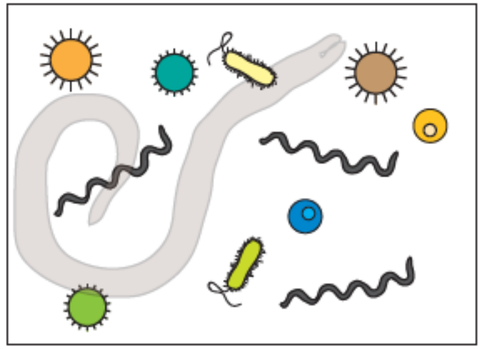
Metagenomics and disease

Mark Stenglein, GDW 2017

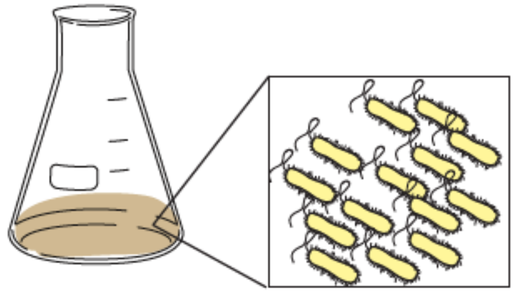
Genomics of Disease in Wildlife

What is metagenomics?

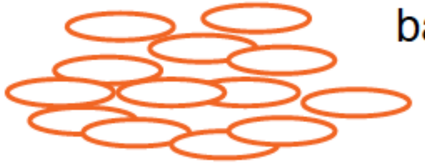
soil community



bacterial isolate



soil ‘metagenome’



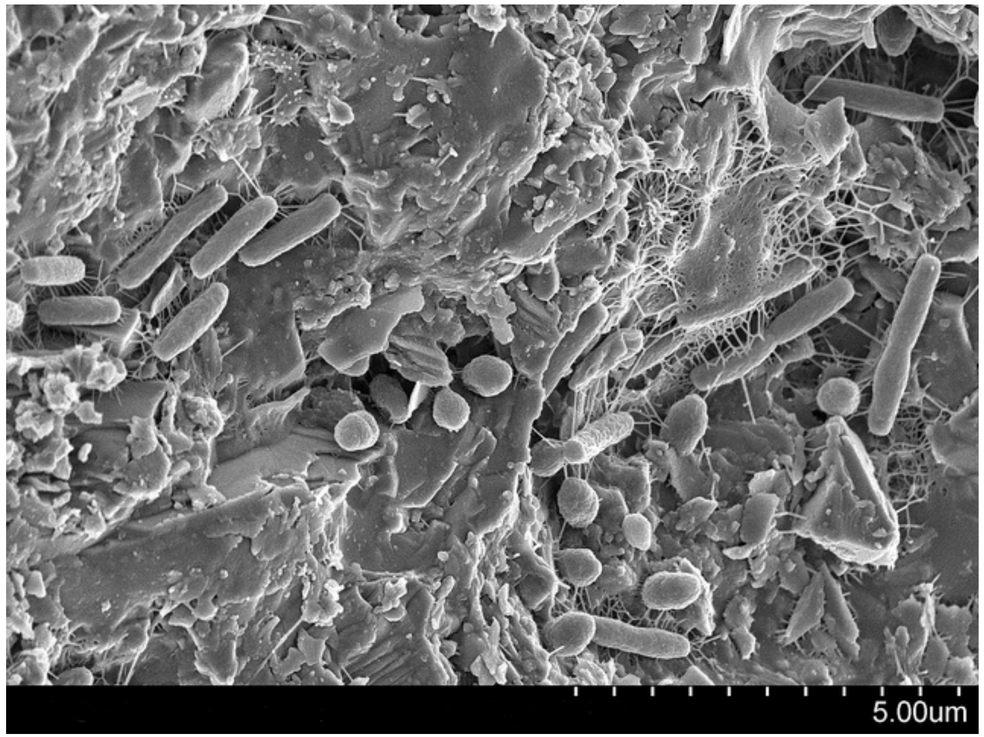
bacterial genome

Metagenomics emerged in response to the observation that most micro-organisms can't be cultured

Morphological diversity typical of microorganisms cultured from soil on a broad spectrum medium, tryptic soy agar.

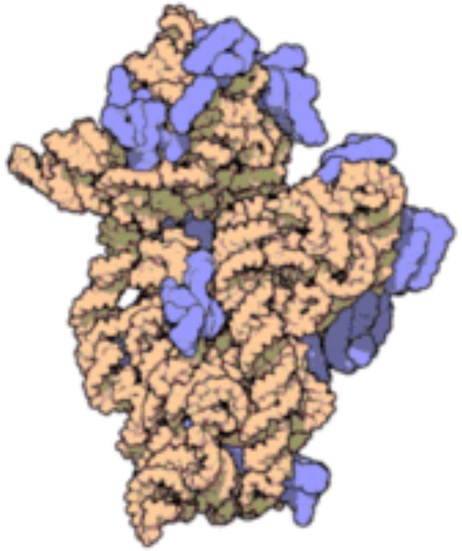
Handelsman et al (1998) Chem & Biol

Estimated: 10^8 bacteria per gram of soil of 6000-8000 different species
Only ~1% culturable (?)

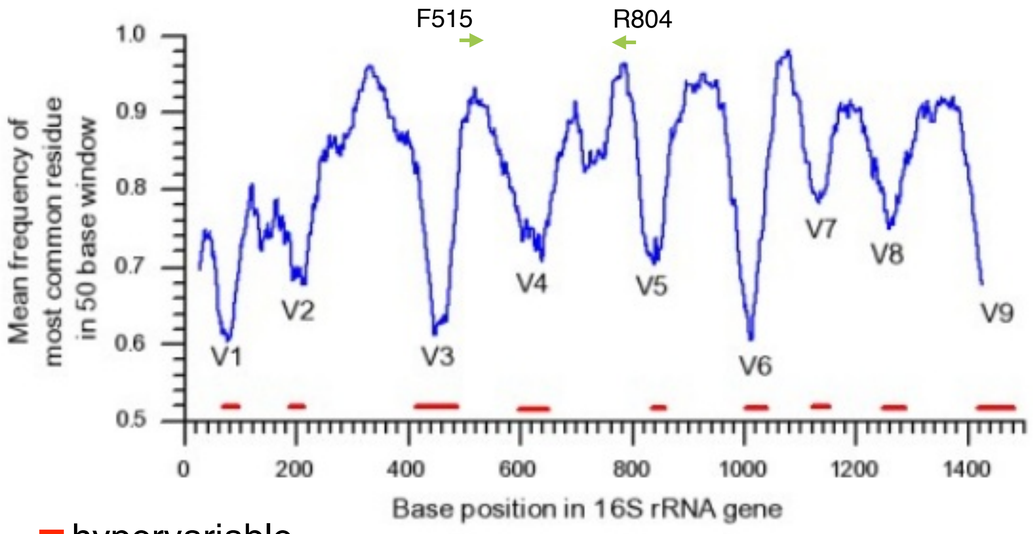


EM: Kim Lewis, Northeastern Univ.

PCR using primers targeting conserved regions of the 16S rRNA gene and sequencing enables genotyping of bacteria and archaea without having to culture them



bacterial 30S ribosomal subunit
16S rRNA is in orange
(purple: ribosomal proteins)
image: wikipedia



hypervariable

One of the earliest “metagenomics” paper, based on 16S sequencing

JOURNAL OF BACTERIOLOGY, July 1991, p. 4371–4378
0021-9193/91/144371-08$02.00/0
Copyright © 1991, American Society for Microbiology
Vol. 173, No. 14

Analysis of a Marine Picoplankton Community by 16S rRNA Gene Cloning and Sequencing

THOMAS M. SCHMIDT,† EDWARD F. DELONG,‡ AND NORMAN R. PACE*

Department of Biology and Institute for Molecular and Cellular Biology, Indiana University, Bloomington, Indiana 47405

Received 7 January 1991/Accepted 13 May 1991

The phylogenetic diversity of an oligotrophic marine picoplankton community was examined by analyzing the sequences of cloned ribosomal genes. This strategy does not rely on cultivation of the resident microorganisms. Bulk genomic DNA was isolated from picoplankton collected in the north central Pacific Ocean by tangential flow filtration. The mixed-population DNA was fragmented, size fractionated, and cloned into bacteriophage lambda. Thirty-eight clones containing 16S rRNA genes were identified in a screen of 3.2 × 10^4 recombinant phage, and portions of the rRNA gene were amplified by polymerase chain reaction and sequenced. The resulting sequences were used to establish the identities of the picoplankton by comparison with an established data base of rRNA sequences. Fifteen unique eubacterial sequences were obtained, including four from cyanobacteria and eleven from proteobacteria. A single eucaryote related to dinoflagellates was identified; no archaebacterial sequences were detected. The cyanobacterial sequences are all closely related to sequences from cultivated marine *Synechococcus* strains and with cyanobacterial sequences obtained from the Atlantic Ocean (Sargasso Sea). Several sequences were related to common marine isolates of the γ subdivision of proteobacteria. In addition to sequences closely related to those of described bacteria, sequences were obtained from two phylogenetic groups of organisms that are not closely related to any known rRNA sequences from cultivated organisms. Both of these novel phylogenetic clusters are proteobacteria, one group within the α subdivision and the other distinct from known proteobacterial subdivisions. The rRNA sequences of the α-related group are nearly identical to those of some Sargasso Sea picoplankton, suggesting a global distribution of these organisms.

plankton

image: Smithsonian magazine

Schmidt et al actually made a shotgun library from pico plankton gDNA, identified rDNA clones (38/32000 clones) by colony hybridization, and Sanger sequenced them

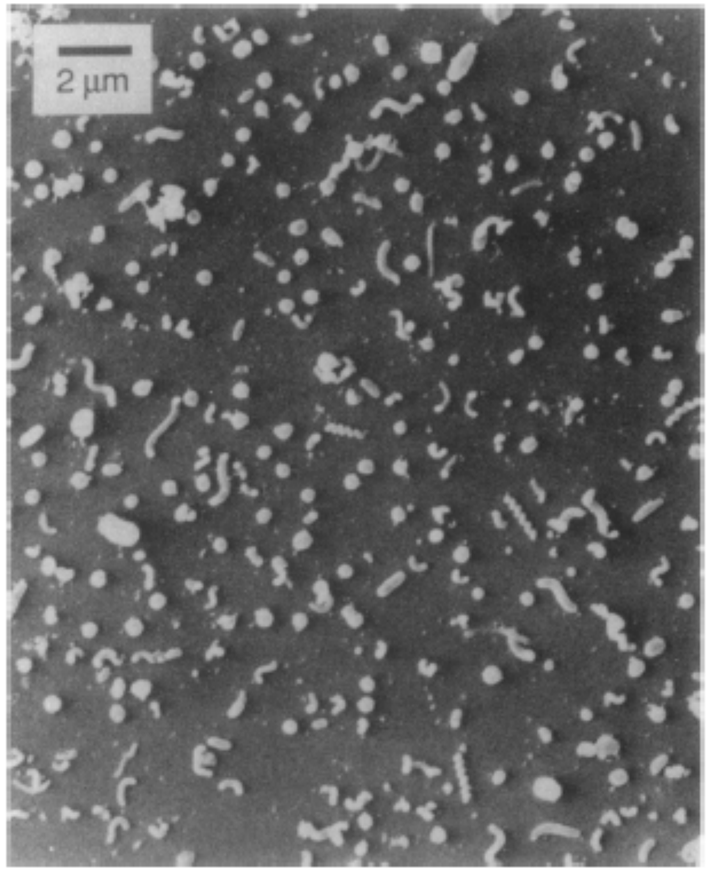


FIG. 2. Scanning electron micrograph of picoplankton represented in the clone library. Picoplankton concentrated from the ALOHA collection site were fixed and prepared for scanning electron microscopy as detailed in Materials and Methods.

