Second, tissue culture experiments in our laboratories using specimens from the extensive *Euphorbia* collection at the Huntington Botanical Gardens consistently were accompanied by microbial growth, while the controls remained sterile, ruling out the possibility of contamination. These observations led us to hypothesize that microbial communities of low phylogenetic diversity are harbored in *Euphorbia* latex, despite its composition and supposed protective function. The objective of the current investigation was to test this hypothesis by analyzing the microbial diversity in latex from a variety of *Euphorbia* species using both preliminary cultivation and deep sequencing approaches.

MATERIALS AND METHODS

Sampling sites—Latex samples were collected from *Euphorbia* plants in cultivation at The Huntington Botanical Gardens (San Marino, California, USA [CA], 34°7.636N, 118°6.624W), with the exception of a single sample that was collected at The Fullerton Arboretum (Fullerton, CA, 33°53.238N, 117°52.992W). The climate at The Huntington is type Csa (dry summer type) according to the classification of Köppen (1948), with an average temperature of 24.9°C and an average annual rainfall of 0.51 m (http://wrcc.dri.edu/wrccpub/).

Sample collection—Latex from two individuals of *Euphorbia mandravioky* was collected aseptically: (1) a parent tree planted in the ground at the Fullerton Arboretum; and (2) a rooted cutting growing in pot located at the Huntington Botanical Gardens (Fig. 1). The collection site on the plant was cleaned thoroughly with a sterile alcohol swab and an incision made with a presterilized surgical knife. The white, opaque latex that beaded at the incision was aspirated into a sterile micropipette tip and dispensed into a sterile microfuge tube. Samples were stored and transported on ice (4°C).

Isolation of bacteria and fungi—Latex samples were inoculated into the following liquid media: minimal medium (Baum et al., 2009) and Terrific Broth (TB) (22711-022, Life Technologies, Grand Island, NY). Samples were incubated at 30°C, 150 rpm for 48 h. Samples from the TB cultures were subcultured at 35°C on Sabouraud agar (SA) plates (R01760, Thermo Fisher Scientific, Waltham, Massachusetts, USA) selective for fungi.

DNA isolation, 18S and 16S rRNA gene sequencing, and sequence analysis of isolates—Cells of the microbe (100 mg of the colonies transferred from the agar plate) were suspended in sterile phosphate-buffered saline (PBS, 1×, 100 μL), flash-frozen using liquid nitrogen, and macerated with a micropestle for 10 s. Genomic microbial DNA was extracted from the resulting lysis solutions using the MasterPure DNA Purification kit (Epicentre, Madison, WI, USA) according to the manufacturer's instructions. PCR amplification was carried out with BMB-'A' and BMB-'B' primers (Vilgalys, 2015) for the fungal small subunit (SSU) 18S rRNA gene. Universal bacterial primers (1369F/1492R) (Suzuki et al., 2000) were employed for SSU 16S rRNA gene amplification using Premix Ex Taq (TaKaRa, Shiga, Japan). Gene fragments were amplified using a MyCycler thermal cycler (Bio-Rad Laboratories, Hercules, CA). The PCR conditions (Gunawardana et al., 2014) consisted of an initial denaturing step of 94°C for 2 min; followed by 7 cycles of 94°C for 30 s, 48°C for 30 s, and 72°C for 1 min, 28 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 1 min; and a final elongation step of 72°C for 10 min. Replicate reactions were pooled, and the amplicons were separated by electrophoresis on 1.0% agarose gels. The amplicons were purified using QIAquick Gel Extraction kit (Qiagen, Valencia, CA) according to manufacturer's instructions.

Sanger sequencing of the above amplicons was carried out on an Applied Biosystems 3730 DNA Analyzer (Life Technologies, Carlsbad, CA) at the City of Hope's Integrative Genomics Core (Duarte, CA). Sequences were aligned to the NCBI BLAST nucleotide collection (NCBI, National Institutes of Health, Bethesda, Maryland, USA; http://blast.ncbi.nlm.nih.gov/Blast.cgi) using the discontiguous MEGABLAST program to determine the identity of the isolates.

Sample processing, microbial DNA isolation and amplification, and sequencing of 16S rRNA and ITS gene amplicons—Latex (0.2–0.5 mL, typically one collection per plant per time) was collected as described from 38 *Euphorbia* species (one individual of each), representing a broad phylogenetic sampling of the genus (see Appendix 1). The plants sampled were from indoor (container) and outdoor (ground) sites at The Huntington Botanical Gardens and the Fullerton Arboretum. Samples were transported and stored at 4°C before processing. DNA was extracted from latex samples using the PowerSoil DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA) according to the manufacturer's instructions. Sample contamination from the kit was not a concern given the high amounts of biomass extracted (average yield 1.2 μg total DNA, 0.2–2.0 μg range, from 0.2–0.5 mL of latex) (Salter et al., 2014; Weiss et al., 2014); see also below for more detailed discussion.

The V4 hypervariable region of the bacterial 16S rRNA gene was amplified and sequenced using the validated, region-specific bacterial/archaeal primers 515F and 806R according to previously described methods (Caporaso et al., 2012; Ursell et al., 2014) optimized for the Illumina MiSeq platform. For fungi, the internal transcribed spacer (ITS) region 1 of nuclear DNA was targeted using ITS1-F

FIGURE 1 Isolation of microorganisms from the latex of Euphorbiaceae. (A) Diseased *Euphorbia mandravioky* parent. (B) Healthy *Euphorbia mandravioky* offspring. (C) Liquid medium after inoculation with plant latex from offspring: (i) Terrific Broth (TB) medium, control "inoculated" with an unused (sterile) pipette tip; (ii) minimal medium is clear (= no microbial growth), and (iii) TB medium is turbid (= microbial growth). (D) Microbial growth after latex from healthy offspring of *E. mandravioky* was added to Terrific Broth (TB) medium and the resulting culture was streaked onto fungi-selective Sabourand agar to isolate single colonies (arrows). (E) Fungal spores entwined in a network of external hyphae were observed by light microscopy using a methylene blue stain (scale bar, 100 μm).

