Monitoring of extreme ecological niches for novel and reoccurring zoonoses

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Introduction

Caves offer an environment with extreme conditions – lack of light, fewer nutrients, high humidity, low temperature, and others, which implies a high level of adaptation of the organisms.

Bats are common inhabitants of caves. Bats are mammals of the order Chiroptera and include about 1460 species. They are the only mammals able to fly. Bats possess some peculiar characteristics, such as unique physiology, metabolism and immune system.

In Europe 47 bat species are encountered and 33 of these are found in Bulgaria and 11 of these bat speices inhabit caves.

All bat species encountered in Bulgaria are prevalently insectivores.
Bats can eat up to 20 000 insects per night.
Bats are our most important natural predators of night-flying insects consuming mosquitoes, moths, beetles, crickets, leafhoppers, chinch bugs, and much more! Many of these insects are serious agricultural or forests pests, and others spread disease to humans or livestock. Every year bats save us billions of euros in pest control by simply eating insects.

Bats have been identified as an important reservoir hosts for numerous emerging infectious viral, bacterial, fungal and protozoa pathogens.

It should be underlined that bats are main or occational reservoirs of many viral (rabies, Marburg, Ebola, Hendra, Nipah, SARS-CoV, etc.), bacterial (Bartonella, Leptospira, Mycoplasma, Brucella, Borrelia, Coxiella, Ehrlichia, Francisella, and Rickettsia), fungi (*Pseudogymnoascus destructans* - white-nose syndrome, *Histoplasma capsulatum* and others) and protozoa (malaria, toxoplasmosis, leishmaniasis, trypanosomiasis) infections.

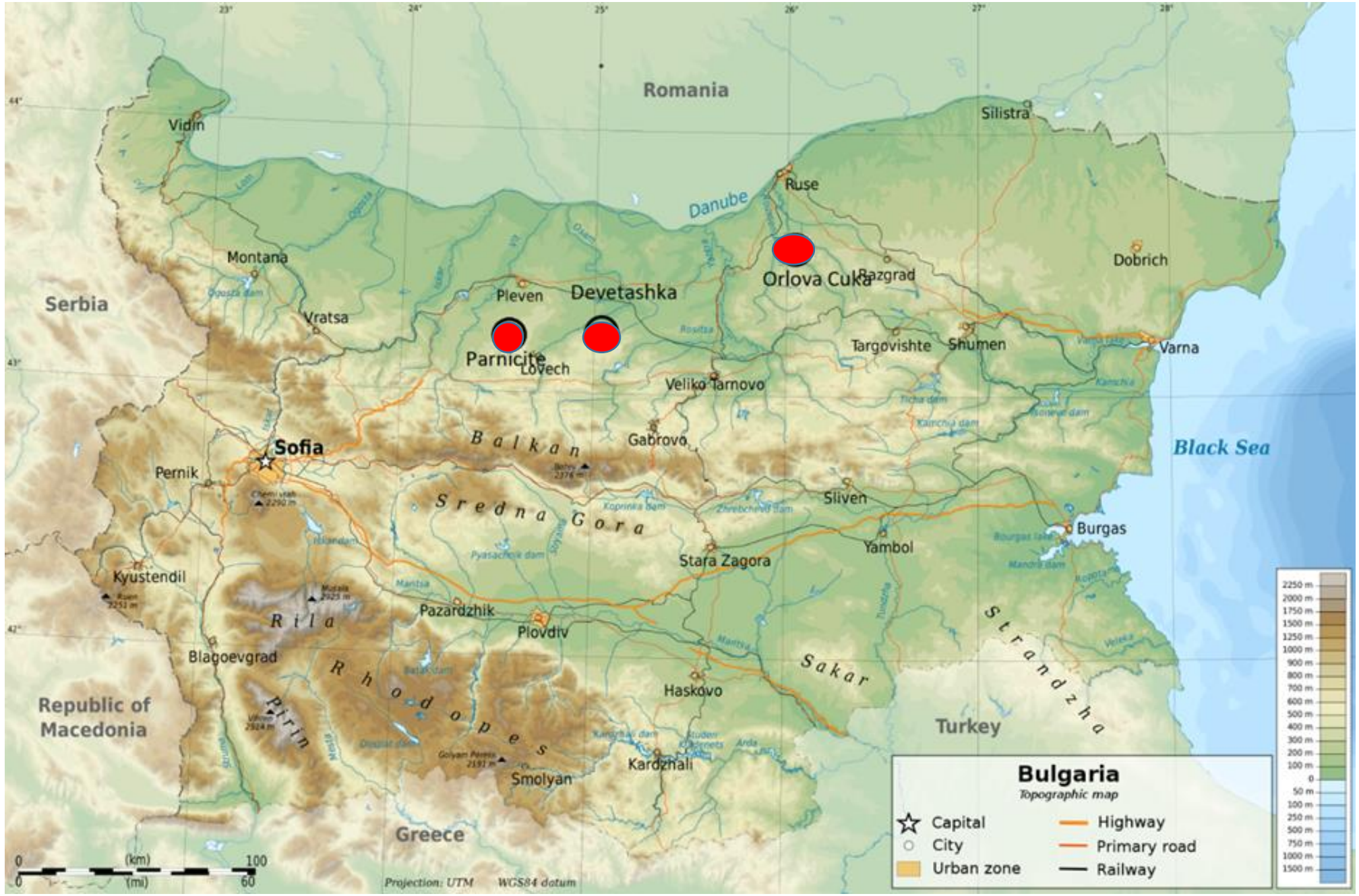
Aim of our study was to monitor bats and environmental samples from Bulgarian caves for novel or reoccurring zoonoses.