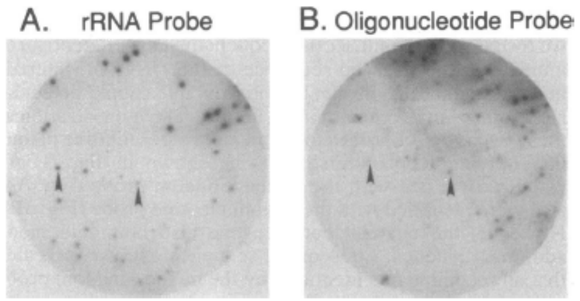


FIG. 3. Identification of rDNA-containing clones. Filter lifts of the recombinant bacteriophage library were probed as detailed in Materials and Methods with a mixed-kingdom rRNA probe consisting of 16S rRNAs from *O. linum* (eubacterium), *S. solfataricus* (archaebacterium), and *S. cerevisiae* (eucaryote) or an oligonucleotide probe complementary to a universally conserved 16S rRNA sequence.

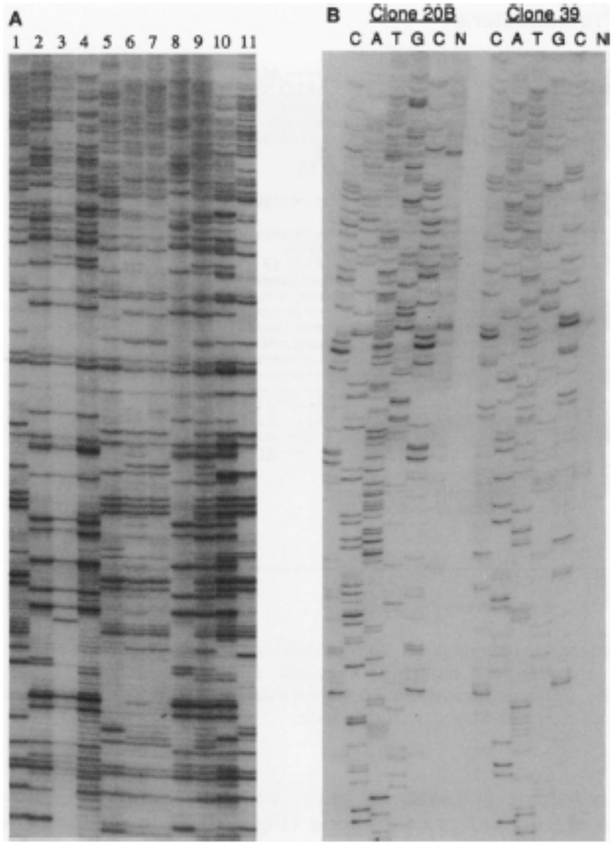
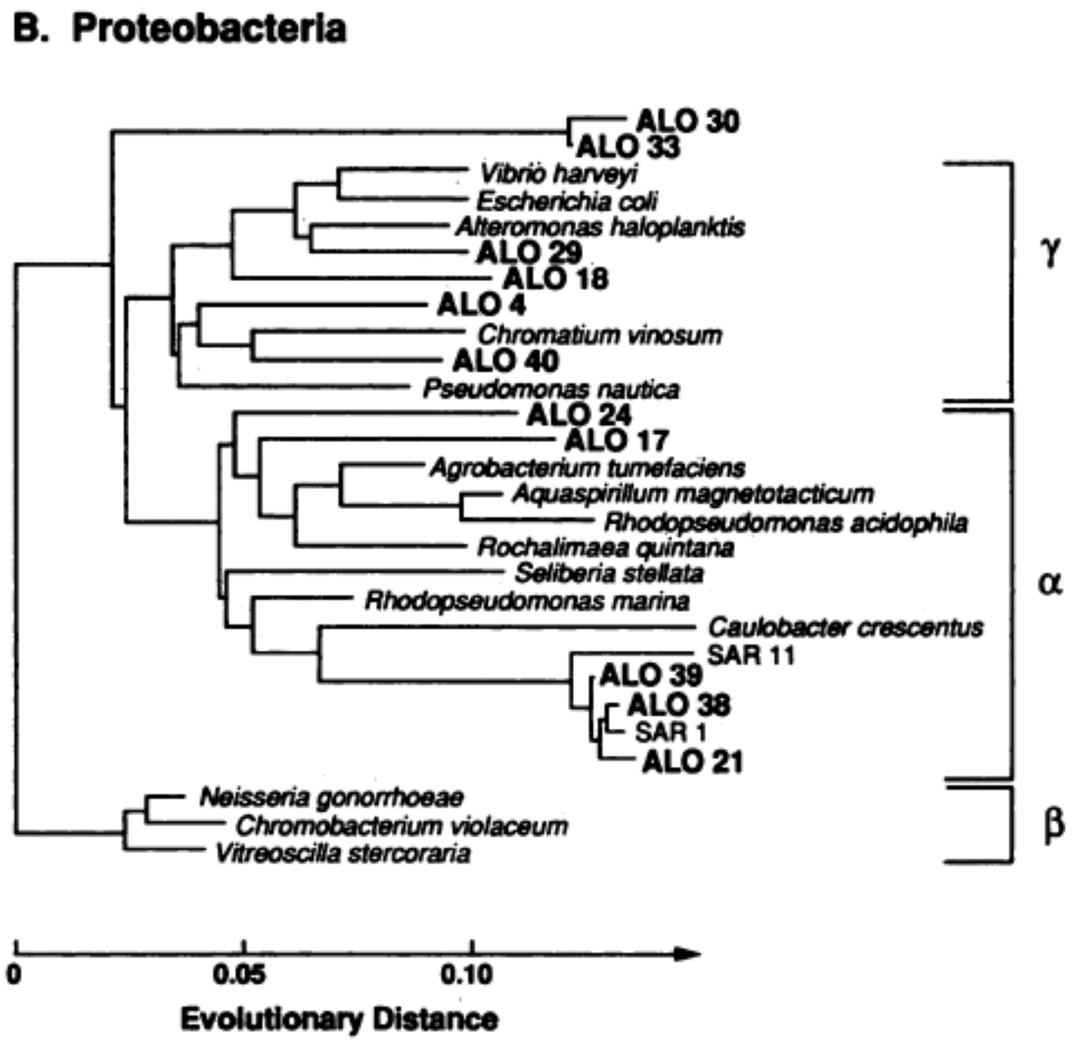
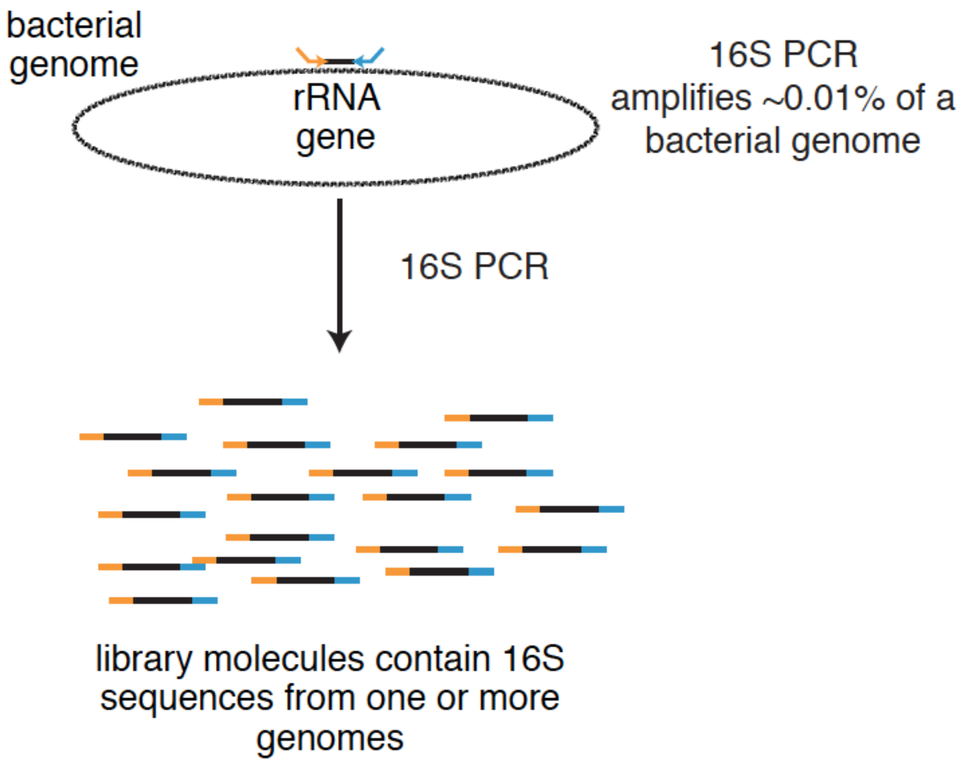


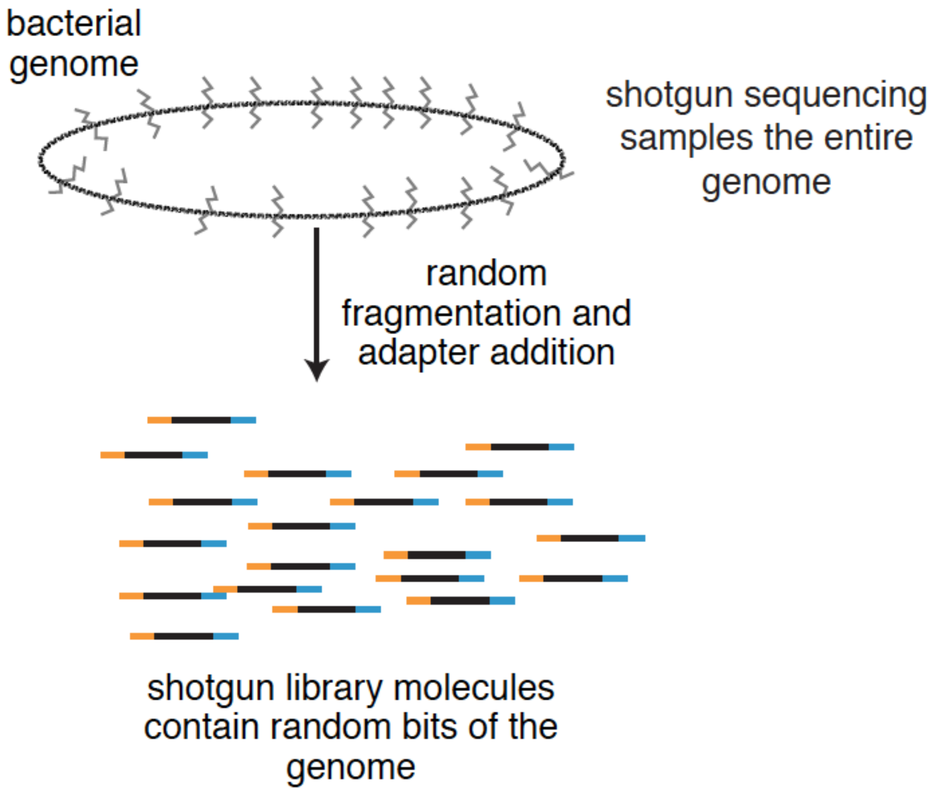
FIG. 5. Sorting and sequence analysis of rDNA-containing clones. (A) Single-nucleotide sequence pattern for 11 picoplankton clones, produced as detailed in Materials and Methods by using a PCR-amplified template, primer 519R, and a single dideoxynucleotide (ddA). (B) Sequence determination by dideoxynucleotide chain termination, using a PCR-generated, single-stranded template from two of the picoplankton clones and primer 519R.