(CTTGGTCATTTAGAGGAAGTAA) and ITS2 (GCTGCGTTCTTCATCGATGC) (Bellemain et al., 2010) as the forward and reverse primers, respectively. Although the reverse primer is named "ITS2", it amplifies the ITS1 region, not ITS2, i.e., a subset flanked by ITS1-F and ITS2 (Bellemain et al., 2010). 5′-Barcoded amplicons were generated in duplicate using Premix Ex Taq (TaKaRa) and a MyCycler thermal cycler (Bio-Rad Laboratories). The PCR conditions consisted of an initial denaturing step of 94°C for 2 min; followed by 7 cycles of 94°C for 30 s, 48°C for 30 s, and 72°C for 1 min; 28 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for

1 min; and a final elongation step of 72°C for 10 min. Negative controls were included to identify environmental contamination (Salter et al., 2014; Weiss et al., 2014). Replicate reactions were pooled, and the amplicons were separated by electrophoresis on 1.0% agarose gels. The amplicons were purified using QIAquick Gel Extraction kit (Qiagen) according to manufacturer's instructions. The A_{260}:A_{280} absorbance ratio was acquired with a SpectraMax Plus Absorbance Microplate Reader (Molecular Devices, Sunnyvale, CA, USA) and used as an indicator of DNA purity. Amplicon DNA was quantified using the Quant-iT PicoGreen dsDNA Assay Kit (Life Technologies). Samples (100 ng) from 35 bacterial and 30 fungal amplicons were pooled (of the 38 individuals, three did not yield bacterial and eight did not yield fungal amplicons) and further purified with the UltraClean PCR Clean-Up Kit (MO BIO Laboratories). The purified, pooled samples (A_{260}:A_{280} ratio of 1.70 for bacterial and 1.77 for fungal amplicons) were quantified (75.2 ng·μL^{-1} for 16S rRNA gene amplicons and 71.3 ng·μL^{-1} for ITS gene amplicons) using Quant-iT PicoGreen dsDNA Assay Kit and were submitted for sequencing using the MiSeq platform (Illumina, San Diego, CA) at the Advanced Genomics Facility, University of Colorado, Boulder. Each set of bacterial and fungal amplicons was sequenced as a dedicated lane per run using a paired-end, 2 × 150-bp strategy. The reads were not stitched together because the quality falls off significantly toward the end of the read, causing paired reads to be truncated at or before the stitch point during quality trimming. The sequence data have been submitted to the European Molecular Biology Laboratory European Bioinformatics Institute (EBI) under the EBI accession number ERP010423.

Microbial sequence analysis—The 16S rRNA and ITS gene sequences obtained from the MiSeq platform were processed through the open source software pipeline Quantitative Insights Into Microbial Ecology (QIIME) version 1.7.0-dev (Caporaso et al., 2010). Sequences were quality filtered (-p, default; -r, default; -q, 19) using established guidelines (Bokulich et al., 2013). Quality-filtered reads were demultiplexed, yielding 11 876 513 16S and 6674 ITS sequences.

Bacterial sequences were binned into operational taxonomic units (OTUs) based on 97% identity using the UCLUST algorithm (Edgar, 2010) against the Greengenes reference database (McDonald et al., 2012) May 2013 release. The representative sequences for each OTU were compared against the Greengenes database for taxonomic assignment.

The OTU table was filtered at a minimum fraction of 0.0005 to remove noise from sequencing error, resulting in 10 399 347 reads, with an average of 253 642 reads per sample (maximum 550 256 reads; minimum 37 reads). This filtering step removes spurious OTUs, such as chimera formation during PCR or sequencing errors. The expected rate of these errors is low, so a filter value was chosen to remove those "low abundance" taxa that are likely spurious and not true representatives of biological diversity. In most samples, the majority of taxa were classified as chloroplast, while domain Archaea was detected in one sample at a relative abundance of 1%. A large number of reads corresponded to Rickettsiales and/or mitochondria, which could not be distinguished conclusively. Filtering out these taxa (i.e., host contamination) resulted in 9 samples from different *Euphorbia* species that each had more than 1000 reads. Two strategies were adopted to analyze these data sets in an effort to maximize sampling depth and the number of samples included in downstream analyses. One approach was more robust and removed all samples with fewer than 1000 reads. The second approach was less robust, but conserved more data, and included all samples with at least 100 reads. With this compromise, the OTU table was rarefied to two levels: (1) 1088 reads per sample (9 samples); and (2) 117