Our working hypothesis is that bats are infected, but do not get ill from the above mentioned microbial infections. These infections remain latent. Blood, tissue, urine form bats and environmental samples (guano, water and soil) would be suitable to monitor for infectious pathogens.

Here, we report analysis of bacterial pathogens identified in the blood of bats by targeted and shotgun sequencing and bioinformatic analysis.

Materials and Methods

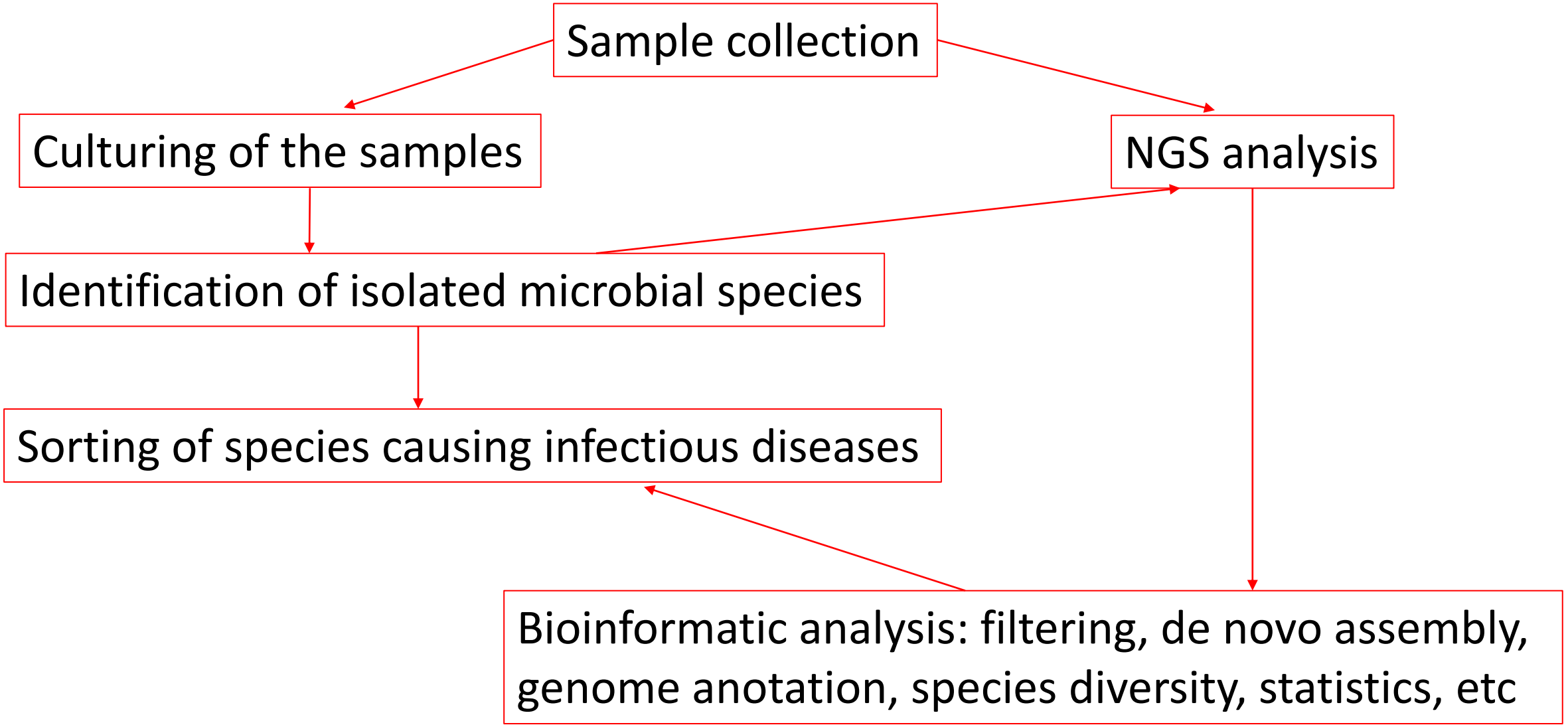
Samples were collected from Orlova Chuka, Devetashka, and Parnitsite in 2022. Bats were caught and blood and tissue samples were obtained. Bat parasites such as ticks and mosquitoes were also collected. Blood of 120 bats was collected and pooled in 13 samples. From each bat 30 µl of blood was collected. Soil, water and guano samples were also collected. Total DNA was isolated from the samples, measured, and prepared for NGS analysis. Sequencing was performed using specific targeting 16S and ITS NGS analysis was applied using Illumina next-generation sequencers. Shotgun sequencing was performed at >6 GB depth. Taxonomic identification of the cleaned raw reads was performed by Kraken2 tool with Refseq NCBI database for Prokaryote, Protozoa, Fungi and viruses in www.usegalaxy.eu environment. OTU table was generated with Bracken tool and visualized by Pavian software.


Romania
Serbia
Republic of Macedonia
Greece
Turkey
Black Sea
Danube
Vidin
Montana
Vratsa
Pleven
Devetashka
Parnicite
Lovech
Ruse
Orlova Cuka
Razgrad
Silistra
Dobrich
Targovishte
Shumen
Varna
Veliko Tarnovo
Gabrovo
Sofia
Pernik
Kyustendil
Blagoevgrad
Pazardzhik
Plovdiv
Stara Zagora
Sliven
Yambol
Burgas
Haskovo
Kardzhali
Smolyan
Balkan
Sredna Gora
Rila
Rhodopes
Pirin
Sakar
Strandzha
Bulgaria
Topographic map
Capital
City
Urban zone
Highway
Primary road
Railway
Projection: UTM
WGS84 datum
0
100
(km)
0
60
(mi)

