



**Revealing the Dynamics of the Microbiome  
Using Bioinformatic Approaches**

**Filipa Godoy-Vitorino, Ph.D.**  
Assistant Professor and Researcher  
PI Microbial Ecology and Genomics Lab  
Department of Natural Sciences  
Inter American University of Puerto Rico - Metropolitan Campus

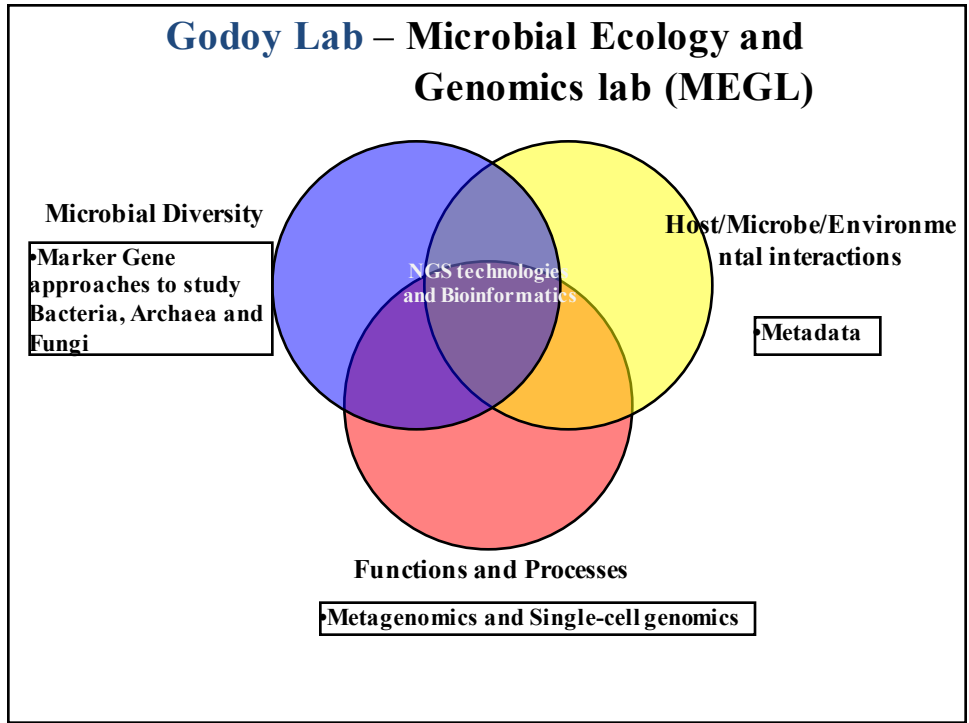


TIDES: UPR-Humacao  
May 5 2015



## TALK OUTLINE

- Microbial Ecology- Definition and Brief History
- What is Microbiome Research?
- NGS Applications to the study of Microbial Communities (Profiling)
- Metagenomics
- The Hoatzin: A Single-cell metagenome project
- Three new Microbiome Research Projects in MEGL:
  - *Tabebuia heterophylla* rhizosphere microbial communities
  - Black and Red Mangrove microbiome
  - HPV and cervicovaginal microbiota



## Main IAUPR Projects

**Collaborators:**  
 Susannah Tringe, DOE-JGI  
 Eugenio Santiago, UPR-PP

**Microbial community contributions to stress adaptation and plasticity in the tropical tree *Tabebuia heterophylla***  
**SEQUENCING FUNDED BY DOE**

**Collaborators:**  
 Janet Jansson, LBNL/DOE-JBEI  
 Matias Cafaro, UPR-RUM  
 Ernesto Torres, UIPR-CECIA

**Bioprospecting for the lignocellulolytic capabilities of the Mangrove ecosystems in Puerto Rico**  
**SEQUENCING FUNDED BY DOE**

**Collaborators:**  
 Josefina Romaguera, UPR-RCM  
 Martin Blaser, NYU  
 Larry Forney, Univ. Idaho  
 Rob Knight, U Colorado  
 Maria Dominguez, NYU/UPR

**Relating the genital microbiota to HPV infections in latin women**  
**SEQUENCING HMP-NYU**



## Other Collaborative Projects

  <p><b>Collaborators:</b>          Maria G. Dominguez, NYU/UPR          Maria A. Garcia Amado, IVIC</p> <p><b>The Hoatzin Microbiome</b></p> <ul style="list-style-type: none"> <li>• Fiber and epithelial metagenome</li> <li>• single genomics of a ciliate cell</li> <li>• fungal community structure</li> <li>• Characterization of crop isolate collection</li> <li>• Whole genome sequencing of 3 crop isolates</li> </ul>	 <p><b>Collaborators:</b>          PI: Pedro Santos, Univ do Minho - Braga          Co-PI's Univ Aveiro, PT</p> <p><b>Cetaceans as marine ecosystem health sentinels</b>          Funded by the FCT (EU)</p>	 <p><b>The microbiome of oropharyngeal squamous cell carcinoma</b>          With John Hopkins Univ</p>
 <p><b>Collaborators:</b>          PI: <a href="#">Rodrigo Costa</a>, U. Algarve, PT          Co-PI: Ulisses Nunes, LBL          Co-PI: Joerg Overmann, Leibniz Institute, Germany          Co-PI: Cymon Cox, Duke Univ</p> <p><b>Uncovering the life-style of Acidobacteria symbionts of marine sponges</b>          Funded by the FCT (EU)</p>	 <p><b>Phylogenomics of Prevotella among different ecosystems</b></p>	




## Research Group

Room 432 – 4<sup>th</sup> Floor IAUPR-MC











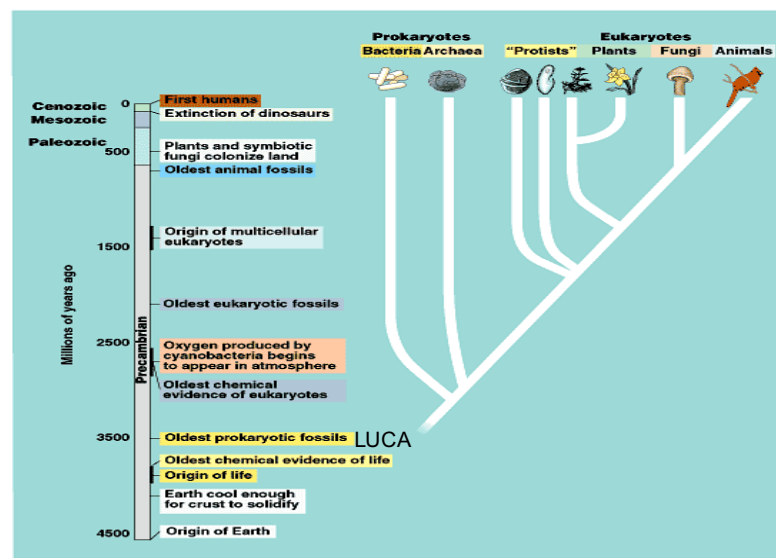
## MEGL IS FOCUSED ON

- **Microbial ecology and evolution of host-associated ecosystems (invertebrates, vertebrates, humans, plants)**

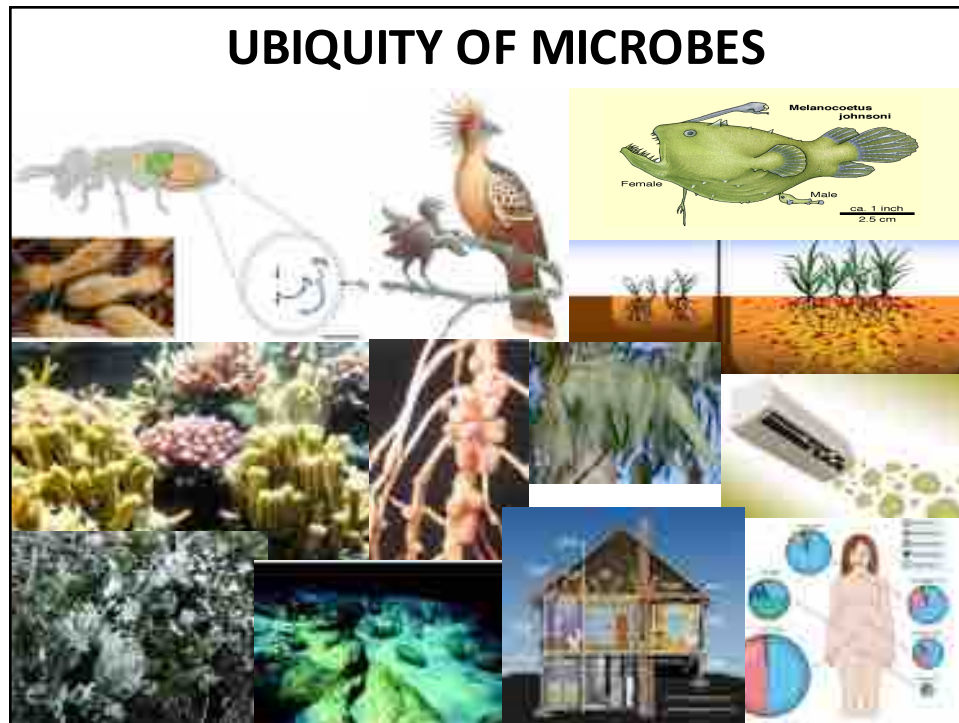
**Approaches:** culture-independent molecular methods to characterize microbial communities including marker gene and shotgun (metagenomic) approaches



## Microbes are the oldest organisms on earth







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## A brief historical summary on Microbial Ecology

**Sergei Winogradsky** (Russia, 1856 – 1953)

- Founder of the study of microbial diversity through his columnar experiments
- worked with soil bacteria and discovered that they could oxidize iron, sulfur, and ammonia to obtain energy;

**Martinus Beijerinck** (The Netherlands, 1851-1931)

- isolated aerobic nitrogen-fixing soil bacteria (*Azotobacter* and *Rhizobium*) and sulfate reducing Bacteria

**Beijerinck and Winogradsky**

- pioneered the use of enrichment cultures and selective media



**Albert Jan Kluyver** (The Netherlands, 1888-1956)

- First to describe the metabolic capacity of microbes to adapt to changing environments in “*The Microbe's Contribution to Biology*”

**Robert E. Hungate** (USA, 1906– 2004)

- descendent from the Delft school of microbiology (Van Niel's first American Ph.D. student. Pioneer of Anaerobic Microbial Ecology and father of Rumen Microbiology -hungate tubes)

## Other important scientists that impacted Microbial Ecology

- Carl Woese (USA, July 15, 1928 -December 30, 2012)
- Proposed that life should be classified in **3** domains and discovered Archaea
- Used **ribosomal RNA** sequence as an evolutionary measure
- **Reconstructed the phylogeny** of Bacteria and Archaea, providing a phylogenetically valid system of classification for prokaryotes.