This unbiased survey revealed that the picoplankton contained many previously unknown bacterial species



16S sequencing vs. shotgun metagenomics





- Only bacteria and archaea surveyed
- Deeper sampling of bacterial diversity per $
- Relatively easy to make libraries and interpret results
- Appropriate if all you care about is microbial diversity / ecology

- All organisms studied*
- Decreased sampling depth per $
- Enables analysis of other genomic features of organisms, e.g. antimicrobial resistant genes
- Analysis is <u>significantly</u> more difficult

Warning! Some will object if you refer to 16S-based studies as “metagenomics”

THE TREE OF LIFE

Blog of Jonathan Eisen, Professor at UC Davis.

Wednesday, August 22, 2012

Referring to 16S surveys as "metagenomics" is misleading and annoying #badomics #OmicMimicry

Aargh. I am a big fan ~~if~~ of ribosomal RNA based surveys of microbial diversity. Been doing them for 20+ years and still continue to - even though I have moved on to more genomic/metagenomic based studies. But it drives me crazy to see rRNA surveys now being called "metagenomics".

Here are some examples of cases where rRNA surveys are referred to as metagenomics:

Deep 16S rRNA metagenomics and quantitative PCR analyses of the premature infant fecal

http://phylogenomics.blogspot.com/2012/08/referring-to-16s-surveys-as.html

Viral metagenomics

JOURNAL OF VIROLOGY, July 2010, p. 6955–6965 Vol. 84, No. 14
0022-538X/10/$12.00 doi:10.1128/JVI.00501-10
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Bat Guano Virome: Predominance of Dietary Viruses from Insects and Plants plus Novel Mammalian Viruses[▽]

Linlin Li,[1,2] Joseph G. Victoria,[1,2] Chunlin Wang,[3] Morris Jones,[4] Gary M. Fellers,[5] Thomas H. Kunz,[6] and Eric Delwart[1,2]*

Blood Systems Research Institute, San Francisco, California[1]; Department of Laboratory Medicine, University of California, San Francisco, California[2]; Stanford Genome Technology Center, Stanford, California[3]; Clinical Investigation Facility, David Grant USAF Medical Center, Travis Air Force Base, California[4]; U.S. Geological Survey, Western Ecological Research Center, Point Reyes, California[5]; and Center for Ecology and Conservation Biology, Department of Biology, Boston University, Boston, Massachusetts[6]

Received 5 March 2010/Accepted 30 April 2010

Bats are hosts to a variety of viruses capable of zoonotic transmissions. Because of increased contact between bats, humans, and other animal species, the possibility exists for further cross-species transmissions and ensuing disease outbreaks. We describe here full and partial viral genomes identified using metagenomics in the guano of bats from California and Texas. A total of 34% and 58% of 390,000 sequence

Tadarida brasiliensis

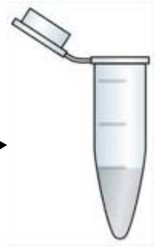
image: Wikipedia/NPS

They found:

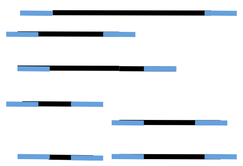
- Known bat viruses
- Putative new bat viruses
- Viruses likely infecting the plants and insects that the bats ate

Pathogen discovery using metagenomics sequencing

case and control tissues

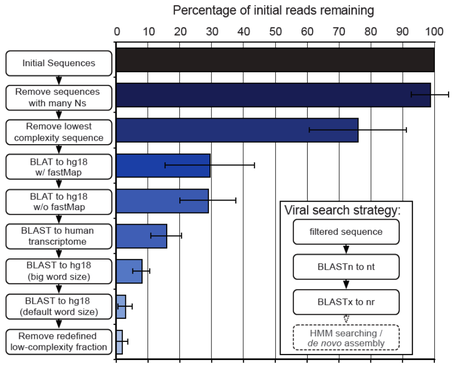


Nucleic acid



Library prep / barcode

Illumina sequencing



Computational Analysis

Follow-up

Inclusion body disease has been a vexing problem in captive snake populations for decades

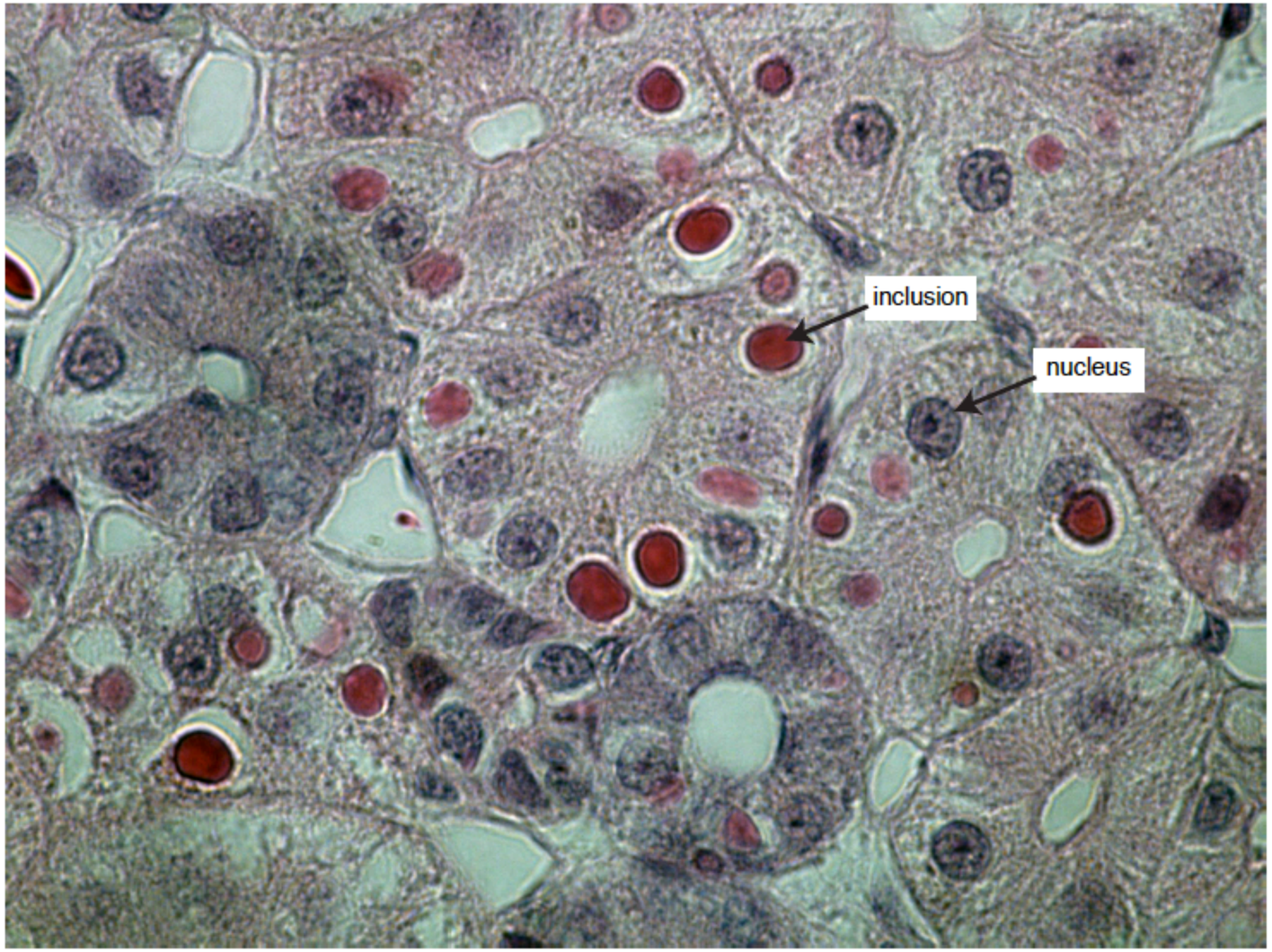
Chang and Jacobson (2010) Journal of Exotic Pet Medicine

‘stargazing’ boa constrictor

https://www.youtube.com/watch?v=Y-lopSetZzw

Diagnosis is based on the presence of eosinophilic inclusions

H&E stained kidney section from a boa constrictor diagnosed w/ IBD



10 μm

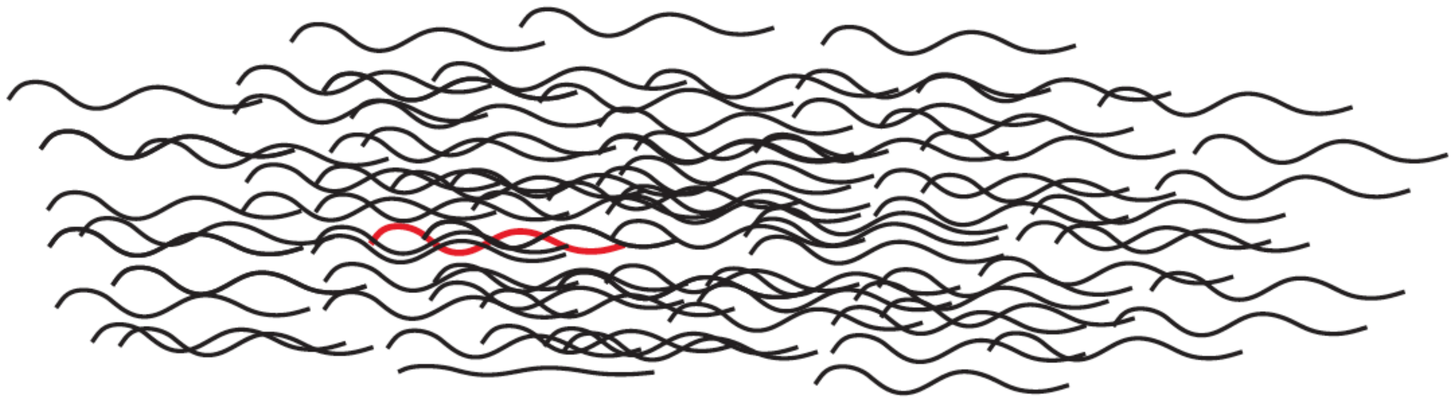
There was an IBD outbreak at the California Academy of Sciences in San Francisco

Annulated tree boas
(*Corallus annulatus*)

Boa constrictors
(*Boa constrictor*)

