Supporting Online Material for

Bacterial Diversity in Tree Canopies of the Atlantic Forest

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Materials and Methods

Sampling location. Samples were collected from mature trees within a semideciduous forest stand within the Atlantic Forest at the Estação Ecológica dos Caetetus (between 22°41'and 22°46'S and 49°10' and 49°16'W), Gália, São Paulo State, Brazil. The experimental site is in a semideciduous mesophytic forest ecotone, which contains 118 different tree species in 85 genera and 45 families, and has been described in relation to its climate and biogeography (http://www.lerf.esalq.usp.br/parcelas/caetetus.html). The sampled trees were located in a preserved experimental parcel (10 hectare) that was established as part of the Biota Program (FAPESP, Brazil).

Leaf sampling. Samples of mature leaves were collected from three individual trees of 9 different species at different locations in the forest (Table S1). To collect the leaves, a 10 m extended pole clipper was used to cut small branches that were thereafter handled only by the branch stems to avoid contamination of the leaves. Leaf samples were collected from the lower shaded, interior part of the canopy from mature trees which ranged in height from 10 to 30 meters tall. Detailed descriptions of the tree species are provided online at http://www.lerf.esalq.usp.br/matrizes/banco.html. The leaves were removed from the felled branches using hand shears to clip individual leaves directly into 4 L plastic bags. Samples were maintained on ice during transportation and immediately processed to extract the bacteria from the leaf surface.

Bacteria isolation. Using axenic procedures, 12 g of leaves (fresh weight) from individual trees were placed into 50 mL polypropylene tubes containing washing solution (0.1 M potassium phosphate buffer, pH 7.0) and sonicated for 10 min at 22.5 kHz in an ultrasonic cell disrupter (Misonix Inc., Atlantic Beach, NY, USA) (*6*). The resulting bacterial suspension was centrifuged at 30,000 *g* for 15 min at 4°C. The pellet was resuspended in 1.5 mL of washing solution and further concentrated by centrifugation at 30,000 *g* for 15 min at 4°C. Bacteria were resuspended in 200 µL of washing solution and frozen at -20°C until processing for DNA extraction.

DNA extraction. Metagenomic DNA was extracted from 100 µL of the bacterial washings using the Fast DNA kit (Qbiogene, Irvine, CA, USA), according to the manufacturer's instructions. DNA integrity was determined by electrophoresis in 0.5X TBE-0.8% agarose gels, after staining with Vistra Green (Amersham Biociences, São Paulo, Brazil). DNA concentration was determined by densitometry, using the Low DNA Mass Ladder (Invitrogen, São Paulo, Brazil), as standard, and the program Fragment Analyses (Amersham Biosciences, São Paulo, Brazil).

PCR-DGGE analyses. Bacterial community structures on different trees were initially surveyed using a low resolution method that separates out 16S rRNA gene sequences based on differences in their GC content. The V3 regions of the 16S rRNA genes were amplified by polymerase chain reaction (PCR) using the metagenomic DNA extracted as template

and primers BA338fGC and UN518r (*S1*). Amplification was performed in 1X Taq Platinum DNA polymerase buffer containing 0.2 mM dNTPs, 3 mM $MgCl_2$, 1 U Taq Platinum DNA polymerase (Invitrogen, São Paulo, Brazil); 5 pmol of each primer and 10 ng of metagenomic DNA. PCR amplification conditions were 5 min at 95°C; 30 cycles 1 min at 95°C, 1 min at 55°C and 1 min at 72°C, and final extension for 10 min at 72°C. The concentration of PCR products (amplicons) was determined by densitometry, after electrophoresis in 0.5X TBE-0.8% agarose gels and staining with Vistra Green (Amersham Biociences, São Paulo, Brazil), using the Low DNA Mass Ladder (Invitrogen, São Paulo, Brazil), as standard, a FluorImager laser densitometer and the program Fragment Analyses (Amersham Biosciences, São Paulo, Brazil).