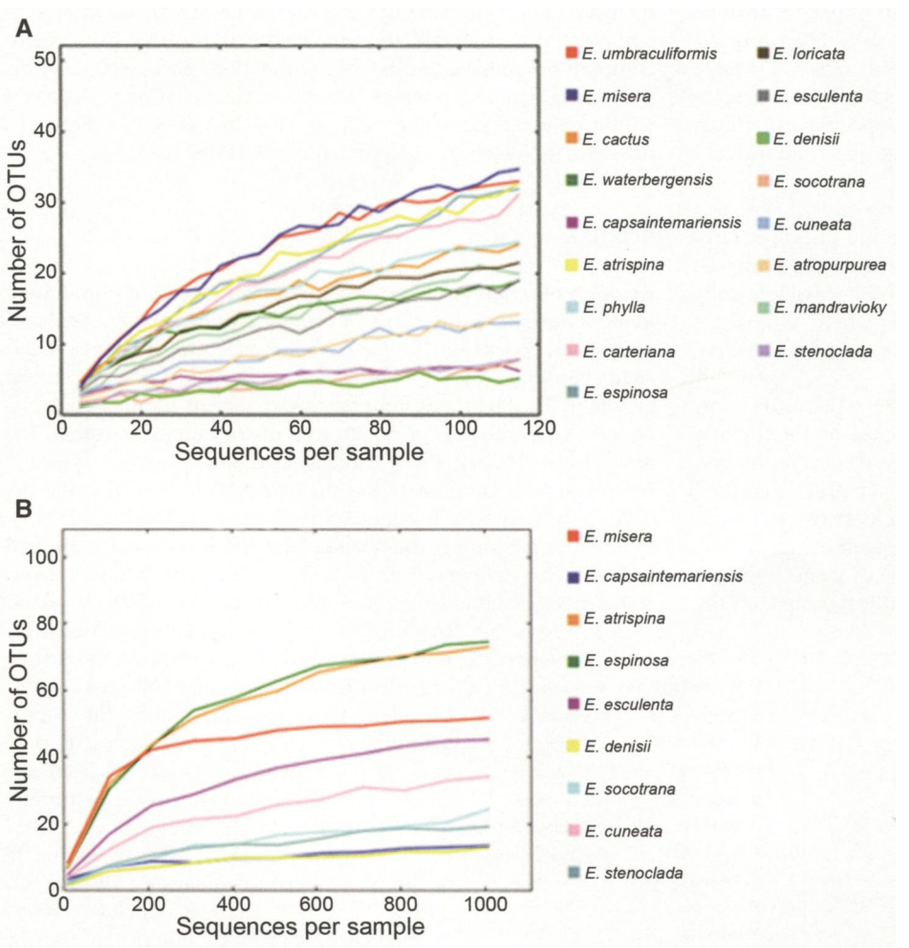

FIGURE 2 Rarefaction curves constructed on the number of bacterial operational taxonomic units (OTUs) detected in *Euphorbia* plant latex at a depth of 117 (A) and 1088 (B) reads per sample. As the number of sequences per sample increased, the number of detected OTUs increased at different rates for different samples. The plateau indicates when the majority of the bacterial community richness and diversity has been captured. The plateau observed for each of the lines in (B) indicates that much of the diversity of the bacterial community has been captured at a sequencing depth of 1000 reads.

reads per sample (17 samples). The thresholds were chosen on the basis that they correspond to the lowest number of reads in any one sample that was equal to or above the chosen cutoff of either 1000 or 100 reads.

Fungal OTUs were selected using an open-reference approach against the May 2013 release (developer version) of the UNITE database (https://unite.ut.ee; Kõljalg et al., 2013). UCLUST (Edgar, 2010) was employed as the clustering algorithm with the optimized parameters and open-reference workflow described by Rideout et al. (2014). Open-reference OTU picking acts as a combination of closed-reference and de novo OTU picking. First, sequences were clustered against the reference database, and any sequences that did not cluster were not discarded because they are used during the closed-reference workflow. Instead, they were clustered de novo against each other and included in the final OTU map. The approach ensures that biologically significant diversity is not lost due to non-existence in the reference databases and is particularly important for some data sets because lesser-studied environments likely contain a considerable proportion of novel taxa. The open-reference fungal OTU table was filtered at a minimum fraction of 0.0005 as done earlier, resulting in 5520 reads with an average of 157 reads per sample (maximum 426 reads; minimum 1 read). The OTU table was then rarefied to a depth of 98 reads per sample (22 samples total) and used for downstream analysis. We speculate that host contamination, as with the bacterial sequences discussed, was responsible for the high proportion of discarded reads.

The number of OTUs detected in each sample, i.e., richness, was calculated for both the bacterial and fungal communities. The phylogenetic diversity of the bacterial samples was assessed using the phylogenetic whole tree metric (Faith, 1992). Unlike taxonomically based metrics, which assume a star phylogeny where all taxa are equidistant from the tree center, phylogenetic metrics conserve true branch lengths on the phylogenetic tree.

The metric unweighted UniFrac (Lozupone et al., 2011) was used to calculate distances between samples based on the fraction of shared branch length between any two bacterial communities on the bacterial phylogenetic tree (McDonald et al., 2012). Principal coordinate analysis (PCoA) plots then were constructed from the distance matrices and interactively visualized using the online tool EMPeror (Vázquez-Baeza et al., 2013) to identify sample groupings. No fungal phylogenetic tree currently accompanies the UNITE database. Therefore, the Bray–Curtis method, which does not use a phylogenetic tree, was employed to construct distances matrices for fungal communities (Navas-Molina et al., 2013). Data were visualized by PCoA similarly to bacterial data. Principal coordinate analysis was used to compare the clustering of microbial communities across samples with the goal of identifying associations between the latex microbiomes and environment (locality and growing conditions), plant height, plant age, original source type of the plant, form of propagation, geographic origin of the plant, indoor and outdoor container plant.

Heatmaps were constructed using QIIME to visualize the abundance distributions in individual samples of the OTUs with the highest sequence representation. Reads were filtered out of the OTU tables according to the following parameters: bacterial communities, all OTUs with fewer than 30 reads (OTU table rarefied to 117 reads per sample), or 100 reads (OTU table rarefied to 1088 reads per sample); fungal communities, all OTUs with fewer than 30 reads.

Data sets were compared using the ANOSIM and PERMANOVA methods in the program QIIME (Caporaso et al., 2010), which specifically wraps around R and uses the vegan and ape packages to compute the statistics, with 999 permutations each. ANOSIM directly tests sample groupings rather than the variation of distances within/between sample groupings (PERMANOVA). These approaches therefore are complementary (QIIME, 2015).

RESULTS

***Isolation of fungal and bacterial organisms from* E. mandravioky *latex*—**One bacterial species from latex collected from diseased and one fungal species from healthy *E. mandravioky* individuals were isolated (Fig. 1). Latex from the parent plant only yielded microbial growth in TB, while latex from the offspring plant afforded microbial growth in TB and on SA. Nothing grew on the minimal medium. The fungal isolate could not be identified based on sequence analysis; *Pyrenophora teres* (strain 0-1) was the best match (BLAST similarity, 68%; query cover, 82%; E-value 8.0×10^{-17}; accession XM 003297539.1).