A couple of the key challenges in metagenomics pathogen discovery

1) Pathogen nucleic acid is typically present in a sea of host nucleic acid

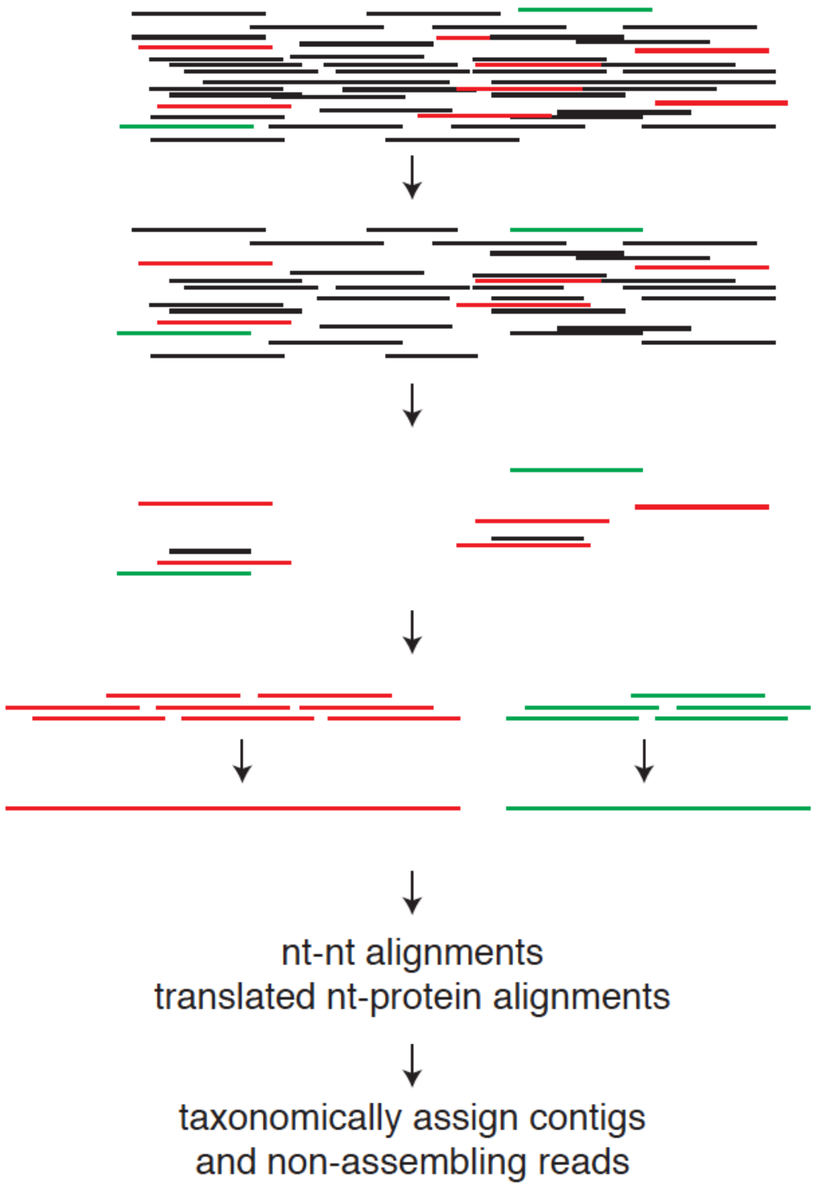


~1 viral nucleic acid per 10^4-10^7 host nucleic acids

2) New pathogens have unknown sequences

TTTCAG?TTT?ACC????TG??AAA?ACATCC??TATAC???T?

Our typical analysis workflow



Quality filter /
remove PCR duplicates
cutadapt
cd-hit-est

Remove host sequences
bowtie2

Assemble remaining reads into contigs
SPAdes

Identify most similar sequences in NCBI databases
BLASTN (gsnap),
BLASTX (rapsearch)

~40 min for a dataset w/ 6M reads

caveat: for a dataset where host filtering removed almost all of the reads

A good reference genome is helpful but not strictly necessary

Ixodes scapularis
Blacklegged tick

genome sequenced
(Gulia-Nuss et al (2015) Nature Comm)

Images: CDC

~3% of reads remaining after filtering

Dermacentor andersoni
Rocky mountain wood tick

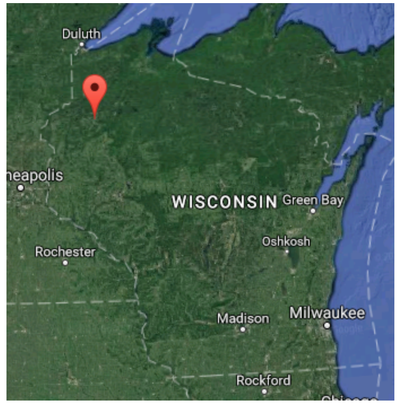
no genome sequence

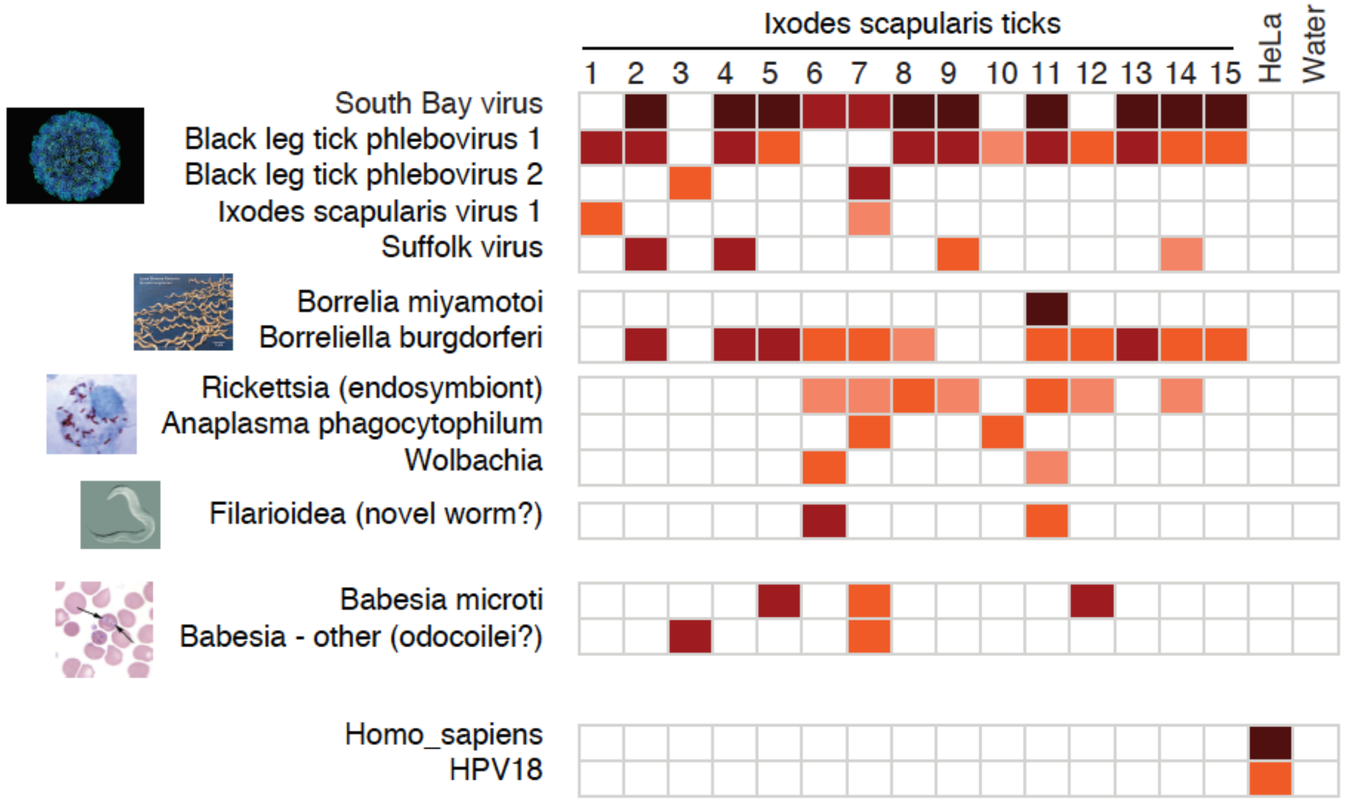
~55% of reads remaining after filtering

- Assembly/downstream steps will go much slower
- The taxonomic assessment will be more difficult (lots of false positive taxonomic assignments)
- Sequence “dark matter”, Eric Delwart

Individual ticks harbor complex communities of organisms

Ixodes scapularis





Jackie Hunter

In theory, a single read is sufficient to identify a pathogen (but that's cutting it a little close)

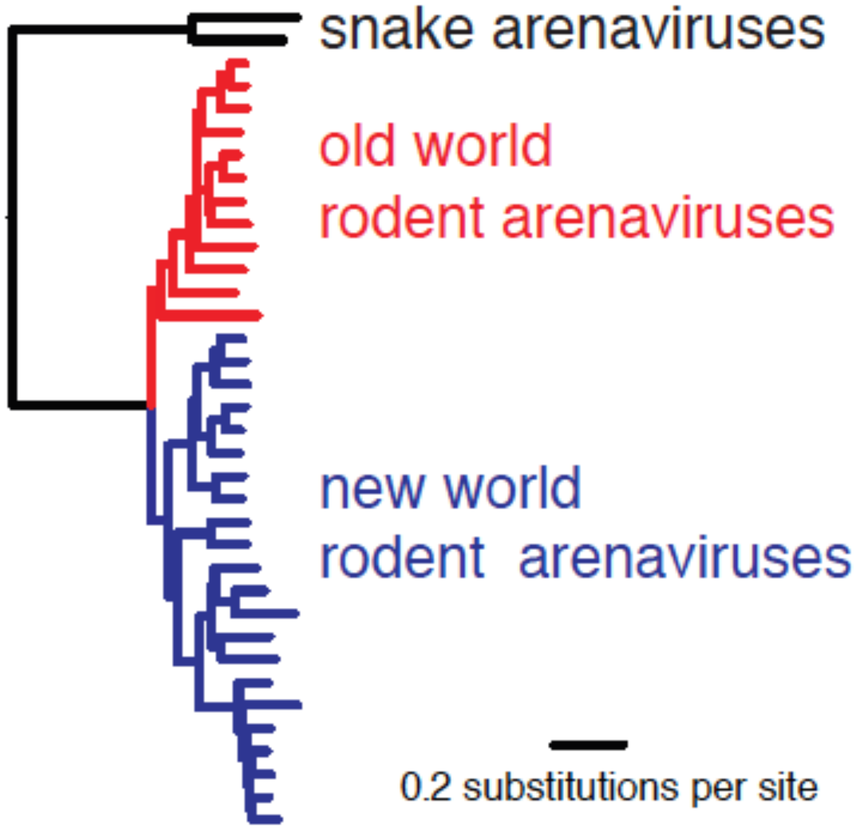
A single read pair aligning to **caprine herpesvirus-2** amongst ~0.5M mule deer reads

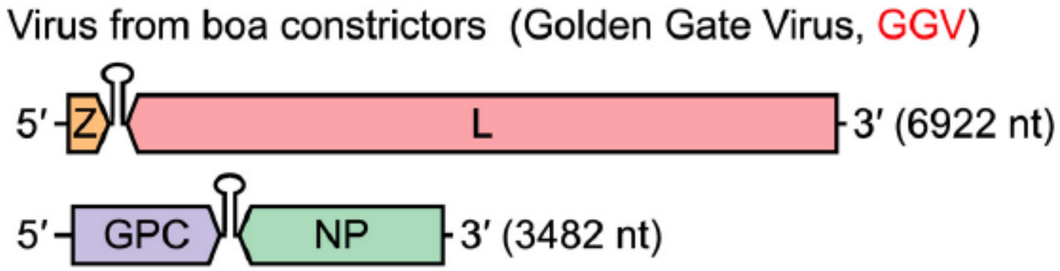
Identification of this pathogen completely consistent with histopathology