Amplicons (300 ng) were analyzed by denaturing gradient gel electrophoresis (DGGE) using 8% (w/v) acrylamide:bisacrylamide (37.5:1, m:m) gels containing a 15 to 55% linear gradient of formamide and urea (100% denaturing solution contained 40% formamide and 7 M urea) (*S1*). Electrophoreses were performed at 200V constant and 60°C, using a DCode System (BioRad, Hercules, CA, USA), in 1X TAE buffer. Gels were stained with Vistra Green (Amersham Biosciences, São Paulo, Brazil) and analyzed by densitometry, using a FluorImager laser densitometer and the program Diversity Database (BioRad, Hercules, CA, USA). Two gels were cast and run simultaneously to compare the communities under uniform electrophoresis conditions. Each gel contained 3 marker lanes that were run with a standard mixture of DNA to allow for alignments between gels.

Statistical analyses of the 16S rRNA gene profiles were conducted to compare the similarities in the community compositions within and between tree species (*5*). The DNA band patterns were analyzed to identify specific bands according to their R_f (relative front) values and pixel intensities. The resulting band profiles were analyzed using linear discriminant analysis and cluster analysis with the software MINITAB version 14 (Minitab Inc., State College, PA, USA). For discriminant analysis, the DNA band data were pruned to discard bands that comprised less than 2% of the total pixel intensity, or that appeared only once across the sample set. The excel data sheet containing the raw data and summary output of the statistical analyses is provided as a supplementary file.

16S rRNA gene cloning and sequencing. The V1-V3 regions of the 16S rRNA genes were amplified by PCR using the metagenomic DNA extracted as template and primers UN518R (*S1*) and BA63F (*S2*). Amplification was performed in 1X Taq Platinum DNA polymerase buffer containing 0.2 mM dNTPs, 3 mM $MgCl_2$, 1 U Taq Platinum DNA polymerase (Invitrogen, São Paulo, Brazil); 5 pmol of each primer and 10 ng of metagenomic DNA. PCR amplification conditions were 5 min at 95°C; 30 cycles 1 min at 95°C, 1 min at 55°C and 1 min at 72°C, and final extension for 10 min at 72°C.

Amplicons were purified using GFX PCR DNA and Gel Band Purification kit (Amersham Biosciences, São Paulo, Brazil) and ligated to pGEM-T Easy Vector (Promega, Madison, WI, USA) at 4^oC overnight, according to the manufacturer's instructions. The ligation products were transformed into *E. coli* DH5α competent cells by heat shock and transformed cells plated on LB-agar, containing ampicilin (100μg mL^{-1}), X-Gal (5-brome-4-chloro-3-indolil-β-D-galactoside). Colonies containing recombinant plasmids were selected and cells were grown in liquid LB medium containing 100 μg ampicilin mL^{-1} at

37°C overnight.

Plasmids were extracted through alkaline lysis. Nucleic acids solutions were incubated with 15 μg RNase A for 20 min at 37°C. DNA was precipitated with equal volume of cold isopropanol for 5 min on ice and centrifugation at 4,000 *g* for 40 min at 4°C. DNA pellet was washed with 70% cold ethanol, dried and suspended in ddH_2O. DNA concentration was determined spectrophotometrically at 260 nm.

Sequencing was performed using 200-500 ng of plasmid DNA, 10 pmol of T7 or SP6 primers, 2 μL of DYEmanic ET Terminator (Amersham Biosciences, São Paulo, Brazil), 2 μL of buffer (200 mM Tris-HCl pH 9.0 and 5 mM $MgCl_2.6H_2O$) and ultrapure H_2O to a final volume of 10 μL, in 25 cycles of 20 sec at 95°C, 15 sec at 50°C and 1 min at 60°C. PCR products were precipitated with ethanol, dried and resuspended in deionized formamide. Electrophoresis was performed using an ABI 3100 automatic sequencer, according to the manufacturer's instructions (Applied Biosystems, São Paulo, Brazil).