Devetashka cave

Orlova chuka cave

General scheme



Results

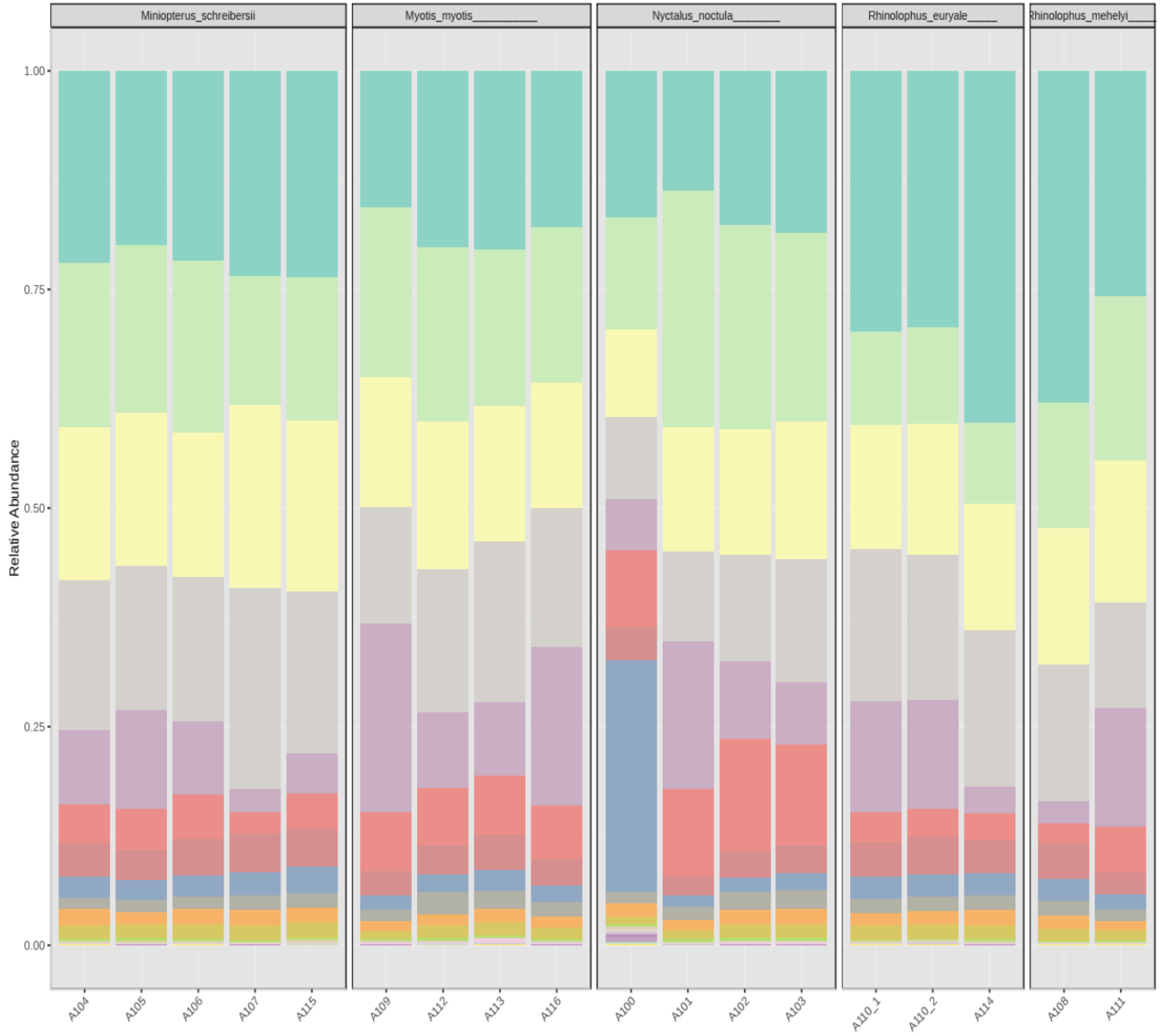
We tested blood samples collected form 5 bat species - *Nyctalus noctula, Rhilophus euryale, Rhinolophus mehelyi, Myotis myotis, and Miniopterus schreibersii.*