case had been tested for *ovine* herpesvirus 2

Laura Hoon-Hanks, DVM
Samples from: Karen Fox DVM, CO Parks & Wildlife

Arenavirus sequences recovered from cases of IBD but not control tissues

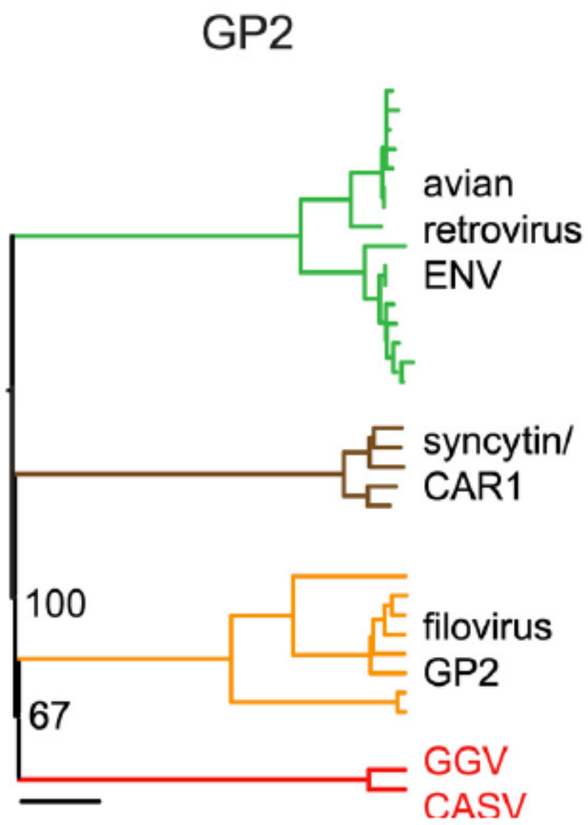




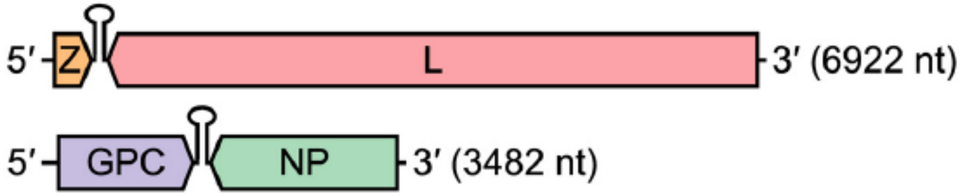
Chang and Jacobson

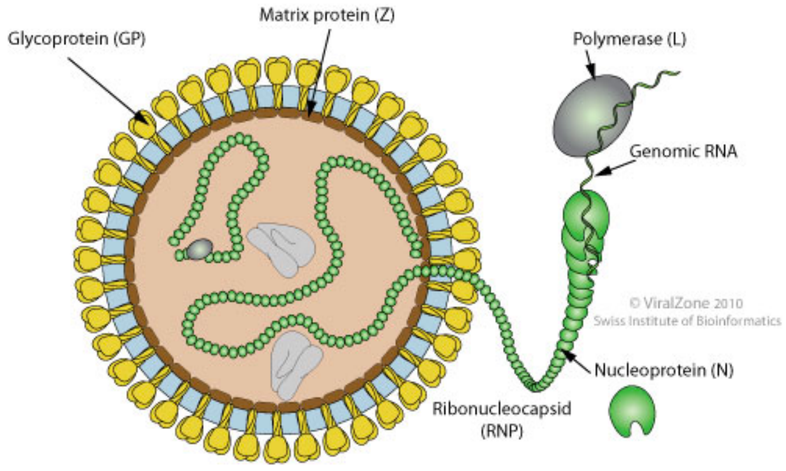
We also had identified contigs that were most similar to filovirus sequences
This was initially confusing. Coinfection by 2 different viruses?

GP gene more closely related to that of filoviruses & retroviruses than to mammal-infecting arenaviruses. Ancient recombination event?



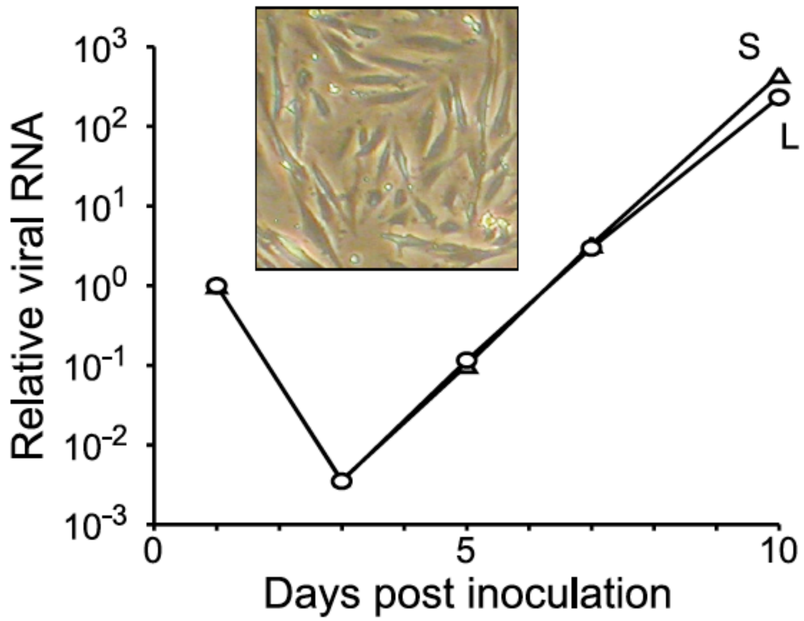
Virus from boa constrictors (Golden Gate Virus, GGV)



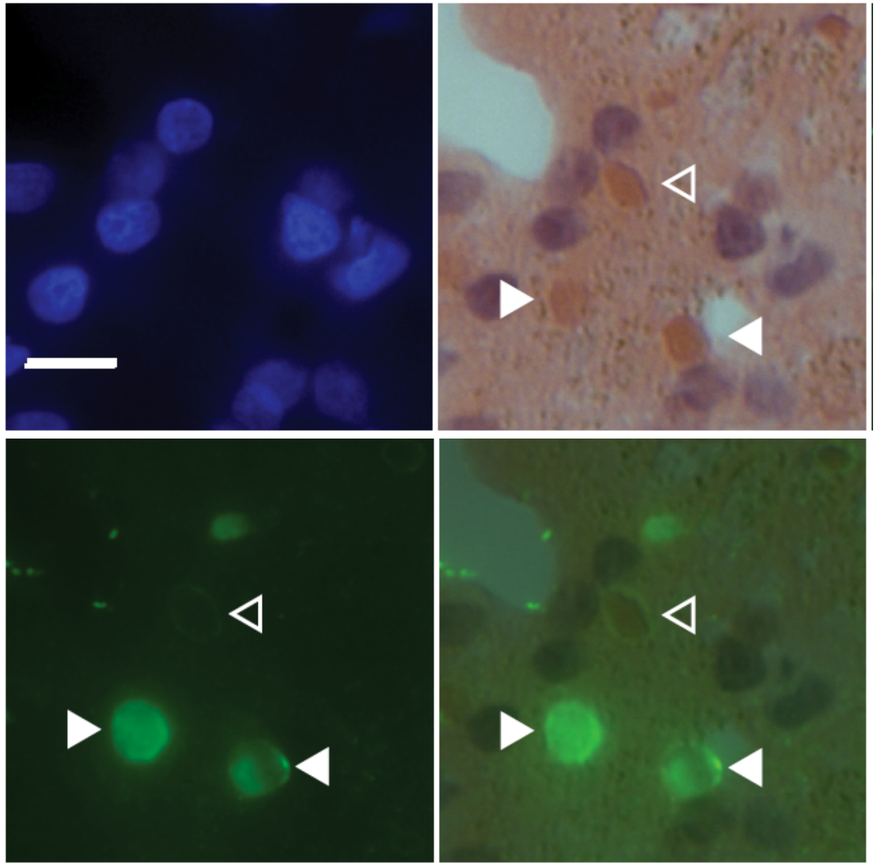


Identification of *sequences* is far from proving that a candidate pathogen causes disease

A boa constrictor cell line to isolate the virus



Inclusions stain with antibody against viral protein



Experimental infection to demonstrate disease causality

$5x10^5$ infectious units of virus intracardiac

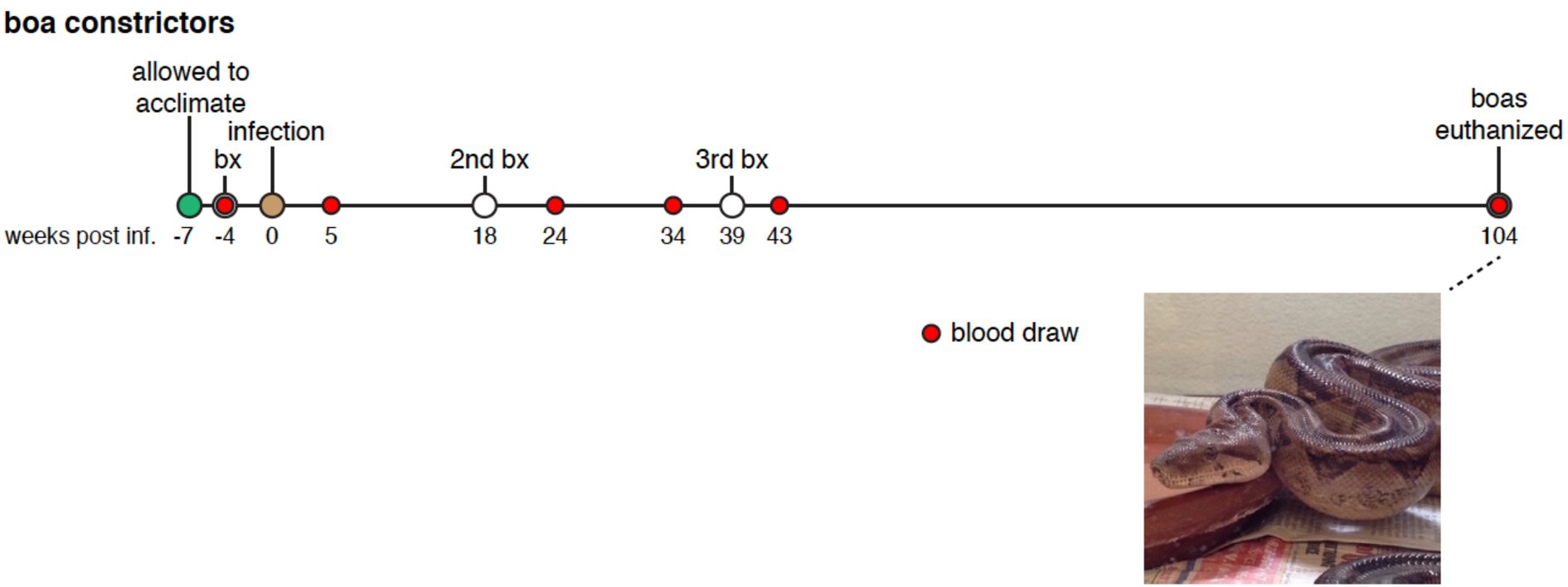
boa constrictor

ball python

Great collaborative team
Joe DeRisi, Val Garcia (UCSF)
Michelle Hawkins, David Guzman, Tracy Drazenovich, Kevin Keel (UC Davis)
Marylee Layton, Laura Hoon-Hanks (CSU)
Scott Boback (Dickinson College)

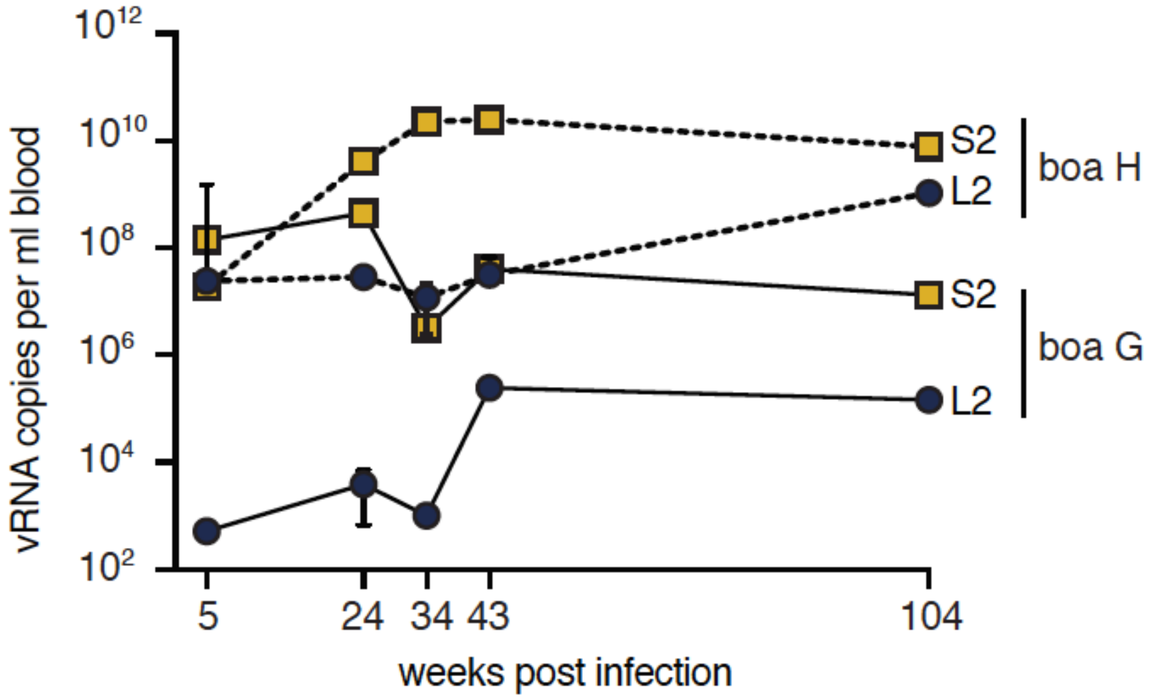
Images: National Geographic, Reptiles Magazine