Sequence analyses and OTU definition. A total of 109, 153 and 166 clones of the V1-V3 region of the 16S rDNA from bacterial communities of the phyllosphere of *Trichillia catigua*, *Trichillia clausenii* and *Campomanesia xanthocarpa* were analyzed. Nucleotide sequences (reads) were trimmed for the removal of low quality bases (quality parameter > 20, i.e. less than one error in 100 nucleotides) and vector sequences using Phred program (*S3*). Valid sequences were then clustered into operational taxonomic units (OTUs) using the program DOTUR and cut-off evolutionary distances of 0.01 or 0.03 (*S4*). Jukes-Cantor evolutionary distances were calculated using DNADIST of the PHYLIP 3.63 Package (J. Felsenstein; http://evolution.genetics.washington.edu/phylip.html), after aligning the sequences using ClustalX 1.83 (*S5*) under the default parameters, except gap-opening penalty of 10.0 and gap-extension penalty of 0.1 for pairwise and multiple alignments. The number of sequences for each OTU was computed and used for diversity estimations. Valid sequences were compared to sequences in the Ribosomal Database Project II for taxonomic affiliation, using the program RDPquery (*S6*).

Diversity indices. The estimated minimum number of OTUs in the samples was determined through ACE and Chao1 non-parametric estimators, using SPADE (Chao & Chen; http://chao.stat.nthu.edu.tw). SPADE was also used for the determination of Shannon's and the reciprocal of Simpson's indices (maximum likelihood estimators) and estimated sample coverage.

Coverage comparisons. Homologous and heterologous coverage comparisons were performed using the program LIBSHUFF 1.22 (*S7*) after aligning the sequences using ClustalX 1.83 (*S5*) under the default parameters, except gap-opening penalty of 10.0 and gap-extension penalty of 0.1 for pairwise and multiple alignments, and calculating Jukes-Cantor evolutionary distances using DNADIST of the PHYLIP 3.63 Package.

Supplementary Text

Bacterial cells are found at an average density of 10^6-10^7 cells cm^{-2} on plants from

temperate regions (*2*), and may be even higher on tropical plants where dense canopies are conducive for bacterial growth. Considering that the estimated leaf area of terrestrial plants is 6.4×10^8 km^2 (*S8*), the number of bacterial cells on leaf surfaces globally is as high as 10^{26} cells. For some time it has been presumed that this harsh environment supports a relatively low bacterial diversity and that those bacteria that colonize plant leaves are transported in wind-borne dust and are broadly distributed. However, there are many conditions that may lead to ecological selection for different microbial communities on leaves from different plant species (*8*). Prior surveys have mainly described bacteria that are associated with agricultural plants and have used culture based methods that typically reveal only a few dozen species of bacteria on any one plant. More recent studies using molecular methods have shown microbial diversity on the leaves of agricultural plants is actually much greater and suggest that different plants harbor distinct bacterial communities (*6*). The discovery of high bacterial diversity for agricultural plants has prompted many questions about the true extent of microbial diversity that is associated with plant canopies in natural ecosystems.

Sequence data obtained from molecular analysis of the microbial communities were used to compare the genetic diversity in the phyllospheres and to identify the predominant taxonomic groups that were present on leaf surfaces. Two lines of evidence were used to ascertain between tree species and within species variation in microbial community composition. The 16S rRNA gene profiles generated by the low resolution DNA fingerprinting method, PCR-DGGE, suggest that there was considerable variation in bacterial community species composition both within and among the 9 tree species studied (Fig. S1). Discriminant analysis of the DNA band profiles for replicates of bacterial communities yielded consistent groups corresponding to tree species (Table S2). Such groupings support the hypothesis that within tree species variation is lower than between tree species, but such data must be viewed cautiously as DNA bands located at the same gel migration distance may not necessarily represent the same bacterial taxa.