The Gram-positive, rod-shaped bacterial isolate was identified as *Bacillus amyloliquefaciens* (BLAST similarity, 100%; query cover, 100%; E-value 2.0×10^{-179}; accession KT368090.1). We suspected that the bacterium could have originated from the organic mulch, but attempts to isolate this organism from hay samples collected around the plant were unsuccessful. An important producer of α-amylase and protease (Priest et al., 1987), *B. amyloliquefaciens* has been isolated from soil infested with plant pathogens where it produces extracellular phytases believed to promote plant growth, especially during phosphorous limitation (Idriss et al., 2002). Plant-associated, beneficial *B. amyloliquefaciens* produces potent antibiotics (Arguelles-Arias et al., 2009; Alvarez et al., 2012; Yuan et al., 2012; Ji et al., 2013; Tanaka et al., 2014), providing protection from phytopathogenic fungi (Danielsson, Reva, and Meijer, 2007; Arguelles-Arias et al., 2009) and bacteria (Lanna et al., 2013). It is not evident what ecological role this organism played in

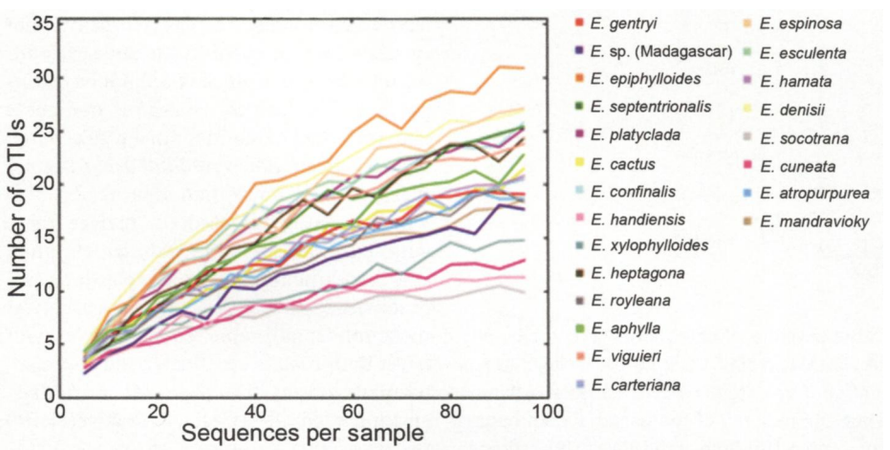

FIGURE 3 Rarefaction curve constructed on the number of fungal OTUs detected in *Euphorbia* plant latex samples calculated at a depth of 98 reads per sample.

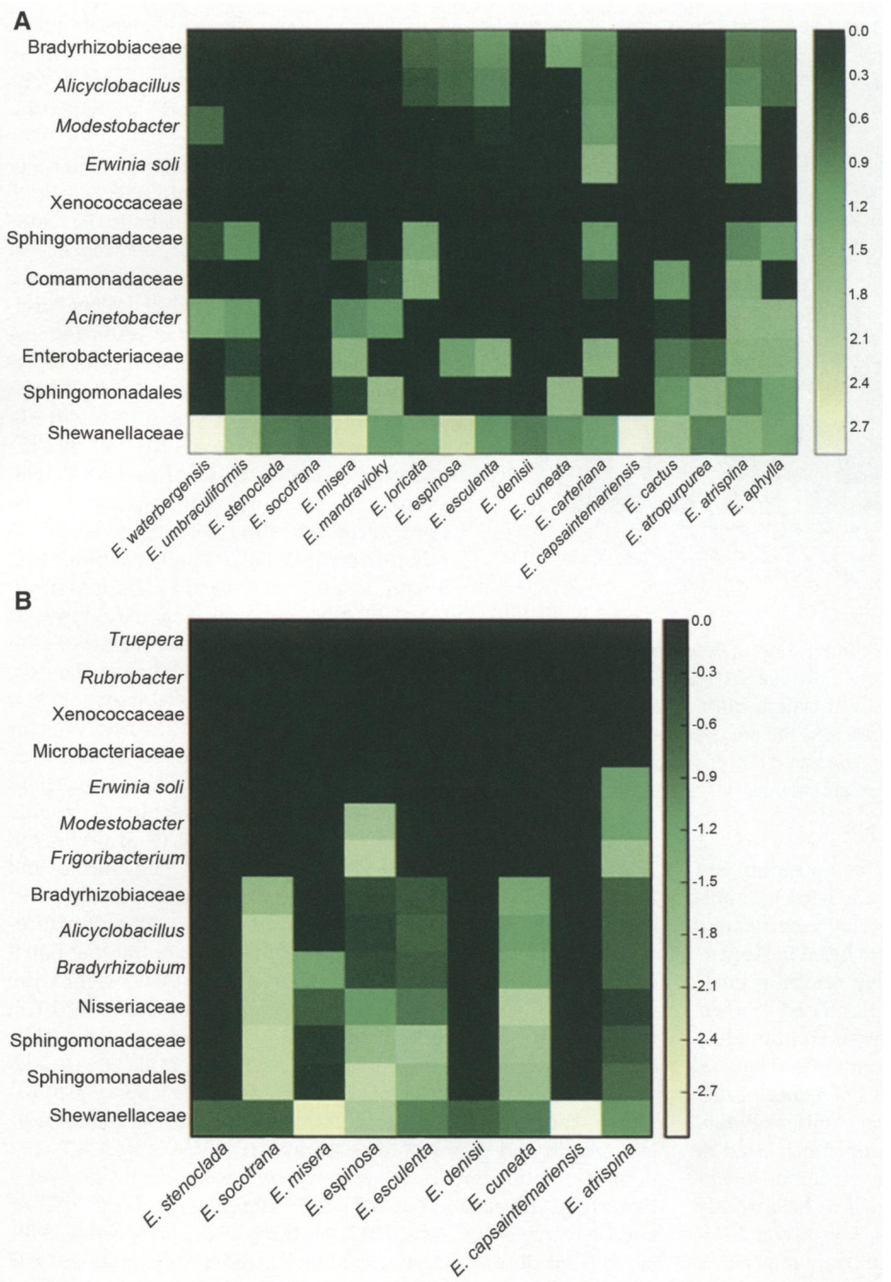

FIGURE 4 Heatmaps showing the bacterial community composition in *Euphorbia* plant latex samples rarefied to (A) 117 and (B) 1088 reads per sample. The *y*-axis denotes individual OTUs; the *x*-axis identifies the *Euphorbia* plant source of these bacteria. All taxa consist of genera, but not all received a genus-level classification. The names of these taxa are not italicized and are classified by their family or in the case of Sphingomonadales, by their order. The color key in the legend indicates relative abundance.

the latex of the diseased plant, nor what other factors (e.g., growth environment, age) contributed to shaping the different latex microbiomes in the two plants.