Dramatically different outcome in the 2 types of snakes

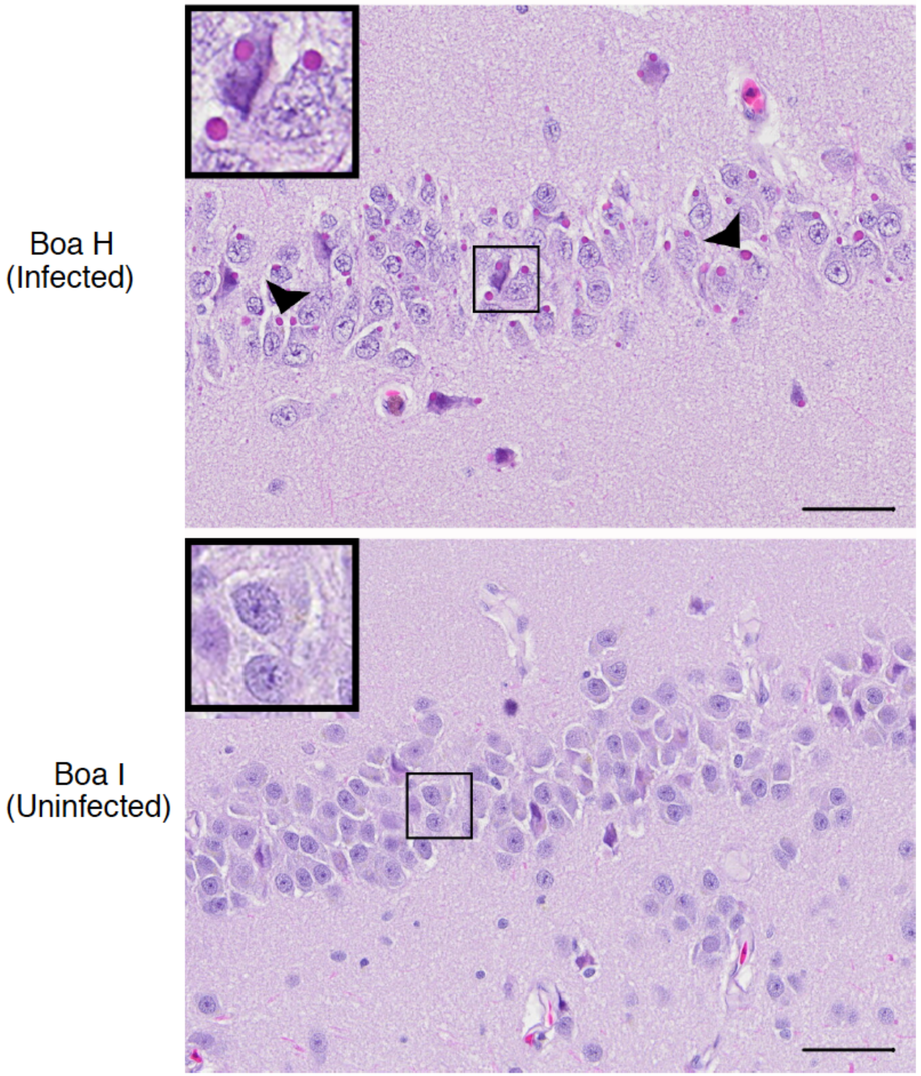


The ball pythons exhibited neurologic signs characteristic of IBD

The boa constrictors maintained high disseminated viral loads over 2 years and there were massive inclusions in every tissue in which we looked

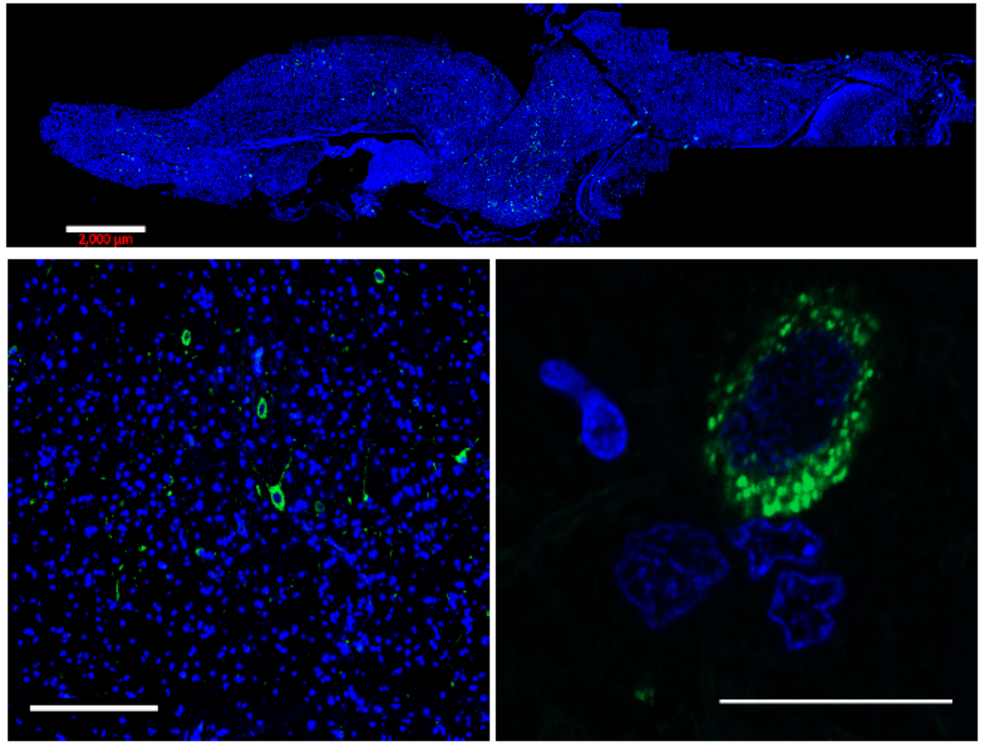


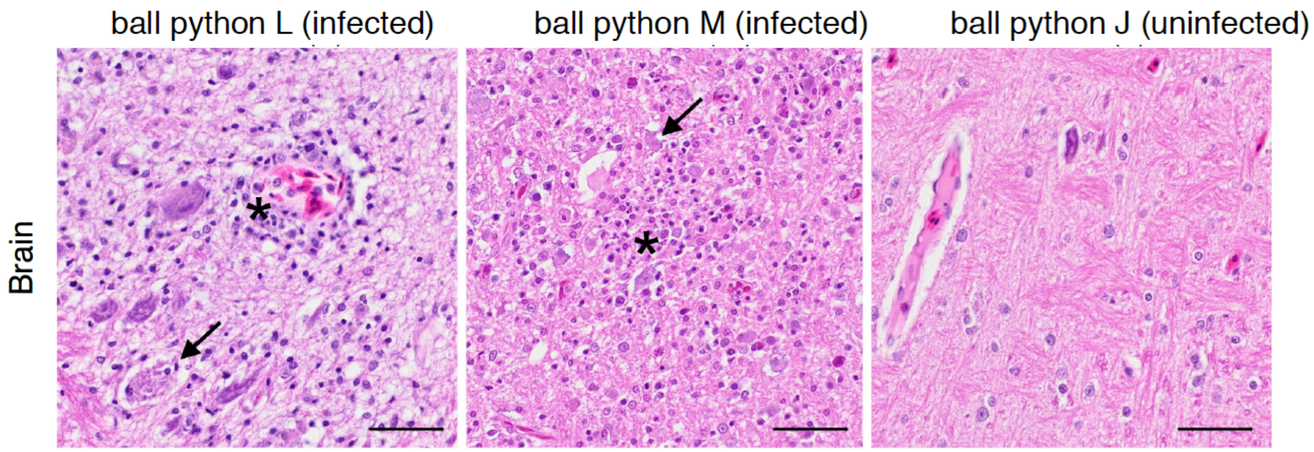
boa brains



In pythons, viral replication was only detected in the CNS, with associated inflammation, but no inclusions

B Ball python L - infected





Some conclusiveness, lots of remaining open questions

- Arenavirus infection causes inclusions and “inclusion body disease”, although inclusions per se are neither necessarily associated with nor required for disease
- Why do the 2 types of snakes respond so differently to infection?
- Would boa constrictors have gone on to develop disease?

The tragic story of Princess Diamond

From Marcio Botelho de Castro <mbcastro@unb.br>
Subject **IBD in Brazil**
To Me <Mark.Stenglein@colostate.edu>

Reply | Forward | Archive | Junk | Delete | More
3/9/16, 3:49 AM

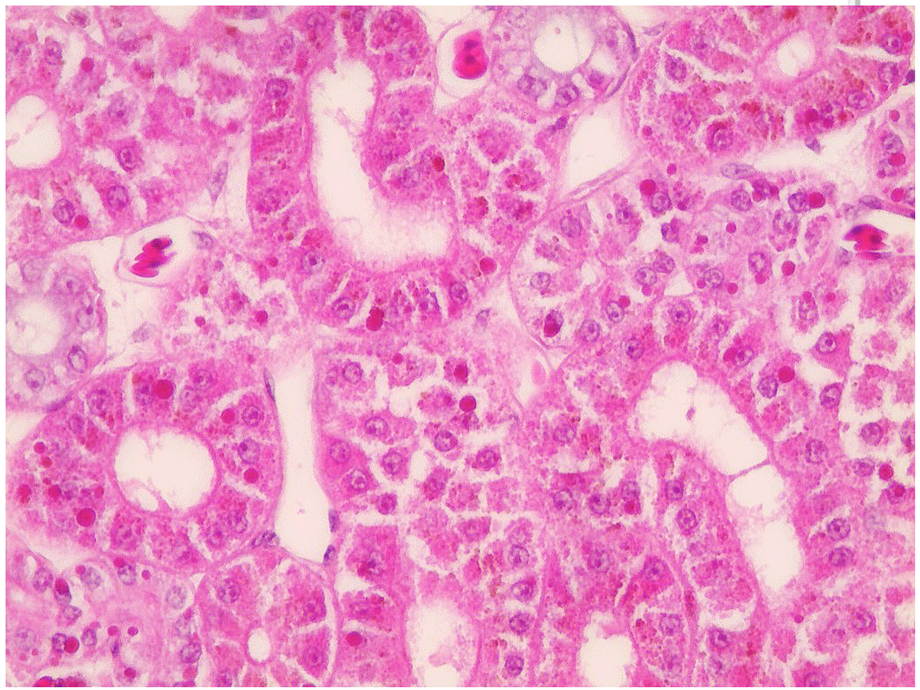
Dear Dr. Stenglein,

I am a veterinary pathology professor at the University of Brasilia, Brazil. We recently diagnose IBD outbreak in boidae snakes (Epicrates cenchria crassus - Princess Diamond) in Brazil. We necropsied one snake with many corpuscles and inclusion bodies in various tissues (attached photo) and we diagnosed in blood smears other 4 live animals with the disease and 2 without inclusions in leucocytes. They are snakes imported from the United States and we are concerned that could spread to wildlife, since IBD disease is unknown in Brazil. We would like your help for the virological diagnosis of the disease. Dr. Jacobson indicated your lab since he said that in rainbow boas (Epicrates cenchria) with inclusions in epithelial cells and circulating white cells that, with H&E staining were found to be Arenavirus negative in PCR. So right now we do not know the nature of these inclusions.
Thank you in advance.