Evidence for high variation between tree species is derived from statistical analysis of the clone libraries that were generated for the three tree species using the program LIBSHUFF (Fig. S2). LIBSHUFF analyses use the approximation form of the Cramér-von Mises statistics to compare homologous and heterologous coverage curves. Results of the analyses indicated that the clone sample sizes were sufficient to obtain good coverage of the bacterial species composition for each tree (67-81%), and that the clone libraries from the three bacterial phyllosphere communities were significantly different ($P = 0.001$). Using RDPquery (*S6*) to assign a phylogenetic affiliation at different taxonomic levels to the 16S rRNA gene sequences, we observed that a majority of the bacteria were from the Proteobacteria (86, 73 and 48% in *T. catigua*, *T. clausenii* and *C. xanthocarpa*, respectively; Table S4). Within this phylum, bacteria of the gamma-Proteobacteria class (*Enterobacteriales* and *Pseudomonadales*) were predominant in the phyllospheres of *T. catigua* and *T. clausenii*, whereas alpha-Proteobacteria (Sphingomonadales) were predominant in the phyllosphere of *C. xanthocarpa*. The taxons identified with the different tree species are described in detail in Table S4.

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Table S1. Tree species at Estação Ecólogica dos Caetetus (Gália, São Paulo, Brazil) selected for analysis of phyllosphere bacterial community structures.

Species	**Family**	**Order**
Aspidosperma polyneuron Müll. Arg.	Apocynaceae	Gentianales
Campomanesia xanthocarpa O. Berg	Myrtaceae	Myrtales
Holocalyx balansae Micheli	Fabaceae	Fabales
Ocotea indecora Schott ex Meisn.	Lauraceae	Laurales
Seguieria floribunda Benth.	Phytolaccaceae	Caryophyllales
Trichilia catigua A.Juss.	Meliaceae	Sapindales
Trichilia clausenii C.DC.	Meliaceae	Sapindales
Trichilia pallida Sw.	Meliaceae	Sapindales
Urera baccifera (L.) Gaudich.	Urticaceae	Rosales

Table S2. Discriminant analysis of bacterial communities associated with different tree species.

	Control	*C. xanthocarpa*	*H. balansae*	*O. indecora*	*S. floribunda*	*A. polyneuron*	*T. pallida*	*T. catigua*	*T. clauseni*	*U. baccifera*
Control	6	0	0	0	0	0	0	0	0	0
C. xanothocarpa	0	3	0	0	0	0	0	0	0	0
Holocalyx balannsae	0	0	3	0	0	0	0	0	0	0
Ocotea indecora	0	0	0	3	0	0	0	0	0	0
Sequiera floribunda	0	0	0	0	3	0	0	0	0	0
A. polyneuron	0	0	0	0	0	3	0	0	0	0
Trichillia pallida	0	0	0	0	0	0	3	0	0	0
Trichillia catigua	0	0	0	0	0	0	0	2	0	0
Trichillia clauseni	0	0	0	0	0	0	0	0	2	0
Urera baccifera	0	0	0	0	0	0	0	0	0	2
Total N	6	3	3	3	3	3	3	2	2	2
N correct	6	3	3	3	3	3	3	2	2	2

Table S3. Estimated diversity indices for bacterial communities in the phyllosphere of *T. catigua*, *T. clausenii* and *C. xanthocarpa*.

Community	NDO	Estimated OTUs		Shannon[a]	1/D[b]	ESC
		ACE	Chao1			
D = 0.01						
T. catigua	58	172 (109, 312)	152 (95, 292)	3.74 (3.58, 3.89)	31.0 (25.1, 40.5)	0.642
T. clausenii	89	253 (178, 390)	221 (154, 357)	4.20 (4.06, 4.33)	46.2 (36.5, 62.8)	0.588
C. xanthocarpa	101	417 (265, 708)	378 (235, 671)	4.28 (4.14, 4.42)	44.1 (31.8, 72.1)	0.527
D = 0.03						
T. catigua	40	95 (60, 190)	84 (54, 180)	3.36 (3.21, 3.50)	22.5 (18.0, 29.8)	0.807
T. clausenii	68	167 (117, 266)	145 (102, 242)	3.79 (3.64, 3.95)	29.2 (23.5, 38.7)	0.719
C. xanthocarpa	83	200 (147, 296)	172 (127, 265)	3.99 (3.83, 4.15)	32.1 (25.0, 45.1)	0,667