Diversity of microbial communities in* Euphorbia *latex by deep sequencing of taxonomically informative genes—The species richness and phylogenetic diversity of the bacterial communities in *Euphorbia* plant latex were relatively high given the composition of the sampled environment: richness, 44.0 ± 29.0 (average number of species per plant ± SD); phylogenetic diversity, 4.46 ± 2.28 (average phylogenetic diversity ± SD). The richness of the fungal communities was also relatively high at 20.9 ± 5.58 (average number of species per plant ± SD). There was considerable variability in microbial richness and diversity across the samples, with many species and high community evenness in some samples and lower values in others (Figs. 2, 3). Hereafter, "sample" refers to latex from an individual *Euphorbia* plant. At a sequencing depth of 117 reads per sample, the bacterial species richness was underestimated based on the rarefaction analyses (Fig. 2A). However, the plateau observed for each of the lines in Fig. 2B indicates that much of the diversity of the bacterial communities has been captured at a sequencing depth of 1000 reads. The complexity of the bacterial communities varied significantly, with the latex of some plants containing fewer than 10 OTUs (e.g., *E. denisii* and *E. capsaintemariensis*) and others containing more than 60 (e.g., *E. espinosa* and *E. atrispina*). The same trend was observed through the phylogenetic whole tree metric (data not shown). Most of the fungal diversity associated with *Euphorbia* latex was not captured at a sequencing depth of 98 reads per sample, as indicated by the rarefaction plots (Fig. 3). This result is not surprising because filtering out low-quality reads removed most of the fungal reads (see Materials and Methods).

Classification of microbiota in* Euphorbia *latex—Overall, the distribution of taxa in representative samples of *Euphorbia* latex was heterogeneous. These results are based on the hierarchically clustered heatmap analysis of the bacterial (Fig. 4) and fungal (Fig. 5) community profiles. Species from the bacterial family Shewanellaceae were the most common taxa across samples (Fig. 4), while a number of fungal taxa, classified to different taxonomic levels, from phylum to species, were common to most samples analyzed (Fig. 5). The UNITE database used here to classify the fungal taxa could lead to biases in the analysis because the database focuses largely on Basidiomycota. In future studies, BLAST searches using fungal sequences will be carried out against a database with a higher representation of Ascomycota, found to be the dominant fungal endophytes in planted and wild rubber trees (*Hevea brasiliensis*) (Gazis, 2012; Unterseher et al., 2013). However, the results will need to be interpreted with the caveat that BLAST searches on small gene segments can be misleading.

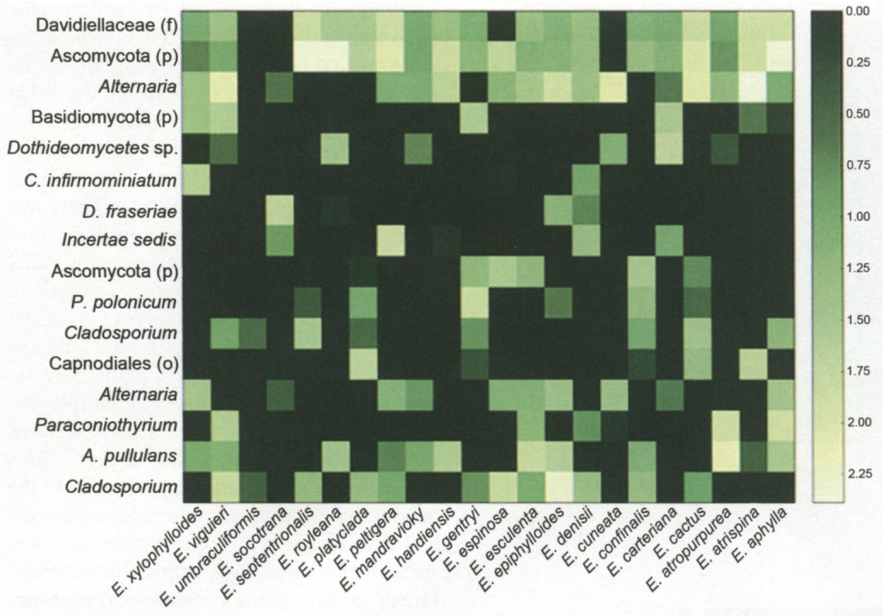

FIGURE 5 Heatmap showing the fungal community composition in *Euphorbia* plant latex samples rarefied to 98 reads per sample. The *y*-axis denotes individual OTUs; the *x*-axis identifies the *Euphorbia* plant source of these fungi. All taxa consist of genera, but not all received a genus-level classification. The names of these taxa are not italicized and are classified by their family (f), order (o), class (c), or phylum (p). "Incertae sedis" refers to a taxon that could not be classified with certainty. The color key in the legend indicates relative abundance.