### Other important scientists that impacted Microbial Ecology

- Norman Pace (USA, 1942 - )
- Used PCR to explore the diversity of ribosomal RNA sequences.
- Propose the idea of cloning DNA directly from environmental samples as early as 1985 and cloned DNA from an environmental sample in 1991
  - Some of his disciples are Edward DeLong, Ruth Ley or Phil Hugenholtz



### Other important scientists that impacted Microbial Ecology

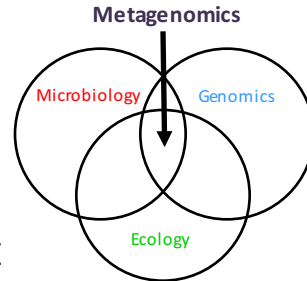
- Philip Hugenholtz (Australia, 1964?-)
- Defined candidate divisions in 1998 finding seqs with less than 85% similarity
- Hugenholtz and Pace have independently named over a dozen phylum-level lineages
- Contributed to the development of Greengenes and continues as a bacterial taxonomy curator





## Other important scientists that impacted Microbial Ecology

- Jo Handelsman (USA, Yale)
- WHITE HOUSE 2013 -Associate Director for Science, Office of Science
- Creator of the definition of METAGENOME
- First usage of the term: "...Methodology has been made possible by advances in molecular biology and eukaryotic genomics, which have laid the groundwork for cloning & functional analysis of the collective genomes of soil microflora, which we term the **metagenome** of the soil."
  - *Handelsman (1998) Chemistry & Biology 5:246*

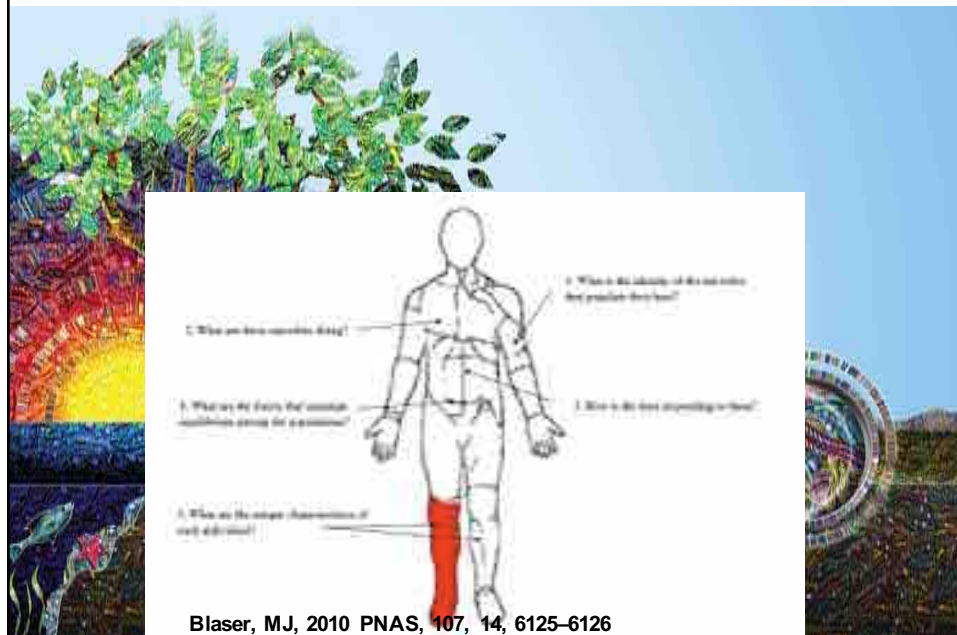


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## Microbes are everywhere



## MICROBIOME

The use of DNA sequencing to study microbial communities present in a given ecosystem

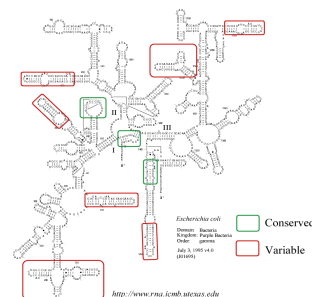
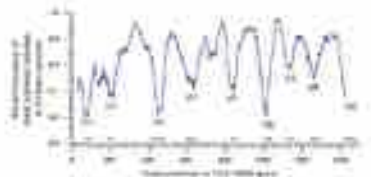
## MICROBIAL ECOLOGY

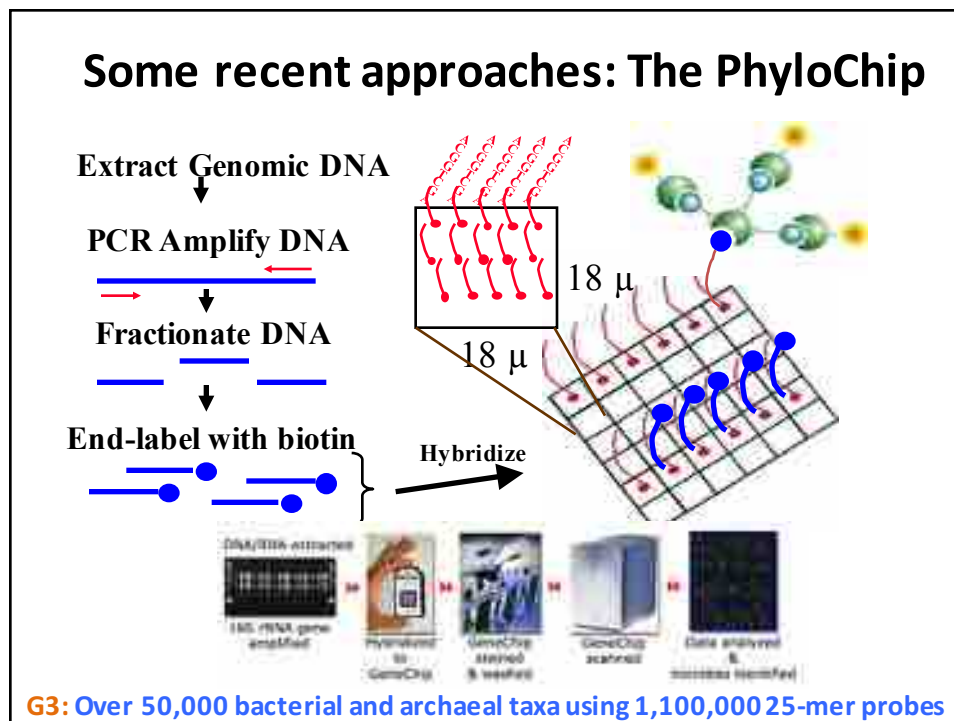
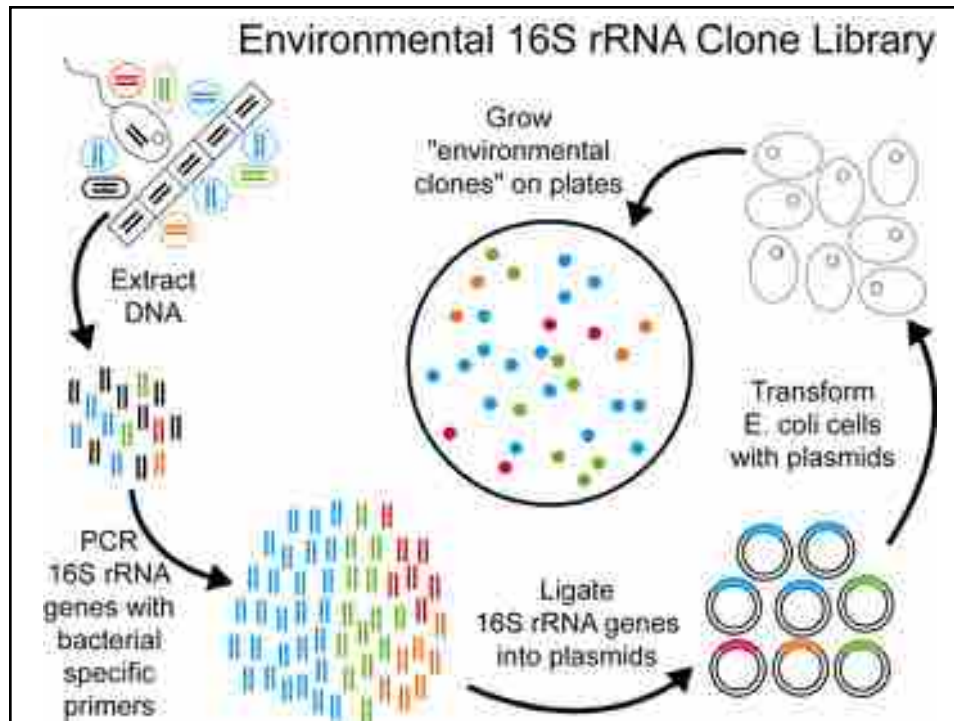
- **Who** is “there”?
- **What** are they doing “there”?
- **How much** are they doing “there”?