[a]Maximum likelihood estimator. [b]Reciprocal of Simpson's index, maximum likelihood estimator. NDO, Number of distinct OTUs; ESC, Estimated sample coverage. Values in parentheses represent the 95% confidence intervals.

Table S4. Frequencies (%) of different bacterial taxons in the phyllosphere of *T. catigua* (Tca), *T. clausenii* (Tcl) and *C. xanthocarpa* (Cxa).

PHYLUM	CLASS	ORDER	FAMILY	GENUS	Tca	Tcl	Cxa
Actinobacteria	Actinobacteria	Actinomycetales	Mycobacteriaceae	Mycobacterium	0.0	0.0	0.6
			Unknown	Unknown	0.0	0.0	0.6
Bacteroidetes	Bacteroidetes	Unknown	Unknown	Unknown	0.0	1.3	0.0
Bacteroidetes	Flavobacteria	Flavobacteriales	Flavobacteriaceae	Chryseobacterium	3.7	0.0	1.2
				Flavobacterium	0.9	1.3	0.0
				Unknown	3.7	0.7	0.6
Bacteroidetes	Sphingobacteria	Sphingobacteriales	Flexibacteraceae	Unknown	0.0	0.7	0.0
Bacteroidetes	Sphingobacteria	Sphingobacteriales	Sphingobacteriaceae	Pedobacter	0.0	3.3	1.2
				Sphingobacterium	0.0	0.0	0.6
				Unknown	0.0	9.2	9.7
Bacteroidetes	Sphingobacteria	Sphingobacteriales	Unknown	Unknown	0.9	1.3	0.6
Bacteroidetes	Sphingobacteria	Unknown	Unknown	Unknown	2.8	5.2	6.1
Bacteroidetes	Unknown	Unknown	Unknown	Unknown	0.9	0.7	0.6
Cyanobacteria	Cyanobacteria	Deferribacterales	Unknown	Unknown	0.0	0.0	10.9
Cyanobacteria	Cyanobacteria	Unknown	Unknown	Unknown	0.0	0.0	3.0
Cyanobacteria	Unknown	Unknown	Unknown	Unknown	0.0	0.0	0.6
Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus	0.0	0.0	8.5
Firmicutes	Bacilli	Bacillales	Staphylococcaceae	Staphylococcus	0.0	0.0	3.6
Firmicutes	Bacilli	Unknown	Unknown	Unknown	0.0	0.0	1.8
Proteobacteria	Alphaproteobacteria	Rhizobiales	Beijerinckiaceae	Beijerinckia	0.0	0.0	0.6
				Unknown	0.9	0.0	0.0
Proteobacteria	Alphaproteobacteria	Rhizobiales	Brucellaceae	Ochrobactrum	0.9	0.0	0.0
Proteobacteria	Alphaproteobacteria	Rhizobiales	Methylobacteriaceae	Methylobacterium	1.8	0.0	1.8
				Unknown	0.0	1.2	0.0
Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	Unknown	0.9	0.0	0.0
				Rhizobium	0.0	0.7	0.6
Proteobacteria	Alphaproteobacteria	Rhizobiales	Unknown	Unknown	0.0	0.0	0.6
Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Sphingomonas	3.7	4.6	20.6
				Sphingopyxis	0.9	0.0	0.0
				Novosphingobium	0.0	0.0	0.6
				Sphingobium	0.0	0.0	0.6
				Unknown	0.0	0.0	1.8
Proteobacteria	Alphaproteobacteria	Unknown	Unknown	Unknown	0.9	1.3	4.8
Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Acidovorax	0.9	0.0	0.0
				Variovorax	0.0	0.7	0.0