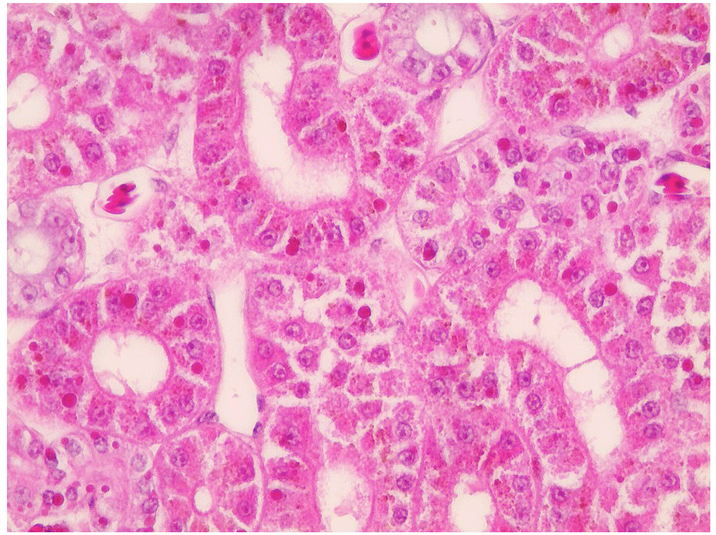
Sincerally,

Márcio B. Castro

Prof. Dr. Márcio Botelho de Castro
Veterinary Pathology Laboratory
Via L4 Norte, sn/ - Veterinary Teaching Hospital - UnB
Universidade de Brasília
Campus Universitário Darcy Ribeiro - CP. 4.508
CEP: 70.910-970 - Brasília - DF, Brazil
phone: +55 61 3107-2808



We hadn’t identified arenaviruses in snakes from Brazil before, and were happy to help



Samples from 5 snakes

Reptarenavirus+ by RT-PCR / Sanger sequencing

Used NGS to obtain complete viral genome sequences

Reported results back to Marcio

Marylee Layton

From Marcio Botelho de Castro <mbcastro@unb.br>

Subject **IBD in Brazil**

To Me <Mark.Stenglein@colostate.edu>

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3/9/16, 3:49 AM

Dear Dr. Stenglein,

I am a veterinary pathology professor at the University of Brasilia, Brazil. We recently diagnose IBD outbreak in boidae snakes (Epicrates cenchria crassus - Princess Diamond) in Brazil. We necropsied one snake with many corpuscles and inclusion bodies in various tissues (attached photo) and we diagnosed in blood smears other 4 live animals with the disease and 2 without inclusions in leucocytes. They are snakes imported from the United States and we are concerned that could spread to wildlife, since IBD disease is unknown in Brazil. We would like your help for the virological diagnosis of the disease. Dr. Jacobson indicated your lab since he said that in rainbow boas (Epicrates cenchria) with inclusions in epithelial cells and circulating white cells that, with H&E staining were found to be Arenavirus negative in PCR. So right now we do not know the nature of these inclusions.
Thank you in advance.

Sincerally,

Márcio B. Castro

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???

Boa constrictor (& related snakes) are almost always imported *into* the US

This is driven in part by a demand for novel ‘color morphs’

~ 98,500 boa constrictors per year were imported into the USA between 2005–2010

[Collis A, Fenili R, 2011]

Citando Mark Stenglein <mark.stenglein@colostate.edu>:

> By the way, Marcio, I also meant to ask: why were these particular Epicrates snakes were brought into Brazil from the USA?
>
> Cheers
>
> Mark

Brazilian Investigators Cracking the Case of Missing One-of-a-Kind Snake

Search continues for a rare boa constrictor stolen from Brazil to breed for the reptile trade.

By **Laurel Neme**, for National Geographic

PUBLISHED MAY 8, 2014

In 2006 firefighters in the Niterói district of Rio de Janeiro stumbled across a boa constrictor. But this was no ordinary boa. It was the world's first known—and remains the only known—wild leucistic specimen of *Boa constrictor constrictor*, better known as the red-tailed boa. The snake was just a few weeks old.

Almost as soon as the video was posted, collectors in online forums expressed their desire for the snake's progeny, with specialists valuing the rare specimen at $350,000 to $1 million.

2006: snake discovered and put in the Niterói Zoo, Rio de Janeiro

2011: zoo closed, the leucistic boa was “nowhere to be found”

http://news.nationalgeographic.com/news/2014/05/140507-brazil-leucistic-boa-zoo-wildlife-trafficking-world/

Totally coincidentally! a snake breeder in Utah obtained a “suspiciously similar looking” white boa constrictor in 2009

“Princess Diamond” offspring begin to be sold in 2011

Stone's initial attempts were unsuccessful, but by 2010 he'd produced a handful of babies. In 2011 he began offering Princess Diamond snakes for sale. Prices ranged from $12,500 to $25,000—ten to a hundred times higher than for other boas—reflecting the commercial value of snakes with the potential to have entirely white offspring.

http://news.nationalgeographic.com/news/2014/05/140507-brazil-leucistic-boa-zoo-wildlife-trafficking-world/

The Brazilian authorities begin to investigate

Searching immigration databases, they found that Stone had traveled to Rio de Janeiro in 2006, probably to visit the Niterói Zoo, and again in January 2009.

The latter trip raised red flags. Entry records showed that Stone and his sister, Keri Ann Stone, had entered and exited Brazil on foot on the same day (January 22) at a little village (Bonfim) in the extreme north of the country, on the border with Guyana.

"That did not make sense for me," said Franco Perazzoni, a special agent with the BFP. "Why would this guy come to Brazil and spend just a day in a little village in the middle of the Amazon?"

http://news.nationalgeographic.com/news/2014/05/140507-brazil-leucistic-boa-zoo-wildlife-trafficking-world/

Wait, what ?!?!

In addition, four days earlier authorities had questioned Stone and his sister at the Manaus airport. Officials had suspected the two of drug trafficking because the sister was wearing a fake pregnancy belly (it had a hollow compartment), which neither she nor her brother could adequately explain. Also, they both had multiple tickets out of Brazil by various modes of transportation, a ploy smugglers often use to avoid surveillance.

!!!!

http://news.nationalgeographic.com/news/2014/05/140507-brazil-leucistic-boa-zoo-wildlife-trafficking-world/

It’s not looking good

Phone records of zoo administrator Candiotto proved she was in Manaus at the same time as Stone, and that her husband, José Carlos Schirmer, who also worked at the zoo, traveled with Stone that same day.

Examination of their banking records showed a deposit at that time of 500,000 reais (about $250,000).

http://news.nationalgeographic.com/news/2014/05/140507-brazil-leucistic-boa-zoo-wildlife-trafficking-world/

Stone pled guilty to unlawfully transporting wildlife into the United States in 2014
The original snake supposedly died, but the offspring were repatriated in 2015

U.S. Attorneys » District of Utah » News

Department of Justice

U.S. Attorney's Office

District of Utah

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FOR IMMEDIATE RELEASE — Wednesday, January 8, 2014

Brother And Sister Charged With Conspiring To Unlawfully Import Rare White Boa Constrictor Into The United States From Brazil

SALT LAKE CITY - A federal grand jury returned an indictment Wednesday afternoon charging two Utah residents with conspiring to unlawfully import a rare white leucistic boa constrictor from Brazil into the United States for the purpose of breeding it with other boa constrictors and selling its offspring for a profit.

Jeremy Stone, age 39, of Lindon, and his sister, Keri Ann Stone, age 34, of Midvale, are charged in the four-count indictment with conspiracy to unlawfully import the snake into the United States; unlawfully importing the snake into the country; transporting the snake knowing it was imported into the United States contrary to law; and making and submitting false records for wildlife imported into the United States.

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JUSTICE NEWS

Department of Justice

Office of Public Affairs

FOR IMMEDIATE RELEASE — Wednesday, June 17, 2015

United States Repatriates Seven Boa Constrictors to Brazil

Seven boa constrictors seized in connection with an illegal wildlife smuggling scheme have been returned to the government of Brazil, announced Assistant Attorney General Leslie R. Caldwell of the Justice Department's Criminal Division and U.S. Attorney John W. Huber of the District of Utah.

"This case exhibited many of the hallmarks that make illegal wildlife trafficking a growing international scourge, including actors motivated by greed who illegally smuggled rare and precious wildlife across international boundaries," said Assistant Attorney General Caldwell. "The return of the precious snakes to Brazil brings to an end this years-long international saga, and serves as an example of our commitment to working with law enforcement partners in Brazil and elsewhere to combat transnational crime."

https://www.justice.gov/opa/pr/united-states-repatriates-seven-boa-constrictors-brazil

Justice served?

By REBECCA KAPLAN / CBS NEWS / June 17, 2015, 2:45 PM

U.S. repatriates seven snakes to Brazil by plane

One of the snakes recovered from a reptile collector and breeder in Utah who illegally transported the snake's mother, a rare white boa constrictor, from Brazil to the U.S. / DEPARTMENT OF JUSTICE

3 Comments / Share / Tweet / Stumble / Email

The Justice Department (DOJ) announced Wednesday that it has repatriated seven rare boa constrictors to the government of Brazil six years after their mother was brought to the U.S. in an illegal wildlife smuggling scheme.

In other words, DOJ sent Brazil a few snakes on a plane.

http://www.cbsnews.com/news/us-repatriates-7-snakes-to-brazil-by-plane/

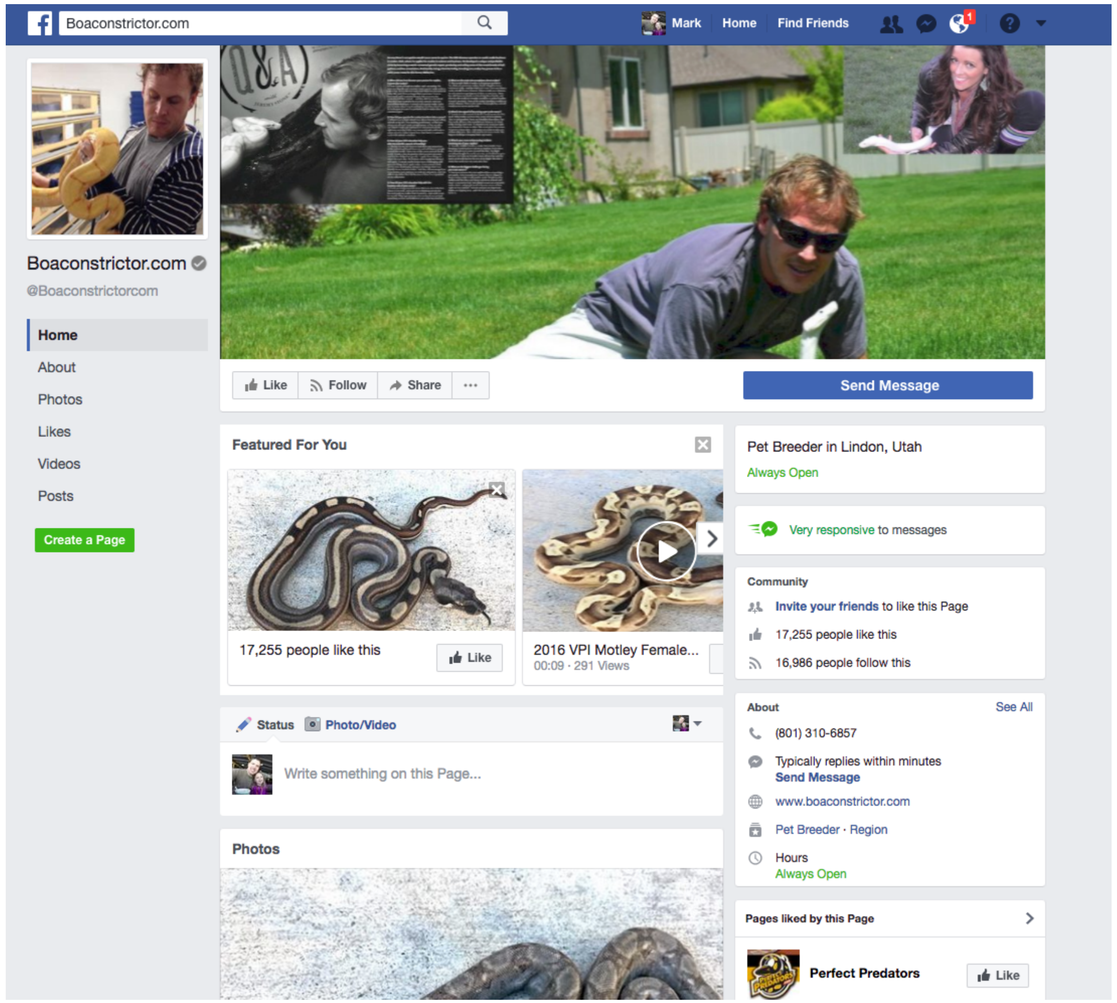
One of the descendent snakes died during quarantine & was diagnosed post-mortem with IBD

All 5 descendents we tested were infected.