## The Star of the Show: SSU rRNA

- Everybody has it
- Contains both highly conserved and variable regions
  - allows making comparisons between different organisms over long periods of time (evolutionary history)
- Not laterally transferred between organisms
- HUGE and growing database

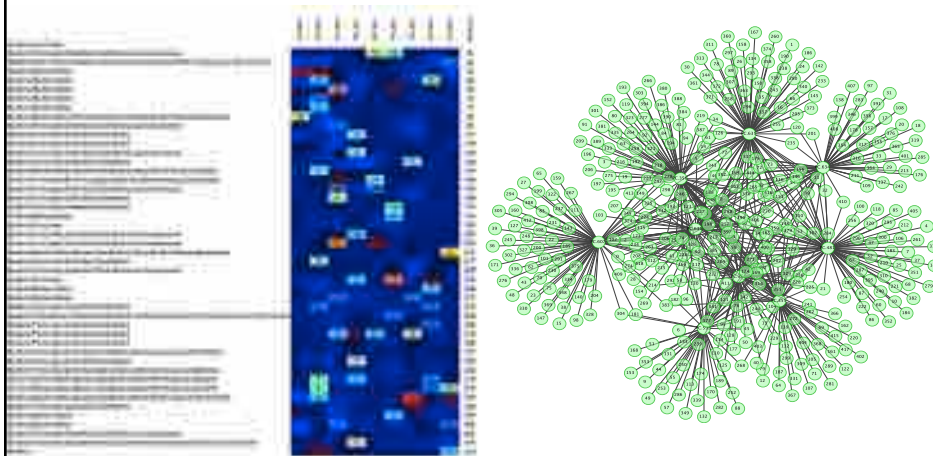




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## NGS Applications to the study of Microbial Communities (Profiling)



Who is there?

## Sequencing then and now: close your eyes and classify!



.. but it takes intense effort and time..

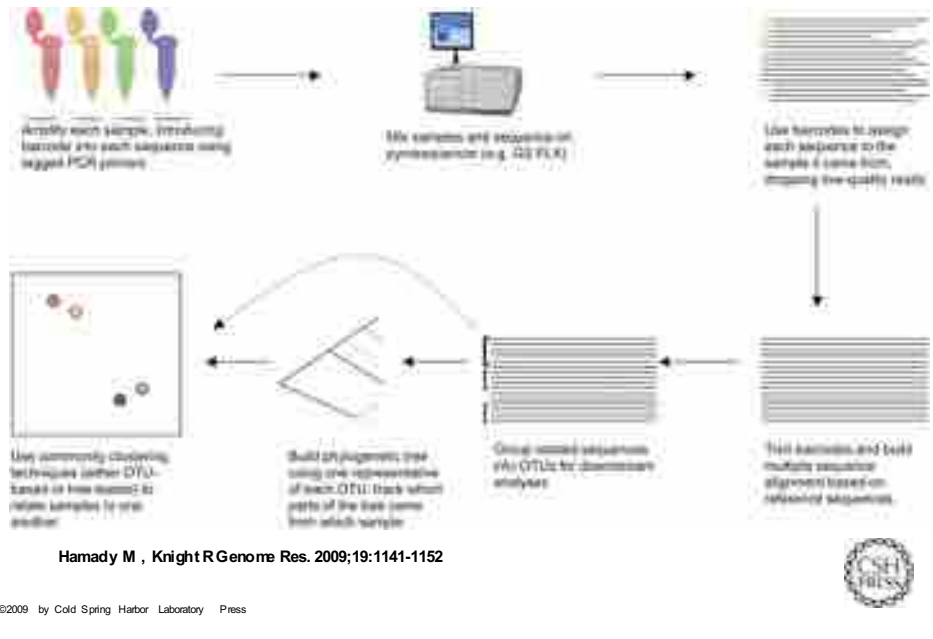


How to make an inventory...with mixed products???



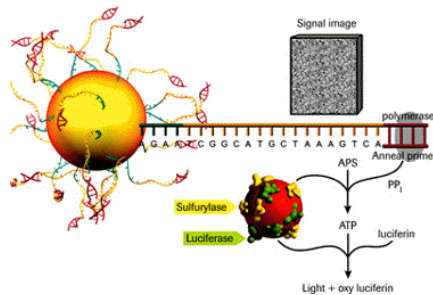


# THE BARCODE STRATEGY



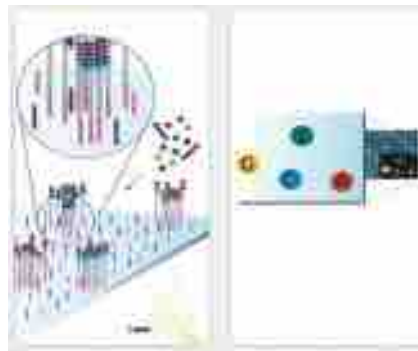
# Next generation sequencing (NGS)

454



0.5M 450-600 bp reads  
\$\$

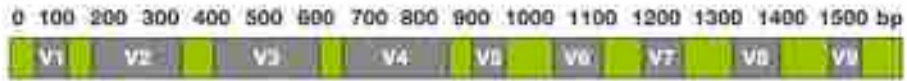
Illumina



10-400M 76-150bp reads/lane  
\$

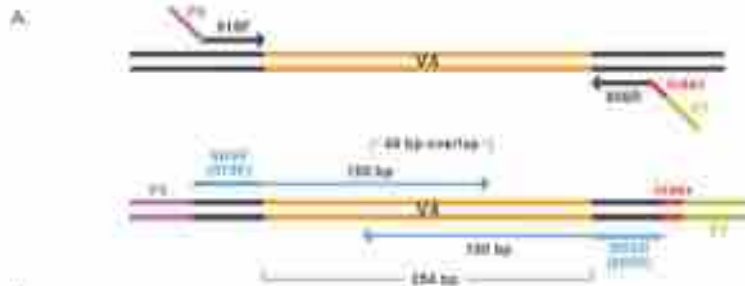


## Preparing Illumina barcodes (PE)



**CONSERVED REGIONS:** unspecific applications

**VARIABLE REGIONS:** group or species-specific applications



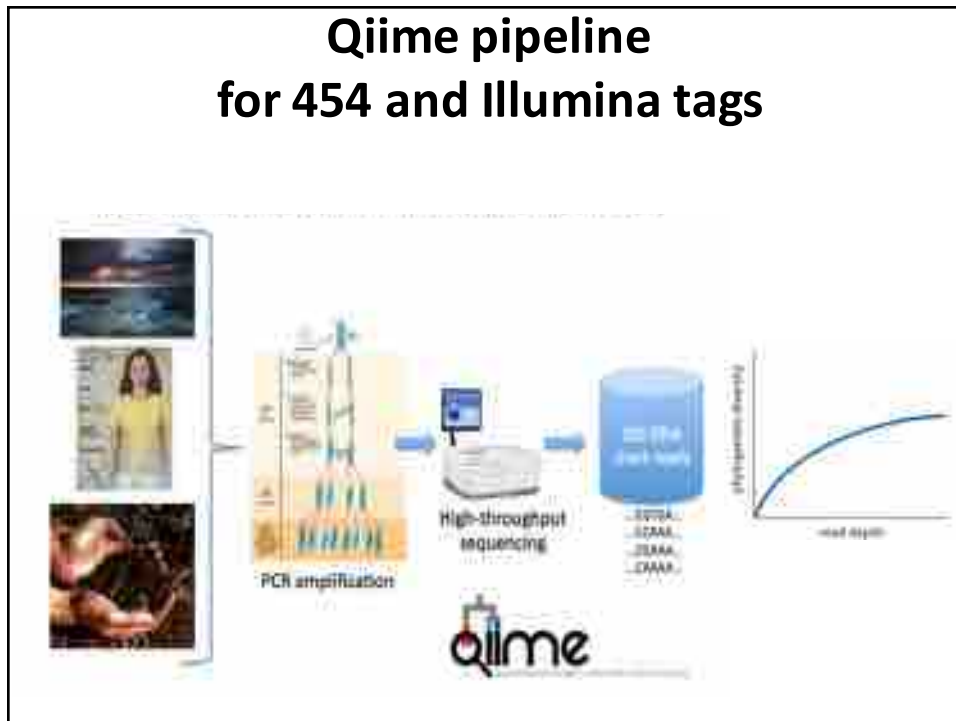
P5: 5' AAT GAT ACG GCG ACC ACC GA 3'

P7: 5' CAA GCA GAA GAC GGC ATA CGA 3'

The collage illustrates the workflow from sample preparation to data analysis. It includes a pipette, a sequencing machine, a petri dish, a sequence alignment, a pie chart, and a phylogenetic tree.