				Delftia	0.0	0.0	0.6
Proteobacteria	Betaproteobacteria	Burkholderiales	Incertae sedis	Xylophilus	0.0	0.0	0.6
Proteobacter	Betaproteobacteria	Burkholderiales	Unknown	Unknown	0.0	0.7	0.0
Proteobacter	Betaproteobacteria	Unknown	Unknown	Unknown	0.0	0.0	1.2
Proteobacteria	Deltaproteobacteria	Myxococcales	Cystobacteraceae	Cystobacter	0.0	0.0	0.6
Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Pantoea	5.5	0.7	0.0
				Enterobacter	12.8	9.2	0.0
				Raoultella	0.0	3.9	0.0
				Erwinia	8.3	10.5	0.0
				Salmonella	4.6	0.0	0.0
				Citrobacter	1.8	0.7	0.0
				Klebsiella	0.0	1.3	0.0
				Kluyvera	0.0	1.3	0.0
				Yersinia	0.0	0.7	0.0
				Unknown	2.8	2.6	0.6
Proteobacteria	Gammaproteobacteria	Enterobacteriales	Unknown	Unknown	0.0	0.7	0.6
Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	Acinetobacter	30.3	1.3	0.0
				Unknown	0.0	0.0	0.6
Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	7.3	19.6	2.4
				Unknown	0.0	3.9	0.0
Proteobacteria	Gammaproteobacteria	Pseudomonadales	Unknown	Unknown	0.0	0.7	0.0
Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	Stenotrophomonas	1.8	2.6	0.0
				Unknown	0.0	0.7	3.6
Proteobacteria	Gammaproteobacteria	Unknown	Unknown	Unknown	0.0	3.3	3.6
Proteobacteria	Unknown	Unknown	Unknown	Unknown	0.0	1.3	0.0
Unknown	Unknown	Unknown	Unknown	Unknown	0.9	3.3	1.8

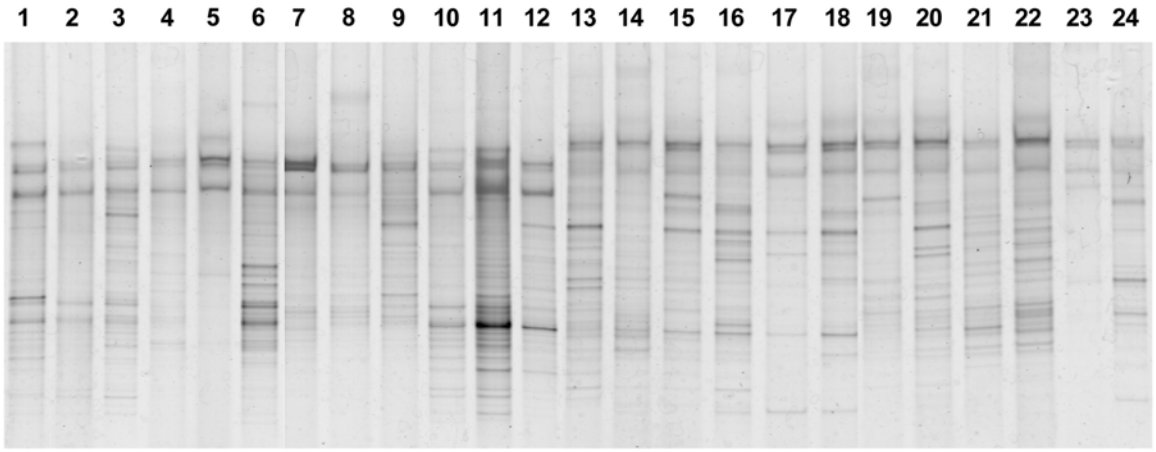

Fig. S1. Bacterial communities associated with the leaves of nine forest tree species from Estação Ecológica dos Caetetus (SP, Brazil) as determined by PCR-DGGE of 16S rRNA genes. Lane headings denote samples as follows: 1-3, *Campomanesia xanthocarpa*; 4-6, *Holocalix balansae*; 7-9, *Ocotea indecora*; 10-12, *Seguieria floribunda*; 13-15, *Aspidosperma polyneuron*; 16-18, *Trichilia pallida*; 19-20, *Trichilia catigua*, 21-22, *Trichilia clausenii*; 23-24, *Urera baccifera*.