The virus genome sequences are most closely related to ones recovered from a boa constrictor in Louisiana in 2010.

United States
North Atlantic Ocean
Gulf of California
Mexico
Gulf of Mexico
Cuba
Dominican Republic
Puerto Rico
Guatemala
Caribbean Sea
Nicaragua
Costa Rica
Panama
Venezuela
Guyana
French Guiana
Suriname
Colombia
Ecuador
Peru
Brazil
Bolivia
Paraguay
Chile
Uruguay
Argentina

Jeremy Stone is going strong,
and still shipping leucistic boa constrictors around the world

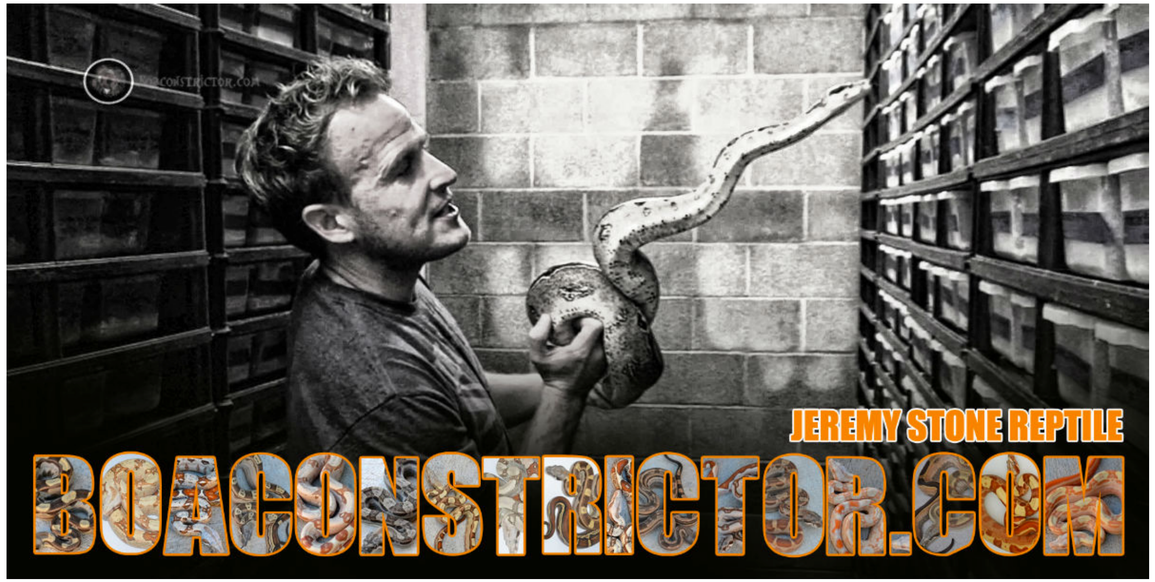


Why does this matter in the bigger picture?

Apparent ongoing, tumultuous reptarenavirus evolution in captive snake populations

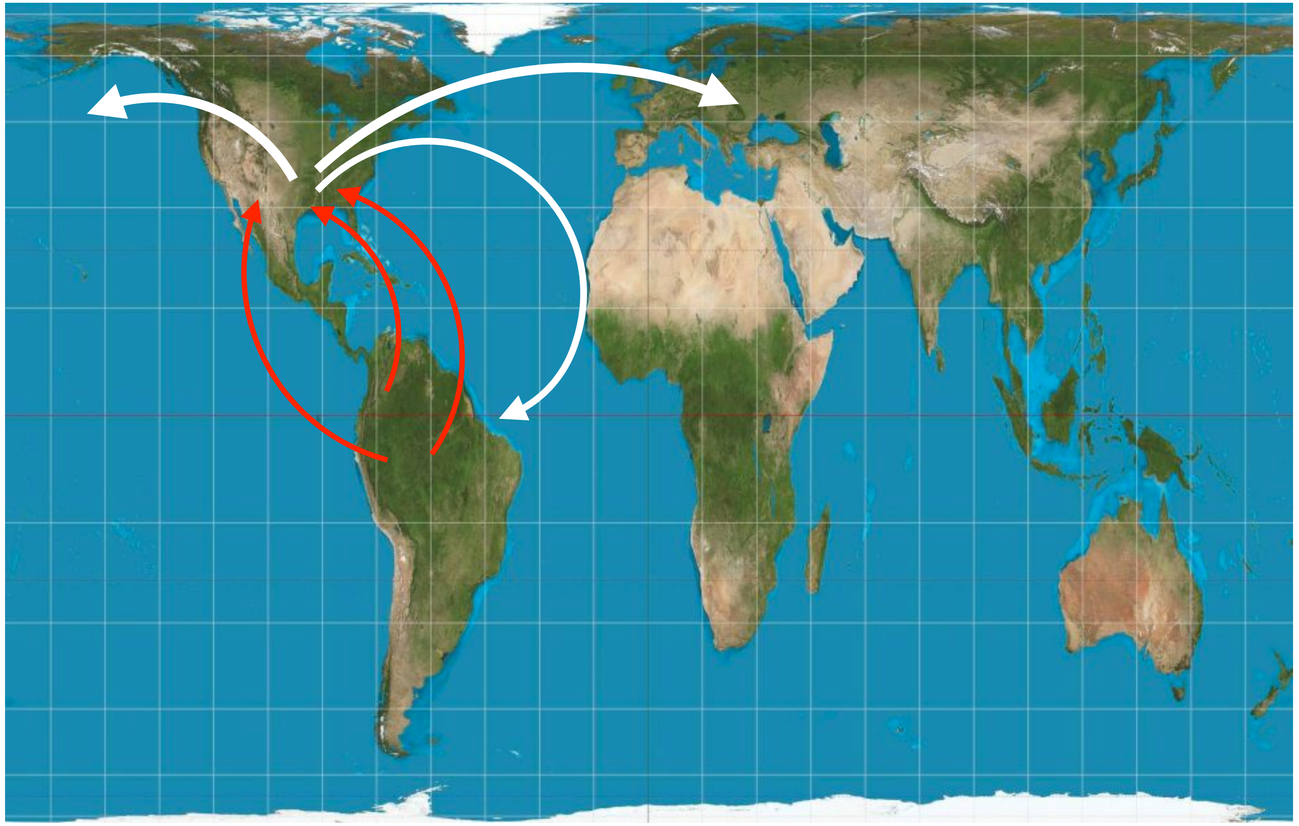
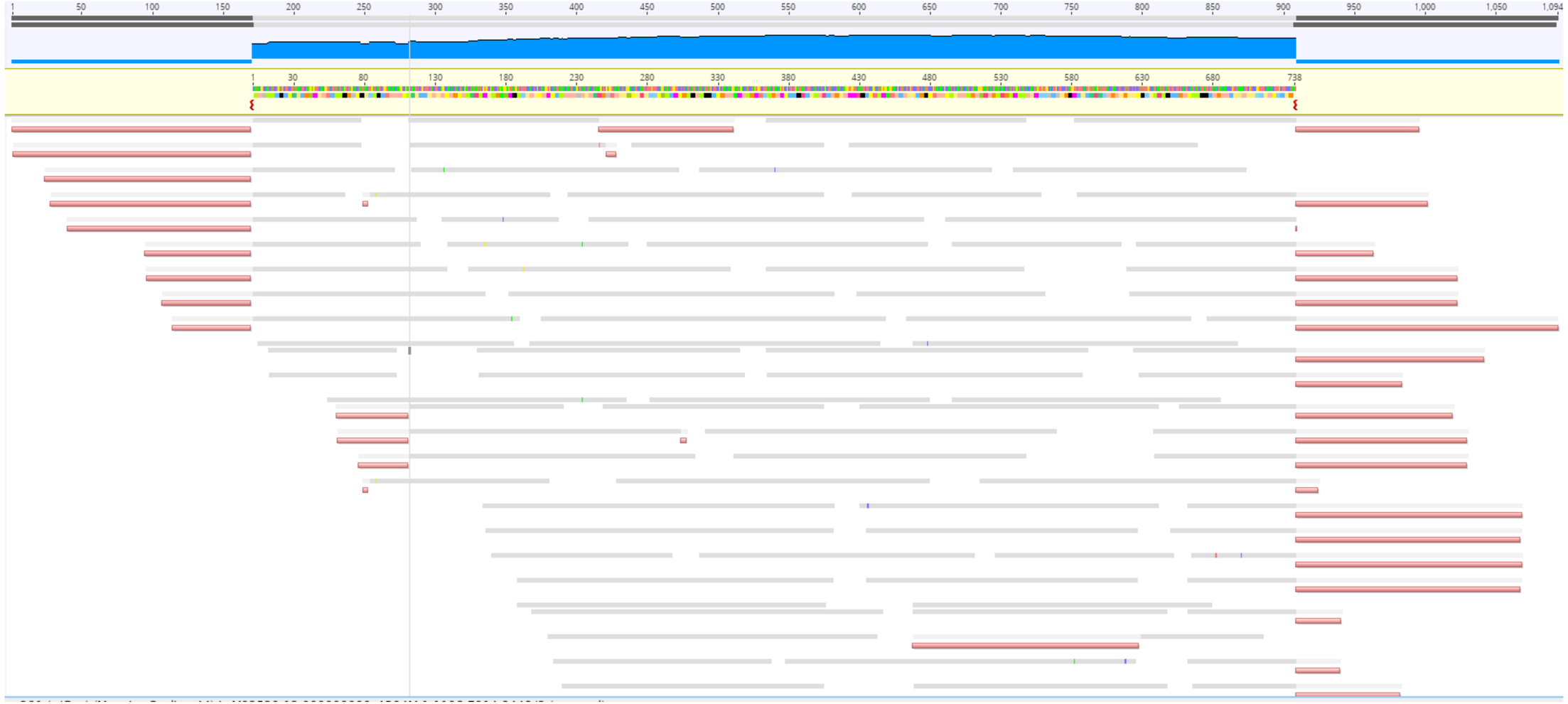
- Widespread recombination
- Reassortment
- High levels of co-infecton
- Apparently different dynamics of S & L segments

Reptile breeders in general don’t follow the best infectious disease prevention & control practices



Are captive breeding populations serving as a crucible for reptarenavirus evolution?

hypothesis: reptarenavirus genetic diversity that existed in wild snake populations is being mixed in captive snake populations



mitochondrial sequences align perfectly to boa constrictor sequences