- From Sample Prep, sequencing (single end or paired-end) to Data Analyses

## Qiime pipeline for 454 and Illumina tags



## DATABASE FOR PROKS: greengenes.lbl.gov



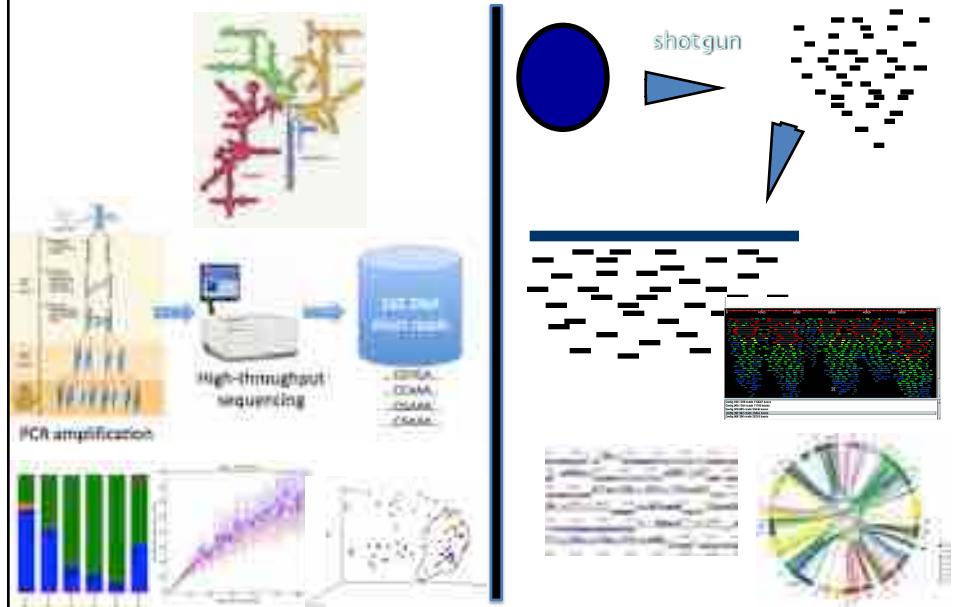
An up-to-date **database** of known 16S rRNA gene sequences  
 A web based **tool** used to classify cloned bacterial DNA sequences  
 Allows users to **compare** sequences to each other and to landmarks in the  
 Greengenes database



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## Community Profiles vs Metagenomics



## What is metagenomics?



**Genomics**



**Metagenomics**

# Metagenomics

The diagram illustrates the metagenomics workflow. It starts with 'Extract DNA' from a microbial community. This is followed by 'Clone (BAC, Fosmid or Small Insert) or Directly Sequence (454 Pyrosequence) Total Environmental DNA'. Below this, a size distribution is shown with three horizontal bars: a top bar of small red segments for 100 bp, a middle bar of yellow segments for 3,000 bp, and a bottom bar of a single long green segment for > 35,000 bp. To the right, a vertical stack of three panels shows a 3D visualization of a microbial community, a 2D map, and a 1D bar chart.

**Metagenomics** is the application of techniques to the study of microbial organisms directly from their natural environments

Access genomes of uncultured microbes:

- Functional Potential
- Metabolic Pathways
- ...

# Sequence and later Assemble Fragments

The diagram shows the process of sequence assembly. It begins with 'sequencer output' represented by a horizontal bar chart. An arrow labeled 'assemble fragments' points to a collection of scattered black line segments representing individual sequence reads. A second arrow labeled 'Closure & Annotation' points from these fragments to a circular genome map, which is a complex, multi-colored ring representing the assembled and annotated genome.

... with some pieces missing



Can we still reconstruct?





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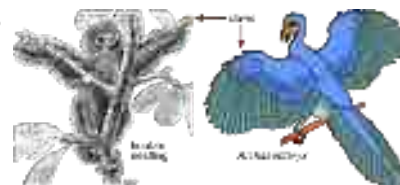


### The Hoatzin project

(Collaboration w Dr. Dominguez-Bello, NYU)



- The *Opisthocomus hoazin* is a South American bird
- Size ~ 60 cm, Weights ~ 700g
- Social animals
- Chicks have functional claws in the wings resembling the Archaeopteryx



# Hoatzin GI Tract

crop

Upper esophagus  
Lower esophagus  
Proventriculus  
Crop  
Gizzard  
Small intestine  
Ceca

10 cm

Hoatzins eat mainly leaves of 17-52 species of plants (Grajal, A. et al, 1989).

(Grajal, A. 1995)

APPLIED AND ENVIRONMENTAL MICROBIOLOGY

Stinked Guts Remove Quarry Bug Follies

## Microbial genomics in the Hoatzin foregut

Differences between fiber and epithelium-attached communities

Community profiling (V8)

Metagenomics

(Grajal, A. 1995)

Epithelium ~ 0.5cm<sup>2</sup>

Fiber ~ 250mg

**1<sup>st</sup> Objective: assessment of microbial diversity in epithelium and fiber using 16S pyrotags**

**2<sup>ND</sup> objective: Assess the diversity of GH's**



# The Self-Contained Microbiome of a Holotrich in the Crop of the Folivorous Hoatzin



**Filipa Godoy-Vitorino**  
**Australian Center for Ecogenomics**  
**Brisbane, AUS**



18S rDNA  
 phylogenetic  
 tree of Ciliate  
 protozoa



The Hoatzin's ciliate is closely related to *Dasytricha ruminantium*





## Crop Holotrichs

- Holotrichs occur in ruminants and are in higher numbers when soluble carbohydrates are readily available (Bonhomme 1990).
- Holotrich ciliates synthesize and store an intracellular reserve polysaccharide during the limited periods when sugars are available in the rumen ecosystem grains (Williams 1986).
- **Hypothesis:** A unique bird anaerobic ciliate (7 x 40 um) acts as a self-contained ecosystem with intimately associated microbes.



## Methods

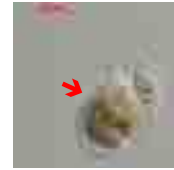
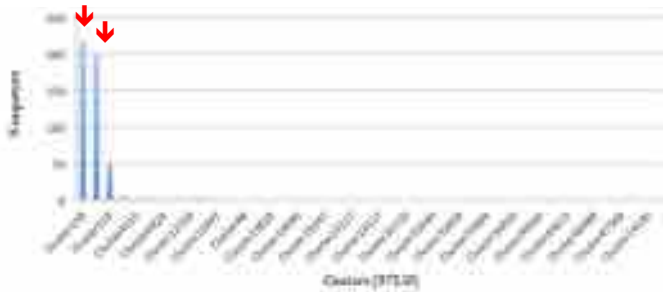
- Micromanipulation
- Filtered 40uM and PBS washing
- 1 Cell transferred to drop of PBS + Lysis solution + MDA mix
- MDA amplification for 20h @ 30C
- 1 channel of Illumina 76PE + pyrotag sequencing with 454-GS-FLX-Titanium (16S V8 region) – 30GB metagenome



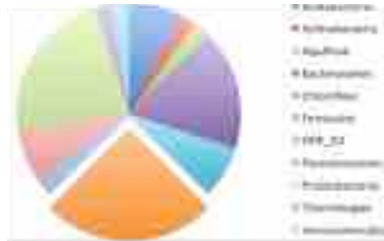
Photos by: Damon Tighe



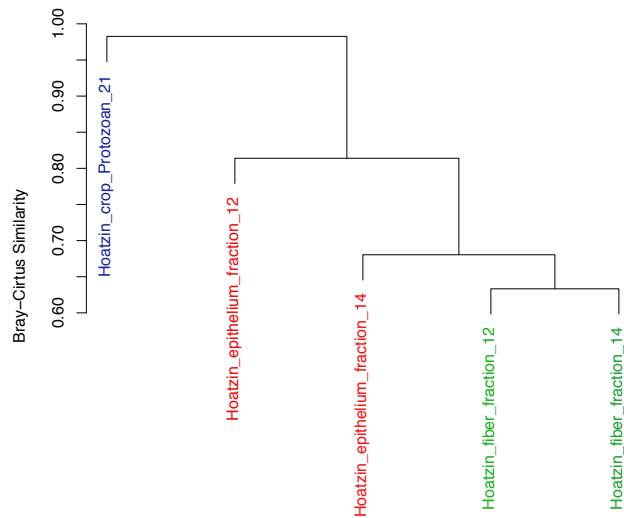
# V8 Community profiling



- 39 OTUs, of which 2 had the highest abundance (42% and 38%) while others had <1%.
- The 2 dominant populations are of unclassified Clostridiales and Bacteroidetes.
- The third most abundant cluster correspond to euk seqs similar to *Dasytricha*