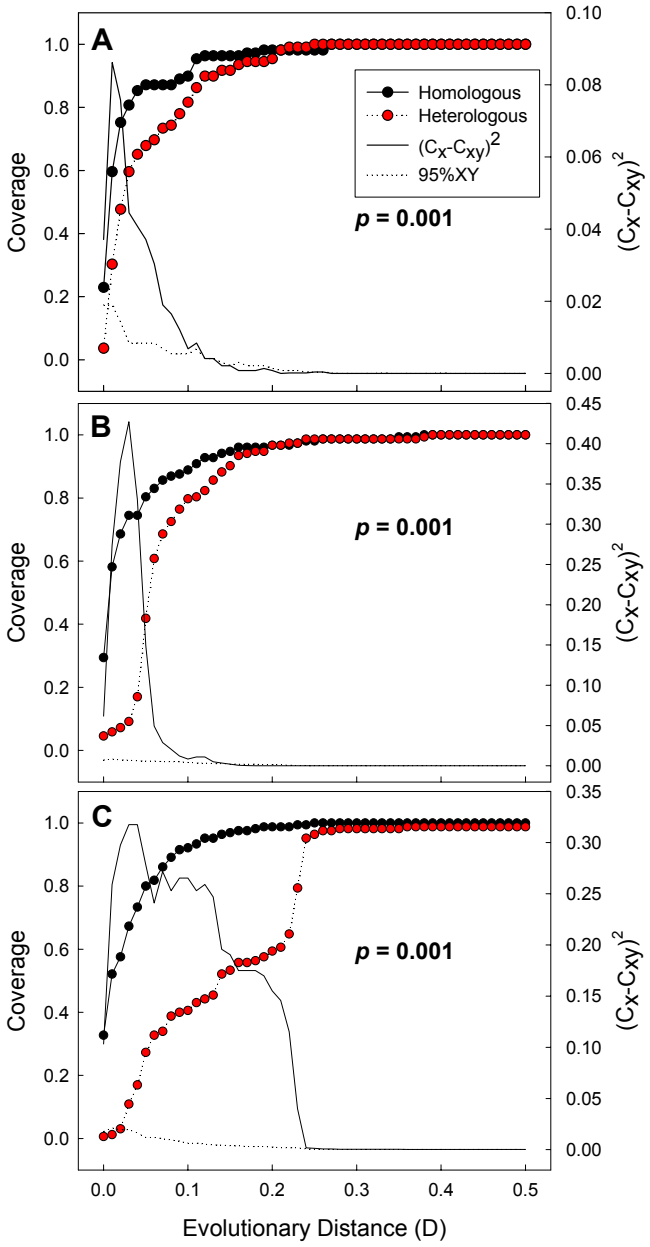

Fig. S2. LIBSHUFF analysis of the bacterial communities of in the phyllosphere of *T. catigua, T. clausenii* and *C. xanthocarpa*. **A**, *T. catigua* (homologous) x *T. clausenii*; **B**, *T. clausenii* (homologous) x *C. xanthocarpa*; **C**, *C. xanthocarpa* (homologous) x. *T. catigua* Communities are significantly different at $P = 0.001$. The distribution of $(C_x\text{-}C_{xy})^2$ as a function of D indicates that the bacterial communities of the phyllosphere of *T. catigua* and *T. clausenii*, *T. clausenii* and *C. xanthocarpa* and *C. xanthocarpa* and *T. catigua* differ mostly at $D < 0.12$, $D < 0.15$ and $D < 0.36$, respectively.