Associations between microbial community composition and plant environment could provide clues on how the latex microbiomes are assembled. However, no significant clustering patterns were observed by PCoA as a function of the parameters listed in Appendix 1, among others (data not shown). However, Fig. 6A shows qualitatively modest clustering of bacterial communities based on their environment (i.e., garden, greenhouse, and nursery), and this clustering pattern is more apparent for fungal communities (Fig. 7). Fungal microbial communities detected in the latex of plants located in the garden and greenhouse environment appear uniformly distributed; however, about half of the samples from plants located in the nursery environment cluster separately from garden or greenhouse samples. Plant environment was determined to be a weakly significant driver of sample clustering (ANOSIM, $R = 0.18$ and $P = 0.02$; PERMANOVA, pseudo-$F = 1.46$ and $P = 0.05$) and may play a role in the transmission of bacteria and fungi between plants.

DISCUSSION

Results from our previous cultivation studies led us to hypothesize that the latex of *Euphorbia* plants commonly harbors microbial communities and that the species richness may be low because they inhabit an environment presumed to be antimicrobial. The hypothesis was tested in a culture-independent study using latex sampled from 38 species of *Euphorbia* representative of the geographic origin before collection and phylogenetic distribution of the genus (see Appendix 1). These studies suggest that our original hypothesis is incorrect. The microbial phylogenetic diversity in the studied samples was surprisingly high, especially in select plants (Figs. 2, 3). The wide range in diversity suggests that a number of factors not studied here could influence the complexity of the bacterial and fungal communities found in *Euphorbia* latex.

The latex samples contained a broad range of polymicrobial communities (Figs. 4, 5) whose ecological roles remain to be elucidated. Members of the family Shewanellaceae represented the most commonly detected bacterial taxa across all analyzed plant specimens (Fig. 4), an unexpected result because these γ-proteobacteria are not typical representatives of plant microbiomes. *Shewanella* generally are thought of as marine and freshwater organisms (Dikow, 2011), with the uncommon ability to use heavy metals as electron acceptors in certain respiratory situations. *Shewanella* also have been isolated from other, diverse habitats, including clinical specimens, meats, butter, marine algae and invertebrates, fish, and even sea ice (Bowman, 2005). Many of the other taxa identified consistently across samples are putative plant endophytes, providing a rational basis for their presence in *Euphorbia* latex. The latex of many *Euphorbia* plants contained bacterial groups that have been shown in symbiotic associations with fungal endophytes in plant root systems, such as *Bradyrhizobium* sp. (Ruizlozano and Azcon, 1993; Chaintreuil et al., 2000; Okubo, Fukushima, and Minamisawa, 2012; Mason et al., 2015; Subramanian et al., 2015) and *Enterobacter* sp. (Nair and Padmavathy, 2014). These diazotrophic bacteria can contribute to nitrogen fixation, promoting nutrition and growth of the host plant (Zhang et al., 2011; Madhaiyan et al., 2013; Terakado-Tonooka, Fujihara, and Ohwaki, 2013; Nimnoi, Pongsilp, and Lumyong, 2014; Teamtisong et al., 2014), including members of the Euphorbiaceae (Dieng et al., 2015).

Most of the fungal taxa identified in *Euphorbia* latex (Fig. 5) belong to the class Dothideomycetes, a diverse clade of pathogenic, endophytic, and saprotrophic Ascomycota (Hyde et al., 2013). Endophytic Dothideomycetes are commonly found in healthy plant tissue (e.g., Arnold and Lutzoni, 2007; Albrectsen et al., 2010; Gazis and Chaverri, 2010; Gazis, 2012; Massimo et al., 2015; Xiong et al., 2015). Taxa observed here, including *Aureobasidium pullulans* and *Cladosporium* spp., have been isolated from the tissue of a broad range of plants (Nair and Padmavathy, 2014) including the cactus *Cereus jamacaru* (Bezerra et al., 2013) and *Cocos nucifera* and *Vitis labrusca* in hypersaline environments (de Oliveira et al., 2014). The diversity, antifungal/antimicrobial activity, and potential applications of fungi such as *A. pullulans*, *Alternaria* spp., and *Cladosporium* spp. are widely known, and strains known from various plant species may produce secondary metabolites with entomopathogenic properties (see Schena et al., 2003; Suryanarayanan, Wittlinger, and Faeth, 2005; Miles et al., 2012; Pancher et al., 2012; Polizzotto et al., 2012; Thakur et al., 2013; Wang et al., 2013; Kaur et al., 2015; Silva-Hughes et al., 2015; Soltani and Moghaddam, 2015). However, these fungal endophytes are not always beneficial to their plant host. For example, some *Alternaria* species are notoriously destructive plant pathogens (Lawrence et al., 2008).

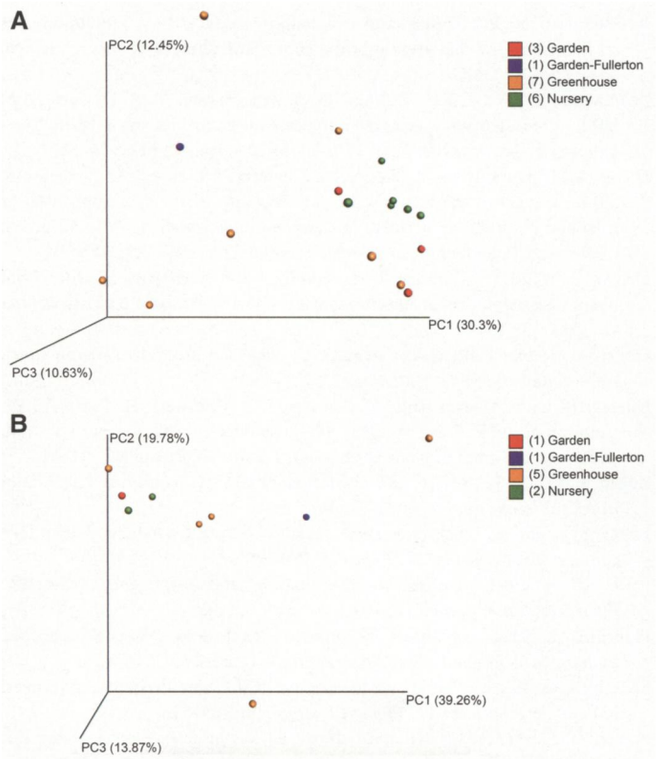

FIGURE 6 Unweighted UniFrac distances, using closed-reference OTU picking, plotted in principal coordinate analysis (PCoA) space to compare the bacterial communities in *Euphorbia* latex with (A) 117 and (B) 1088 reads per sample. Each sphere represents a single microbial community, and the spheres are color-coded based on plant environment. Numbers in parentheses in the legends indicate the number of plants in each group.