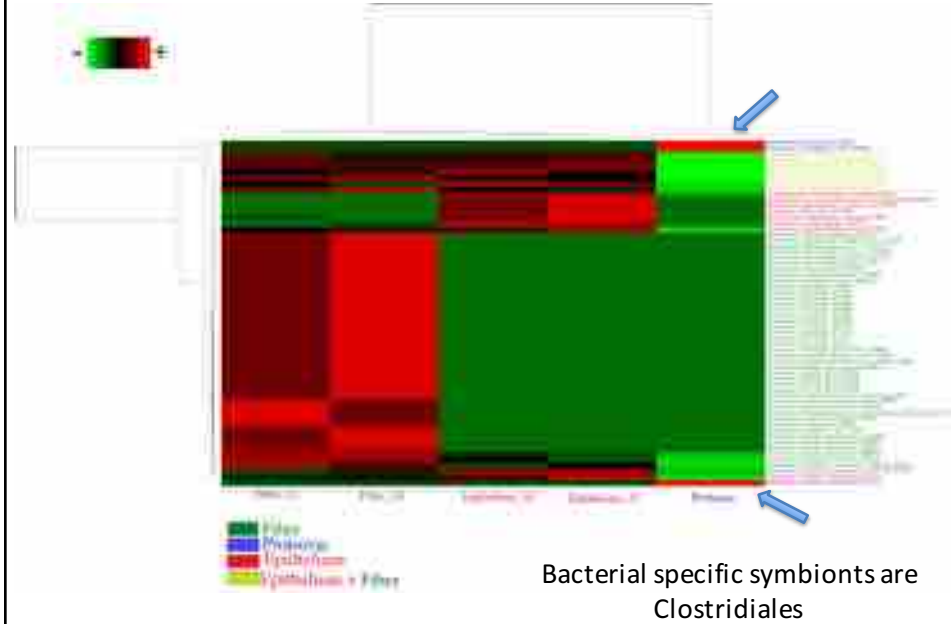


# Bray-Curtis similarity between samples

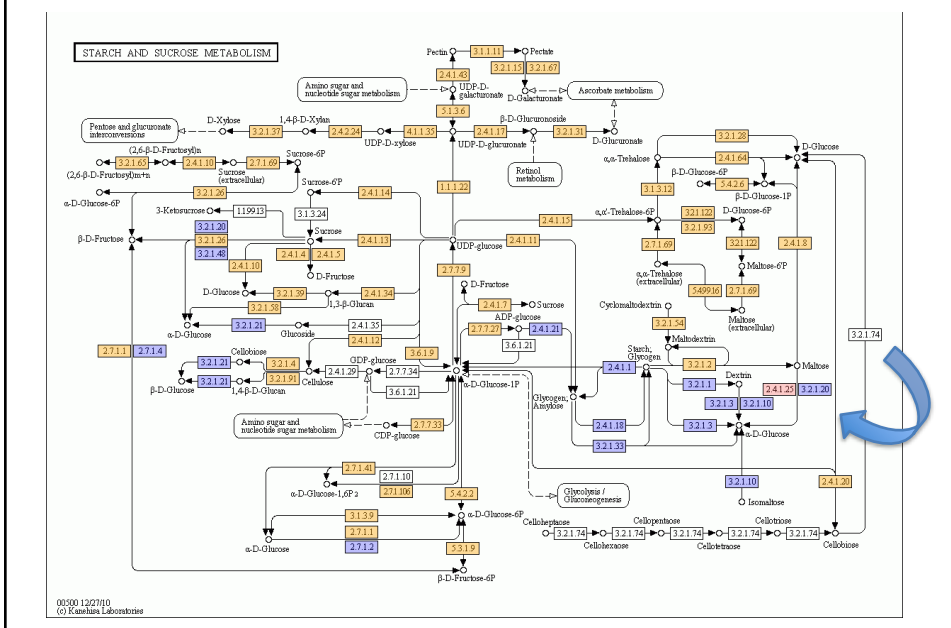


Samples  
Hierarchical Clustering using group average

### Comparison of the significantly different OTUs between the 3 sample types



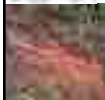
### Bacterial endosymbiotic enzymes involved in glycogen metabolism



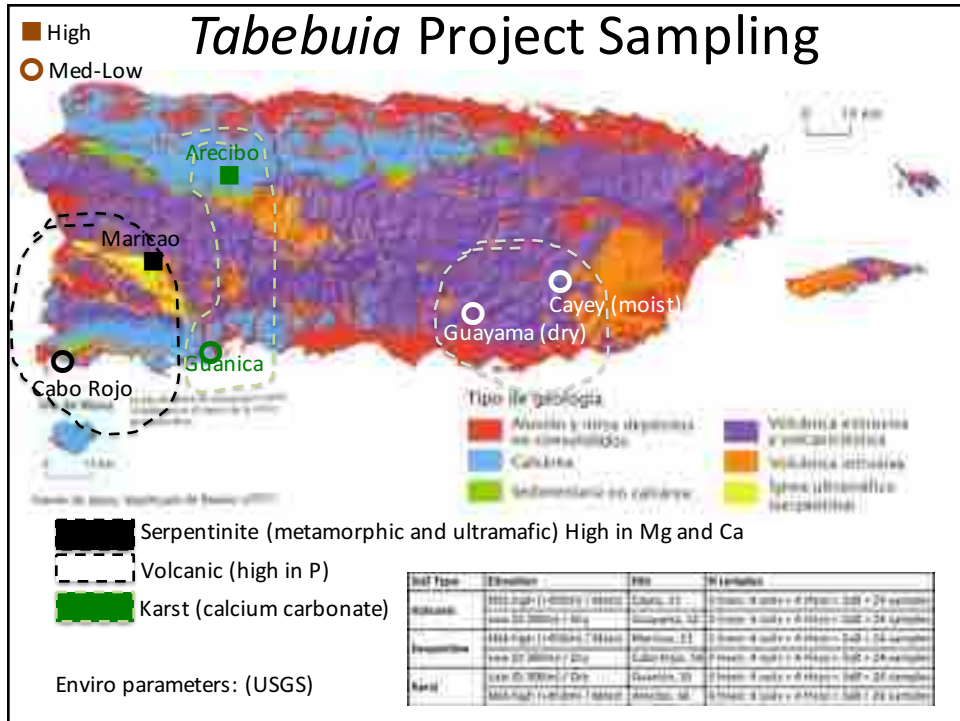
## TALK OUTLINE

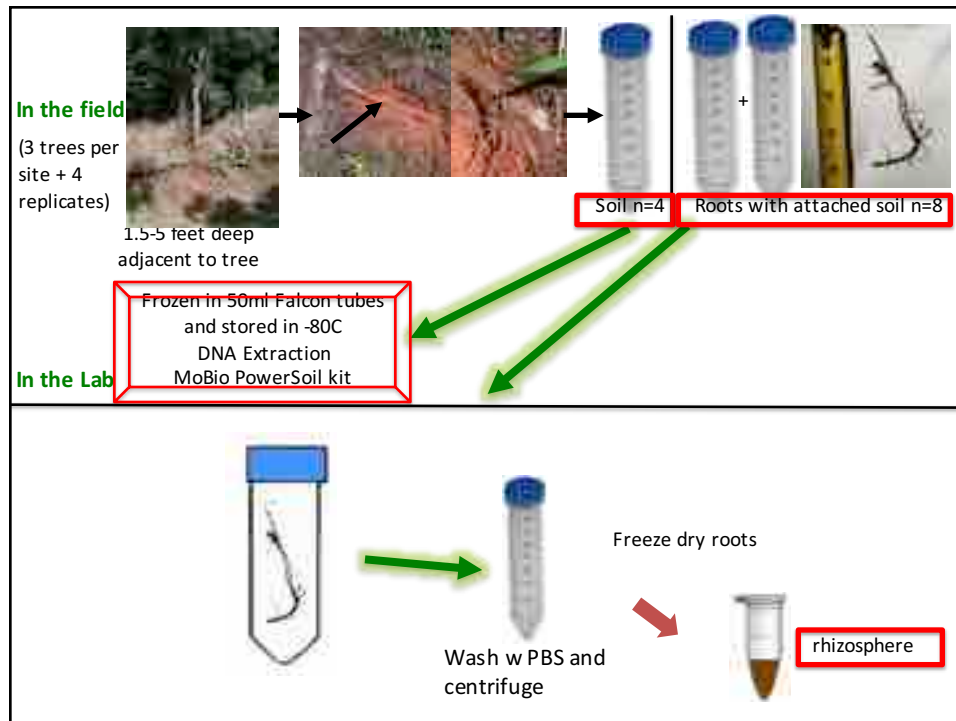
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### Microbial community contributions to stress adaptation and plasticity in the tropical tree *Tabebuia heterophylla*



- **Hypothesis:** *Rhizosphere microbial communities as possible drivers of tree plasticity*
- Understanding the phenotypic plasticity underlying the adaptation of *T. heterophylla*, will be crucial for predicting changes in tree distribution and productivity under global change conditions.
- We will disentangle the precise ecological interactions at the genomic, environmental and edaphic levels that influence community structure and metabolism in the rhizosphere of *T. heterophylla* to understand how they affect tree plasticity.





## Procedures and overall Data

- A total of **196** samples were collected and immediately stored at  $-80^{\circ}\text{C}$ . We extracted gDNA using MoBio Power Soil, and amplified the V4 region of the 16S rRNA for community profiling using Illumina MiSeq platform. Shotgun sequencing was also performed for two selected samples. Data analyses were executed in Qiime, R and IMG considering **samples with >1000 sequences, for a total of 137 samples**.
- We found 14 million sequences ( $\sim 291\text{bp}$ ) from 137 samples (>1000 Operational Taxonomic Units).