Sample ID	Sample_description	Sample_description 2	Sample_description 3
A100	сборна от 14 прилепа Деветашка H. Нок	Nyctalus noctula	
A101	сборна от 7 прилепа мъжки Деветашка H нок	Nyctalus noctula	
A102	сборна H нок 3 прилепа женски Деветашка	Nyctalus noctula	
A103	H нок 1 мъжки Деветашка	Nyctalus noctula	
A104	MSch 4 женски Деветашка	Miniopterus schreibersii	
A105	MSch 4 мъжки Деветашка	Miniopterus schreibersii	
A106	MSch 12 прилепа мъжки и женски Деветашка	Miniopterus schreibersii	
A107	сърце от миниоптерус Деветашка	Miniopterus schreibersii	
A108	далак от Rh Mehely	Rhinolophus mehelyi	
A109	Mdal сборна Орлова чука	Myotis далак?	
A110_1	Rheur сборна Орлова чука	Rhinolophus euryale	Пробата A100 е разделена на две части
A110_2	Rheur сборна Орлова чука	Rhinolophus euryale	Пробата A100 е разделена на две части
A111	Rh meh сборна Орлова чука	Rhinolophus mehelyi	
A112	M meu сборна Орлова чука	Myotis myotis	
A113	M cap сборна Орлова чука	Myotis myotis	
A114	далак от Rheur Орлова чука	Rhilophus euryale	
A115	MSch сборна 22 прилепа Парниците	Miniopterus schreibersii	
A116	сборна от парниците	?кръв ?видове	
A117_control	контрола от реактиви	контрола	

Microbial DNA is < 0.3% of the total.

Name	Number of raw reads	Classified reads	Chordate reads	Artificial reads	Unclassified reads	Microbial reads	Bacterial reads	Viral reads	Fungal reads	Protozoan reads
A100	26,131,251	2,630,974	2,487,390	0	23,500,277	141,661	99,557	1,020	3,917	5,421
A101	24,701,891	2,578,473	2,459,345	0	22,123,418	117,275	78,924	1,164	3,403	3,461
A102	26,280,926	2,718,189	2,613,545	0	23,562,737	103,116	60,031	995	3,451	4,276
A103	23,683,418	4,378,993	4,182,571	0	19,304,425	194,176	131,418	1,939	7,060	6,871
A104	25,489,074	2,904,269	2,826,560	0	22,584,805	76,039	40,118	727	3,107	2,746
A105	24,099,966	4,686,389	4,518,596	0	19,413,577	165,411	100,690	1,604	6,958	7,481
A106	23,153,323	2,630,483	2,551,594	0	20,522,840	77,850	39,038	873	2,935	6,230
A107	21,726,421	4,481,902	4,311,409	0	17,244,519	166,588	93,326	1,738	6,766	8,454
A108	18,901,712	2,916,029	2,848,136	0	15,985,683	65,764	33,386	746	1,954	1,778
A109	23,378,440	4,320,092	4,119,237	0	19,058,348	198,753	131,546	2,222	6,495	16,301
A110_1	18,982,152	4,505,294	4,391,579	0	14,476,858	112,726	72,139	839	3,972	3,998
A110_2	4,190,529	988,668	963,321	0	3,201,861	25,111	16,057	207	875	927
A111	25,945,013	3,567,195	3,479,543	0	22,377,818	85,251	50,220	747	4,246	3,068
A112	26,454,129	5,134,864	4,941,042	0	21,319,265	192,032	115,259	2,818	7,049	20,602
A113	25,070,421	4,667,044	4,491,961	0	20,403,377	173,806	103,044	1,661	6,931	15,561
A114	19,841,952	2,782,037	2,735,167	0	17,059,915	46,231	27,449	388	2,113	1,593
A115	22,304,487	4,376,634	4,232,756	0	17,927,853	142,579	83,741	1,343	6,450	7,876
A116	28,398,263	5,493,549	5,270,868	0	22,904,714	221,352	136,599	2,500	7,584	21,249
A117	4,156,553	1,917,918	586,754	0	2,238,635	1,327,334	1,305,809	1,488	14,054	531