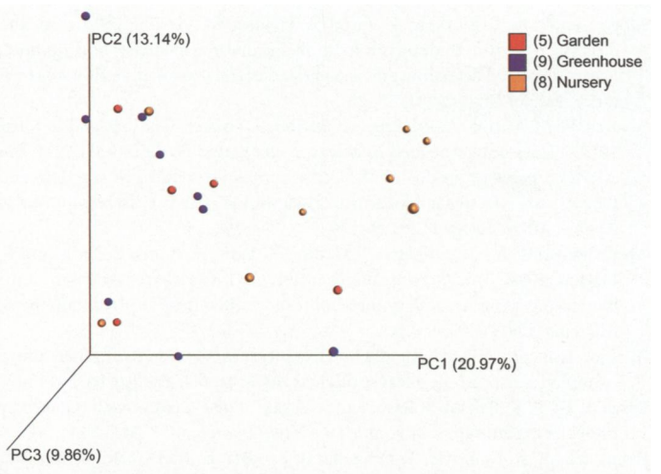

FIGURE 7 Unweighted UniFrac distances, using open-reference OTU picking, plotted in principal coordinate analysis (PCoA) space to compare the fungal communities in *Euphorbia* latex with 98 reads per sample. Each sphere represents a single microbial community, and the spheres are color-coded based on plant environment. Numbers in parentheses in the legend indicate the number of plants in each group.

It is unclear what factors determine the assembly of the resident microbiota in *Euphorbia* plant latex. Horizontal transmission of airborne microbes is a plausible mechanism for a number of the taxa observed here (Fig. 5). Aerobiological studies frequently identify spores from the fungi *A. pullulans* (Punnapayak et al., 2003), *Alternaria* spp. (Recio et al., 2012; de Aldana, Bills, and Zabalgogeazcoa, 2013; Lee and Liao, 2014; O'Connor et al., 2014; Ben Sidel et al., 2015; Kasprzyk et al., 2015), and *Cladosporium* spp. (Recio et al., 2012; de Aldana, Bills, and Zabalgogeazcoa, 2013; Lee and Liao, 2014; O'Connor et al., 2014; van Kampen et al., 2014) in air samples collected across the globe. The latex microbes also could originate from water, soil, and litter through currently unknown mechanisms of entry. Insects are known to mediate horizontal transfer of microbes between plants. *Phytomonas* species, flagellate protozoa known for over 100 yr to inhabit *Euphorbia* latex (Lafont, 1909, 1910), are transferred between plants by various insect vectors (Harvey and Lee, 1943; Troll, 1973; Dollet, 1984; Jaskowska et al., 2015). The whitefly *Bemisia tabaci* transfers *Rickettsia* spp. to the plant, where they move inside the phloem and can be acquired by other whiteflies (Caspi-Fluger et al., 2012). Interestingly, symbiotic, gut-associated bacterial taxa from the Enterobacteriales (Fig. 4A) and Neisseriaceae (Fig. 4B) recently were isolated from honey bees and bumble bees (Kwong and Moran, 2013; Saraiva et al., 2015). These findings suggest a possible insect-driven transfer to *Euphorbia* latex, although the mechanism of transfer has yet to be elucidated.

Vertical transmission of microorganisms from mother plant to offspring via seeds or pseudo-vertical transfer during vegetative propagation cannot be ruled out (Truyens et al., 2015). For example, fungal endophytes from the genus *Alternaria* and *Cladosporium* (Fig. 5) recently have been shown to be transmitted vertically in forbs, likely through fungal growth in the pollen tube as the mode of entry into the developing seed (Hodgson et al., 2014). Vertical transfer would lead to some retention of the microbial signature through time. The relative contributions of these various transmission routes are difficult to unravel, particularly in an exploratory study as presented here. No clear associations were observed between microbial community and indicator metadata such as geographic origin of the plant. However, a weak association was observed between the microbial community composition of the latex and the plant-housing environment (Figs. 6, 7), suggesting that it may have a role in shaping the latex microbial ecosystem.

The unexpected microbial diversity in *Euphorbia* plant latex suggests these microbes play unrecognized roles in the host, such as maintenance of health status, and merit further investigation. Our results are a first step toward elucidating the microbial ecology of this highly unusual niche.

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APPENDIX 1 *Euphorbia* taxa sampled. All accessions are from living collections of the Huntington Botanical Gardens, except for one from the Fullerton Arboretum (FA).