**Table 1.** Detailed geological origin of sites (a), number of samples per soil type and niche (b)

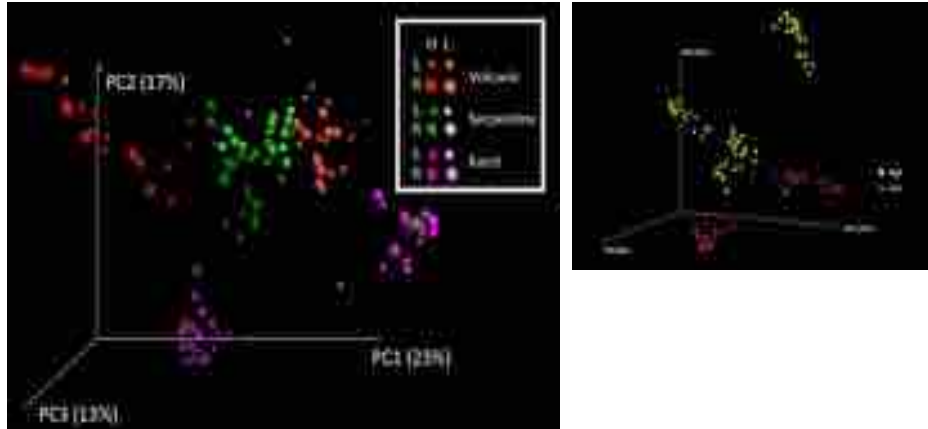
a)

Sample #	Site	Soil type	Distance
1	Yuma River	Sagebrush	Low
2	Lower Klamath of Klamath	Sagebrush	High
3	Yuma Dry channel	Sand	Low
4	Yuma	Sand	High
5	Yuma	Sagebrush	Low
6	Yuma de Salto, Early	Sagebrush	High

b)

Site	Height	Distances	
		Low	High
Yuma	High	11	11
	Low	11	8
Klamath	High	11	11
	Low	11	11
Yuma	High	11	11
	Low	11	11

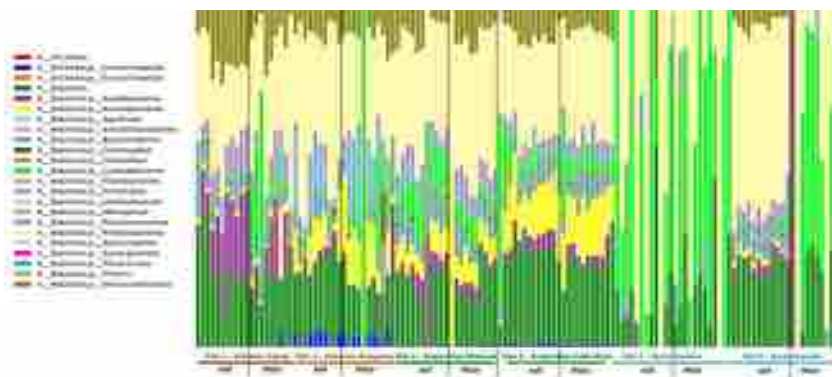
## Beta diversity for 137 samples



- Dry and humid Karst areas differ significantly
- serpentine is similar to dry volcanic areas
- altitude and humidity lead to differences in the microbiota regardless of the geology

## Phyla-level bacterial profiles

- These samples yielded 36,658 OTUs belonging to 71 different phyla.



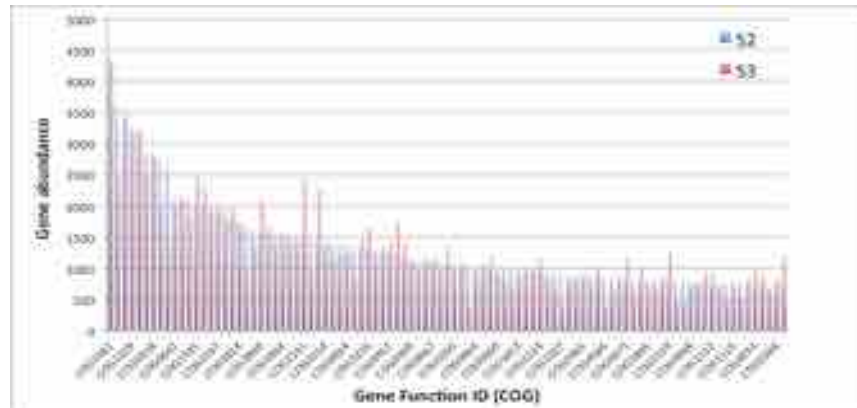
- 2 OTUs from unclassified archaeal and bacterial phyla
- From these, Proteobacteria, Actinobacteria and Verrucomicrobia were the most dominant

Y. Ortiz, H. Herrera (c) Godoy-Vitorino



## COG abundance profiles for volcanic (s2) and serpentine (s3) metagenomes

- COG profiles show abundance of flavin-dependent oxidoreductase, **luciferase family in serpentine** (involved in bioluminescence), and **glycoside hydrolase genes in volcanic soils** (glycolytic enzymes).



## Conclusions

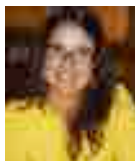
- ◆ Preliminary analyses of microbial data shows that there are differences between soil types and rhizosphere and soil communities.
- ◆ The rhizosphere microbiome of *Tabebuia* seems to be enriched with key bacterial species that may help with nutrient availability at different soil types.
- ◆ Ongoing metagenomic analyses on the three rhizosphere types, will help elucidate the “conversation” between microbes and plants
- ◆ Greenhouse transplantation experiments will help test the role of microbes in inducing plant phenotypic traits

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### “Microbial communities and enzymes from mangrove ecosystems of Puerto Rico”



**Principal Investigator**  
Dr. Filipa Godoy  
IAUPR-MC  
PI-MEG Lab



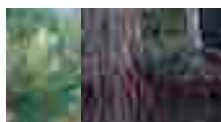
**Co-PI**  
Dr. Janet Jansson  
LBNL  
DOE JBEI/JGI  
Senior Staff Scientist



**Co-PI**  
Dr. Matias Cafaro  
UPR-RUM  
Full Professor



**Co-PI**  
Prof. Ernesto Torres  
IAUPR-MC  
CECIA Coordinator



Collaborative project that includes IAUPR-MC, DOE-JBEI and UPR-RUM

## Importance of mangroves



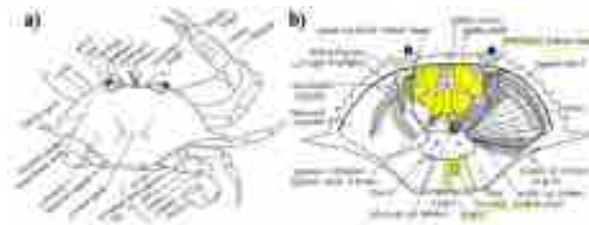
- Mangroves dominate 75% of the world's coast lines (Wilson 1962).
- Protect and stabilize coastal lands and maintain food sources for estuarine and coastal food chains.
- Support a high abundance and variety of wildlife (Ong, 1995).
- Mangrove leaves are very thick (rich lignocellulose and polyphenols).



## Aims and Scientific opportunity

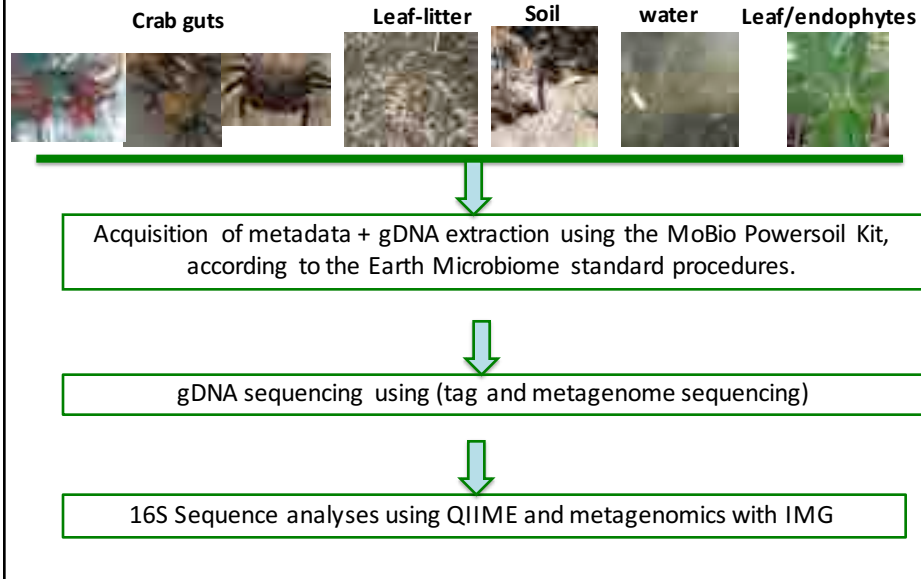
•Soils and Herbivore crabs should have a great variety of Glycosyl hydrolases

•**Aim:** characterize the microbiome of the hindgut of herbivore crabs, leaf litter and soil order to identify lignocellulolytic enzymes



Smithsonian in Panamá  
(STRI) – Punta Galeta  
additional sampling

## Methodology

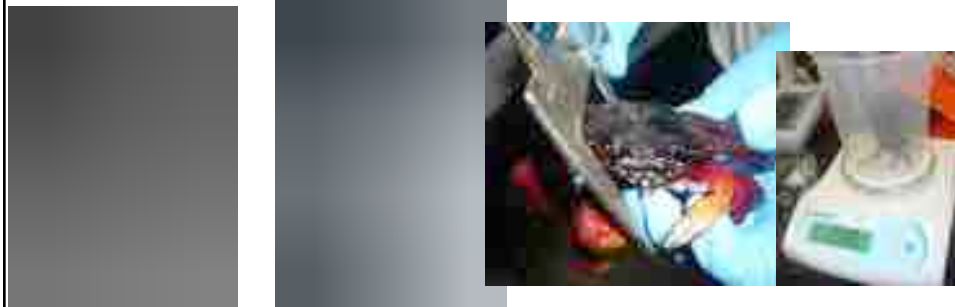


## Sampling site



## Metadata

- Latitude, longitude, elevation, soil temperature, soil pH, soil texture, water temp., salinity, DO, external temp, pluviometry, water pH crab species, crab gender, crab weight, carapace length x wide, leaf diameter



### Sampling and metadata measurements

- Crab sampling in red mangrove – *Goniopsis* (mangrove root crab)
- We sampled water and soil. Then measured the pH of water and soil, salinity, oxygenation, temperature and elevation of the sampling site.