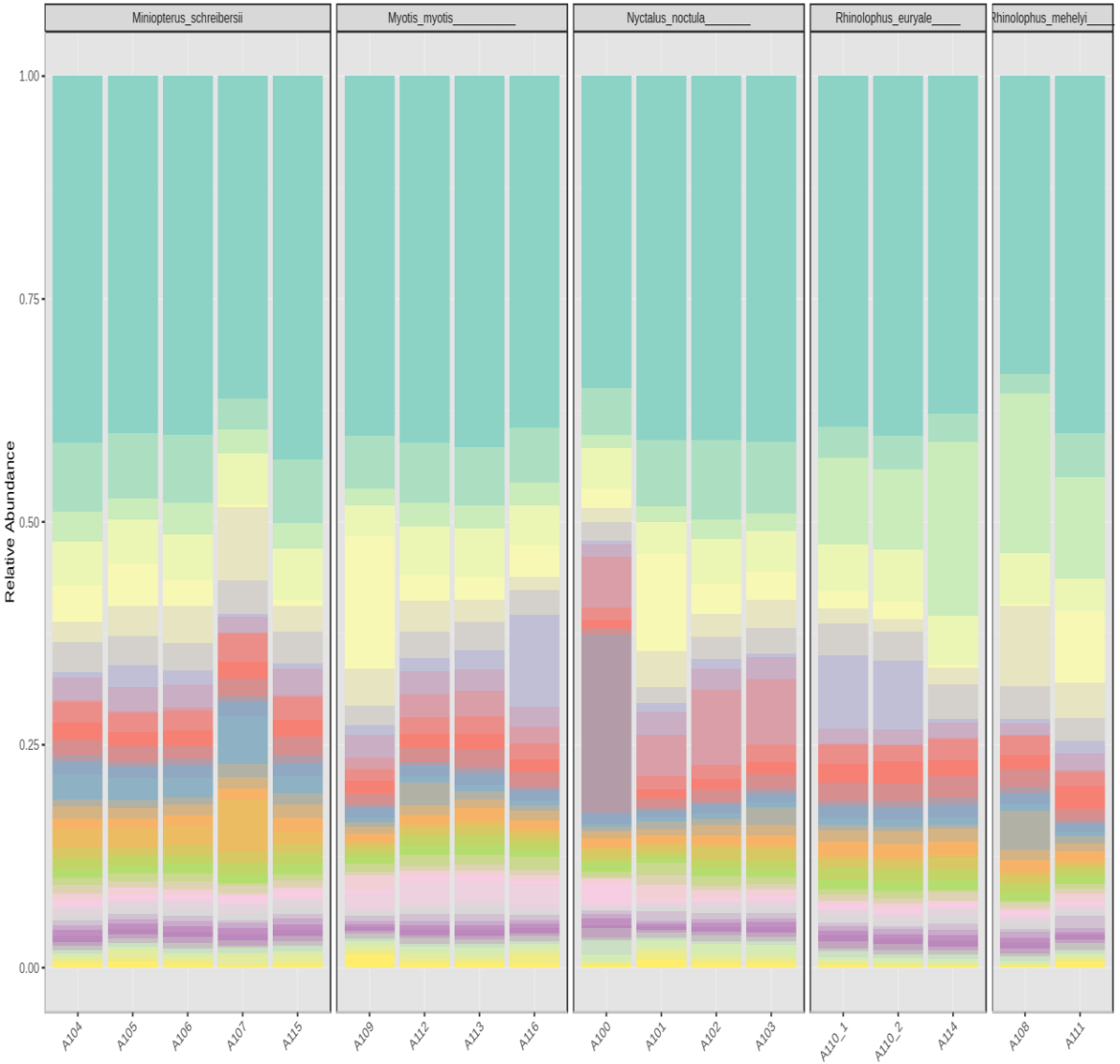
Bacterial phyla/class diversity by bat species



PHYLUM

- Firmicutes
- Actinobacteria
- Gammaproteobacteria
- Bacteroidetes
- Alphaproteobacteria
- Betaproteobacteria
- delta_epsilonB_subdivisions
- Tenericutes
- Spirochaetia
- Fusobacteriia
- Cyanobacteria_MelainabacteriaE
- Deinococcus
- Planctomycetes
- Deltaproteobacteria
- Verrucomicrobia
- Thermodesulfobacteria
- Oligoflexia
- Aquificae
- Endomicrobia
- Cyanobacteria
- Hydrogenophilalia