Taxon	Subgenus	Section	General distribution	Accession	Germplasm[a]	Propagation	Height (m)[b]	Age (yr)[c]	Container/ ground
Euphorbia alfredii Rauh	Euphorbia	Goniostema	Madagascar	82644	P/W	SD	0.15	17	pot
Euphorbia aphylla Brouss. ex Willd.	Esula	Aphyllis	Spain (Canary Is.: N Gran Canaria, NW Tenerife, N Gomera)	59748	P/W	VEG	1.00	28	ground
Euphorbia heptagona L.	Athymalus	Anthacanthae	South Africa (Western Cape, Eastern Cape)	80442-3	P/Z	VEG	0.61	19	ground
Euphorbia atropurpurea Brouss. ex Willd.	Esula	Aphyllis	Spain (Canary Is.: Tenerife)	81103	P/W	VEG	1.00	28	ground
Euphorbia balsamifera Aiton	Athymalus	Balsamis	Spain (Canary Is.), Northern Africa, Arabian Peninsula	59749	P/W	VEG	2.44	28	ground
Euphorbia cactus Ehrenb. ex Boiss.	Euphorbia	Euphorbia	Eritrea, Ethiopia, Arabian Peninsula	39984	P/W	VEG	0.76	37	pot
Euphorbia capsaintemariensis Rauh	Euphorbia	Goniostema	Madagascar	65362	P/Z	VEG	0.10	50	pot
Euphorbia carteriana P.R.O.Bally	Euphorbia	Euphorbia	Somalia	57827	P/W	VEG	0.46	29	pot
Euphorbia confinalis R.A.Dyer	Euphorbia	Euphorbia	Zimbabwe, Mozambique, South Africa (Limpopo, Mpumalanga)	44919	P/W	VEG	0.91[#]	35	pot
Euphorbia cuneata Vahl	Athymalus	Lyciopsis	Northern and Eastern Africa, Arabian Peninsula	95413	P/Z	VEG	0.16	8	pot
Euphorbia denisii Oudejans	Chamaesyce	Denisiae	Madagascar	77286	SD/Z	VEG	0.33	19	pot
Euphorbia epiphylloides Kurz	Euphorbia	Euphorbia	India (Andaman Is., Nicobar Is.)	108282	P/Z	VEG	0.18	7	pot
Euphorbia esculenta Marloth	Athymalus	Anthanthae	South Africa (Eastern Cape)	100713	P/G	SD	0.10	24	pot
Euphorbia espinosa Pax	Chamaesyce	Espinosae	Kenya to South Africa	55560	SD/W	VEG	1.00	30	pot
Euphorbia gentryi V.W.Steinm. & T.F.Daniel	Chamaesyce	Alectoroctonum	Mexico (Sonora, NW Sinaloa)	82714	P/W	VEG	0.60	17	pot
Euphorbia globosa (Haw.) Sims	Athymalus	Anthacanthae	South Africa (Eastern Cape)	100770	P/G	VEG	0.80	12	pot
Euphorbia hadramautica Baker	Athymalus	Pseudacalypha	NE Tropical Africa, Arabian Peninsula	118315	SD/G	SD	0.05	6	pot
Euphorbia handiensis Burchard	Euphorbia	Euphorbia	Spain (Canary Is.: S. Fuerteventura)	23311	P/W	VEG	0.33	47	pot
Euphorbia lamarckii Sweet	Esula	Aphyllis	Spain (W Canary Is.: Tenerife, NW Gomera, La Palma, Hierro)	80823	SD/W	VEG	1.22	18	ground
Euphorbia leistneri R.H.Archer	Chamaesyce	Frondosae	Namibia	107014	P/Z	SD	0.23	10	pot
Euphorbia loricata Lam.	Athymalus	Anthacanthae	South Africa (Western Cape)	100825	P/G	VEG	0.33	12	pot
Euphorbia mandravioky Leandri	Euphorbia	Pachysanthae	NW Madagascar (Anstiranana)	106471	P/G	VEG	0.13	21	pot
Euphorbia milii Des Moul.	Euphorbia	Goniostema	Madagascar	85657-2	P/Z	VEG	0.33	15	ground
Euphorbia misera Benth.	Chamaesyce	Alectoroctonum	W USA (California, incl. Channel Is.), NW Mexico	90222	P/Z	SD	0.23	12	pot
Euphorbia hamata (Haw.) Sweet	Athymalus	Anthanthae	South Africa (S Cape Prov.), Namibia	65394	P/W	VEG	0.15	26	pot
Euphorbia platyclada Rauh	Chamaesyce	Bosseriae	S Madagascar	28718	P/Z	VEG	0.05	44	pot
Euphorbia royleana Boiss.	Euphorbia	Euphorbia	Himalaya: Bhutan, China, Nepal, Myanmar, Pakistan, India	58351	P/W	VEG	3.05	28	ground
Euphorbia septentrionalis P.R.O.Bally & S.Carter	Euphorbia	Euphorbia	Ethiopia, Kenya, Uganda	82104	P/Z	VEG	0.15	17	pot
Euphorbia socotrana Balf.f.	Athymalus	Somalica	Socotra (Yemen)	69269	P/G	VEG	1.00	28	pot
Euphorbia stenoclada subsp. *stenoclada* Baill.	Euphorbia	Tirucalli	Madagascar, Mozambique Channel Is. (Europa I.)	FA s.n.	P/G	VEG	N/A	4	ground
Euphorbia tanquahuete Sessé & Moç.	Euphorbia	Tanquahueteae	Central-Southern Mexico	85160	P/W	VEG	0.61	16	pot
Euphorbia jansenvillensis Nel	Athymalus	Anthacanthae	South Africa (Eastern Cape)	118316	P/G	VEG	0.15	20	pot
Euphorbia viguieri Denis	Euphorbia	Goniostema	Madagascar	81223	P/Z	VEG	0.33	18	pot
Euphorbia wakefieldii N.E.Br.	Euphorbia	Euphorbia	Kenya	40934	P/W	VEG	0.91	36	pot
Euphorbia waterbergensis R.A.Dyer	Euphorbia	Euphorbia	South Africa (Limpopo Province).	44924	P/W	VEG	0.18	35	pot
Euphorbia weberbaueri Mansf.	Euphorbia	Euphorbiastrum	Peru, Ecuador	57183	P/W	VEG	0.61	40	pot
Euphorbia xylophylloides Brongn. ex Lem.	Euphorbia	Tirucalli	Madagascar	57774	P/W	VEG	0.91	30	pot
Euphorbia sp. (Madagascar)	Euphorbia	Denisophorbia	Madagascar	97495	P/Z	SD	0.61	8	pot

[a] Germplasm received as, or provenance type: SD, seed; P, plant; W, wild collected; Z, grown in cultivation, from known provenance (i.e., one generation removed from the wild); G, plant from cultivation, origin unknown.
[b] Measured from soil to highest shoot.
[c] Measured as years in cultivation at the Huntington Botanical Gardens.