## Overall Methodology

A total of 88 samples from red and black mangrove sites

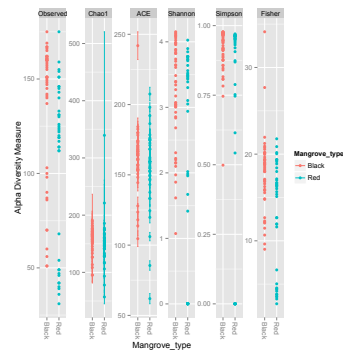
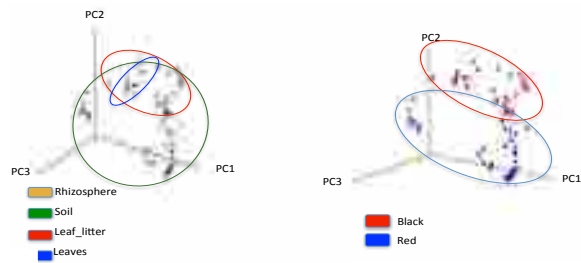


Extraction of gDNA using the MoBio Powersoil kit



16S rRNA V4  
And ITS2  
sequenced with Illumina

## Preliminary Results for 16S:



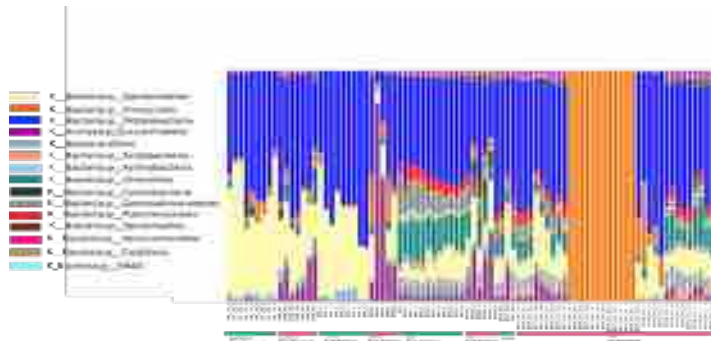
- only soil, rhizosphere and leaf litter were sequenced
- 88 samples, 17 million seqs = ~42K OTUs
- Microbiota is different in the two ecosystems
- **Black mangrove rhizosphere greater richness and diversity of bacteria**



## Taxonomic Differences between red and black

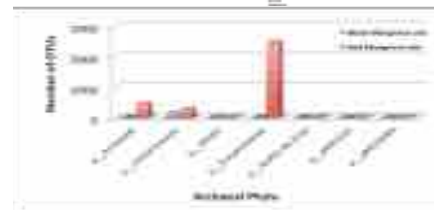
### Red mangrove

samples exhibited significant amounts of Spirochaetaceae, *Staphylococcus* and a higher dominance in Archaea, *Methanobacterium*

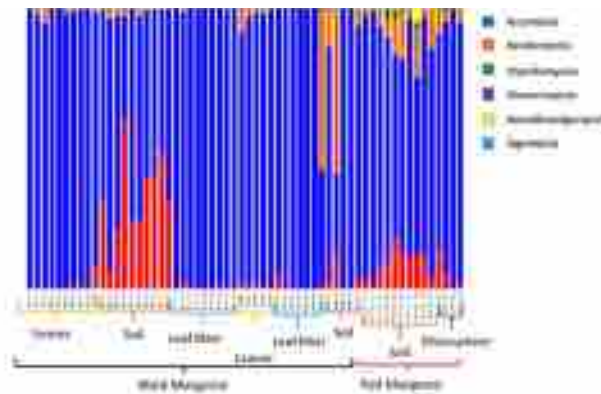


### Black mangrove

samples had abundant *Anaerolineae* (biopolymer degrader), Desulfuromonadales (metal bioremediator)

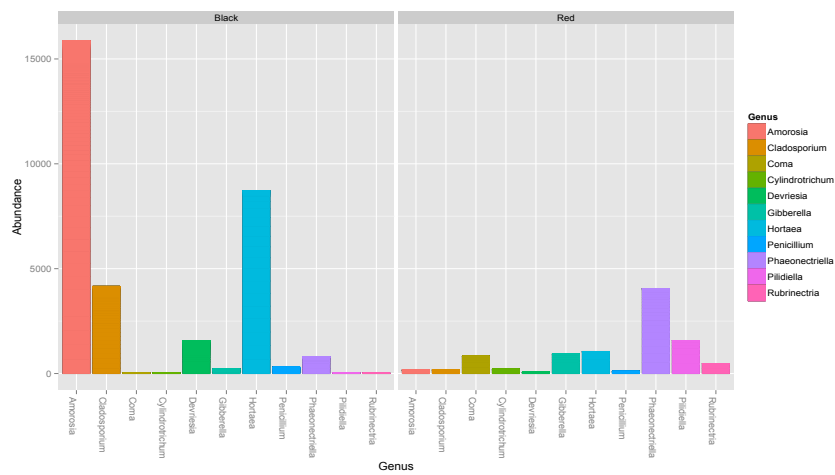


## Phylum level taxonomic differences regarding the ITS2 data (fungi)



- The most abundant phyla is Ascomycota with almost 90% of all the observed species
- There only 5 phyla found in black mangrove samples: Ascomycota, Basidiomycota, Chytridiomycota and Neocallimastigomycota
- **Red mangrove samples display more phyla than black mangrove samples (6):** Ascomycota, Basidiomycota, Chytridiomycota, Neocallimastigomycota, Glomeromycota and Zygomycota

## Genus level taxonomic analysis based on mangrove type



- *Phaeoectriella* spp. is found in both mangroves types in high abundance but significantly higher in red mangrove samples
- Even though black mangrove samples have high abundance of *Amorosia* and *Cladosporium* and *Hortaea*

## Our preliminary analyses shows

- Red mangrove samples have higher richness and diversity of fungi and less diversity of bacteria than the black mangrove samples
- Abundance of methanogenic archaea in red mangrove samples
- The black mangrove bacteria reveal a diversity of taxa with potential for bioremediation while red mangrove bacteria show an abundance in GHs
- This report confirms the great biotechnological potential of the mangrove microbial niches.
- Future analyses include metabolic pathways for lignocellulase degradation and nitrogen fixation through metagenomics

## TALK OUTLINE

- Microbial Ecology- Definition and Brief History
- What is Microbiome Research?
- NGS Applications to the study of Microbial Communities (Profiling)
- Metagenomics
- The Hoatzin: A Single-cell metagenome project
- **Three new Microbiome Research Projects in MEGL:**
  - Tabebuia heterophylla rhizosphere microbial communities
  - Black and Red Mangrove microbiome
  - **HPV and cervicovaginal microbiota**

## Relating the genital microbiota to HPV infections in latin women



### Collaborators



Josefina Romaguera, MD, UPR



Larry Forney, Ph.D. U Idaho



Dominguez-Bello, Ph.D. NYU



Martin Blaser, MD, NYU

Infection with HPV is the most common sexually transmitted infection worldwide, and cervical cancer is the third most common cancer in women worldwide.

Cervical cancer is the fifth most commonly diagnosed cancer among women in Puerto Rico and infection with HPV is known to be a necessary factor for development of invasive cervical cancer. In Puerto Rico the prevalence of HPV is ~34% slightly higher than the ~27% prevalence on the U.S. mainland.

**The purpose of this project is to elucidate the microbial community structure and function among HPV+ and HPV- reproductive age Puerto Rican women through an in-depth and accurate understanding of the composition and ecology of the cervicovaginal microbial ecosystem.**

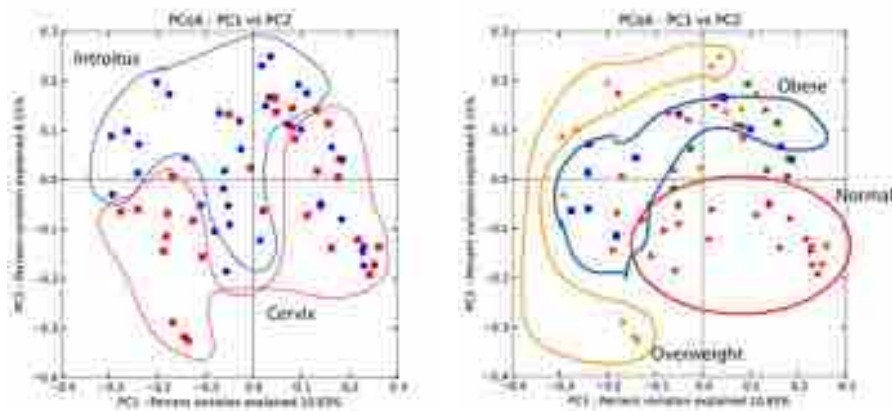


## Scientific Questions/Aims



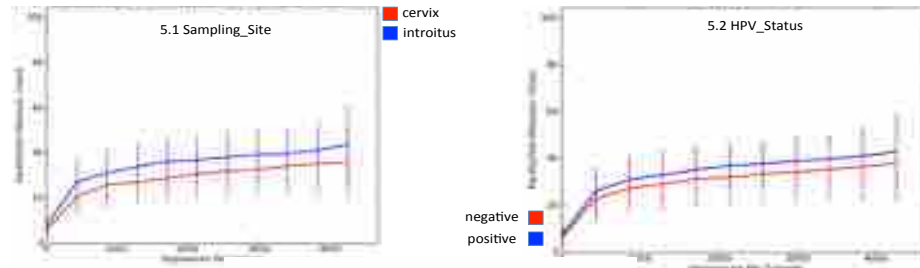
- Relation between vagina microbiota in women affected with HPV and healthy women in different cohorts.
- Are there specific groups of bacteria in the vaginal microbiota of women with hpv/cancer markers?

**Preliminary results:** vagina differs from cervix and obese differs from normal weight (n=72)



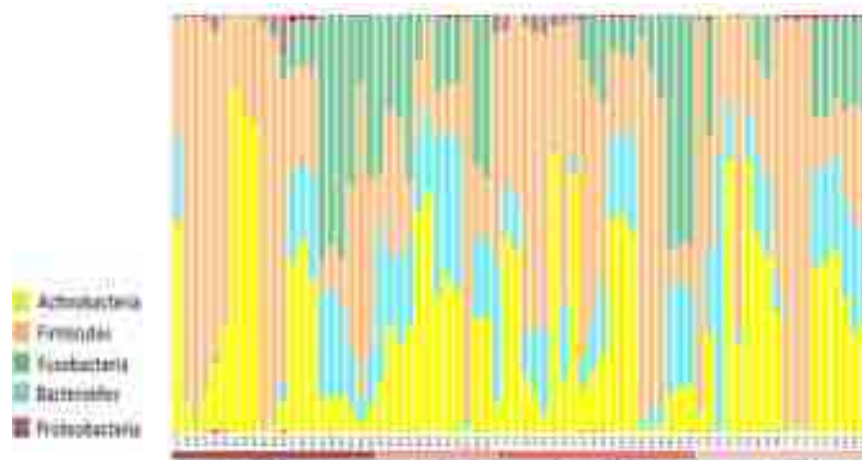
$10^6$  seqs = 5073 OTUs

## Rarefied richness curves for the 72 samples



- Introitus samples have significantly higher richness than cervix as does HPV positive samples (greater microbial diversity = disease)

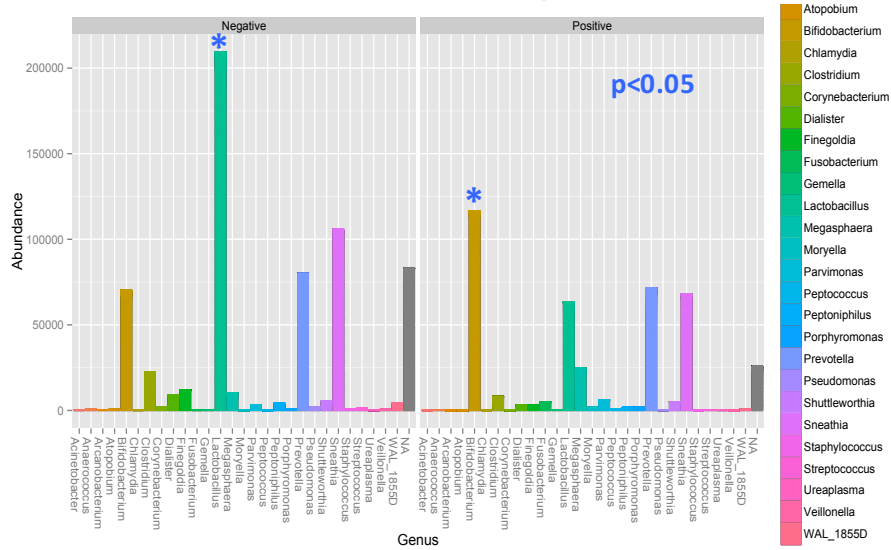
## Phyla-level taxonomic diversity of the 72 samples according to sampling site and HPV status





## Significant differences between HPV+ and HPV-

HPV- = *Lactobacillus*; HPV+ = *Bifidobacterium*



## Preliminary data indicates

...that *Lactobacillus* is significantly associated with HPV negative samples, while *Bifidobacterium*-like is a marker for HPV positive samples.

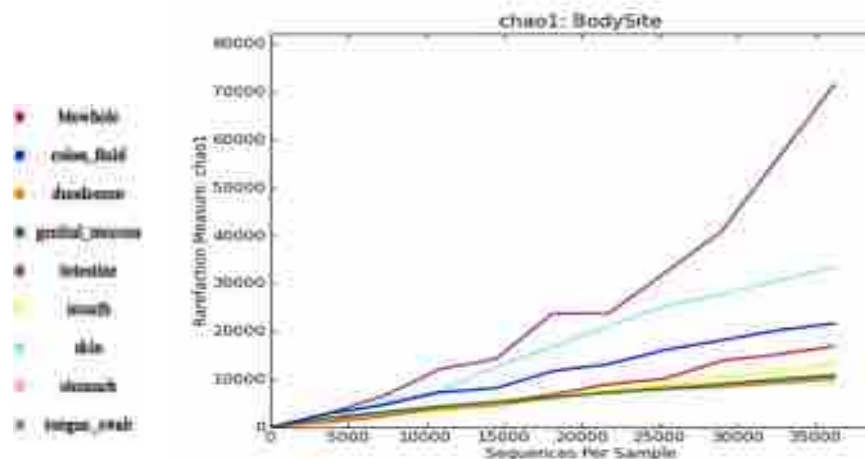
Additional samples are needed to elucidate the differences in microbial community structure to help clarify how the microbiota may serve as a frontier in cervical cancer oncogenesis and prevention.

**A High-Throughput Look at the Microbiome  
of a Striped Dolphin (*Stenella coeruleoalba*)  
Off the Coast of Portugal**



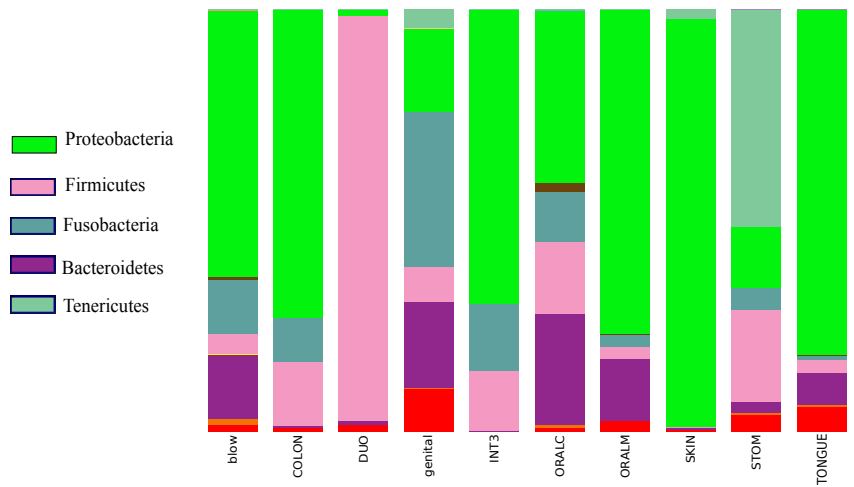
IAUPR AND UNIV. MINHO, PORTUGAL

**Alpha Rarefaction Curves per Body Site**



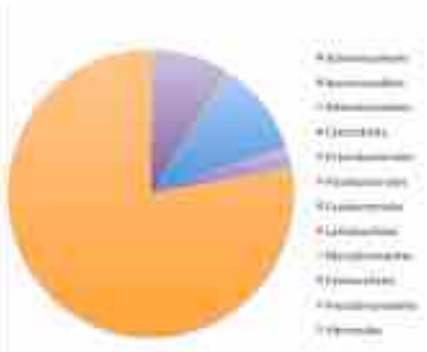
Curves showing number of observed species between body sites. Stomach, oral-associated and skin samples show a higher number of observed species than other body sites.

### Taxa Summary plots at the phylum-level per body site

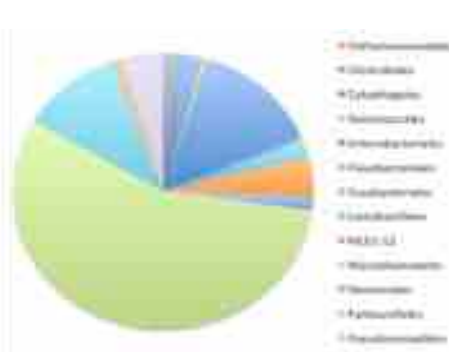


### Pathogen abundance in different organs

intestines = mainly *Vibrio*



stomach = mainly *mycoplasma*



## Preliminary data indicates:


...that the striped dolphin microbiome shows variation in structure and diversity according to the organ type, and a **prevalence of gram-negative anaerobic pathogens in such high dominance confirming the diseased status of the animal.**

Comparisons between healthy and stranded striped dolphins are underway as are between multiple dolphin species.

## Microbiome reseach and Bioinformatics:

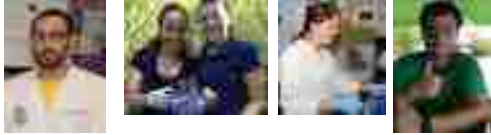

- Microbiome projects can elucidate the functions of different ecosystems;
- Unravel novel beneficial applications to serve mankind (biofuels, human or forest probiotics, etc);
- Cheap science!
- Broad collaborative networks
- Unprecedented amount of data and training opportunities






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- Gilmary Ortiz
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- Beatriz Cabrera Colón
- Haydee Herrera
- Herminio González
- Chardiel Delgado
- Gabriel Vargas




# ***THANKS! GRACIAS! OBRIGADA!***

**FGODOY@INTERMETRO.EDU**



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May 5 2015