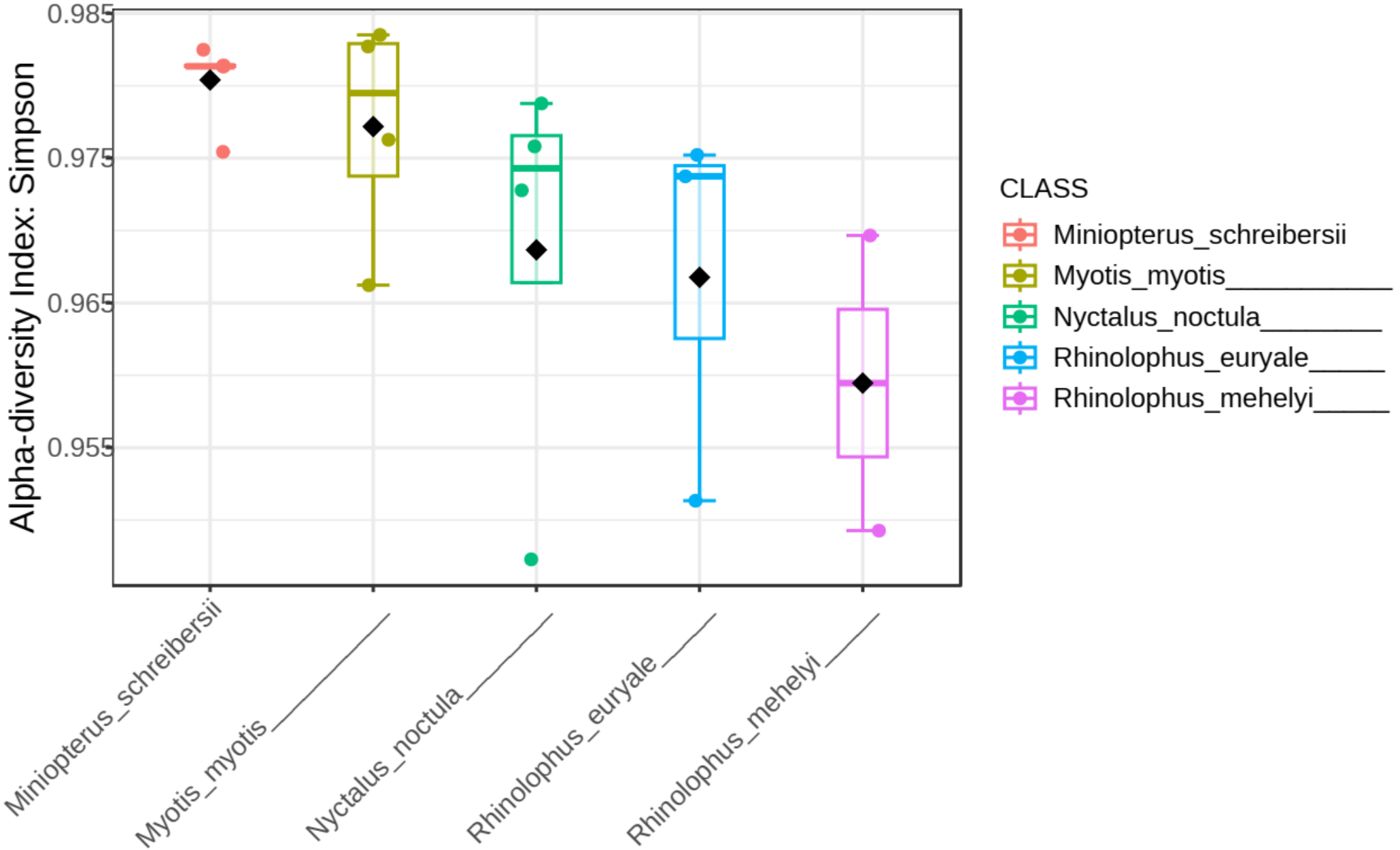
Bacterial genera diversity by bat species



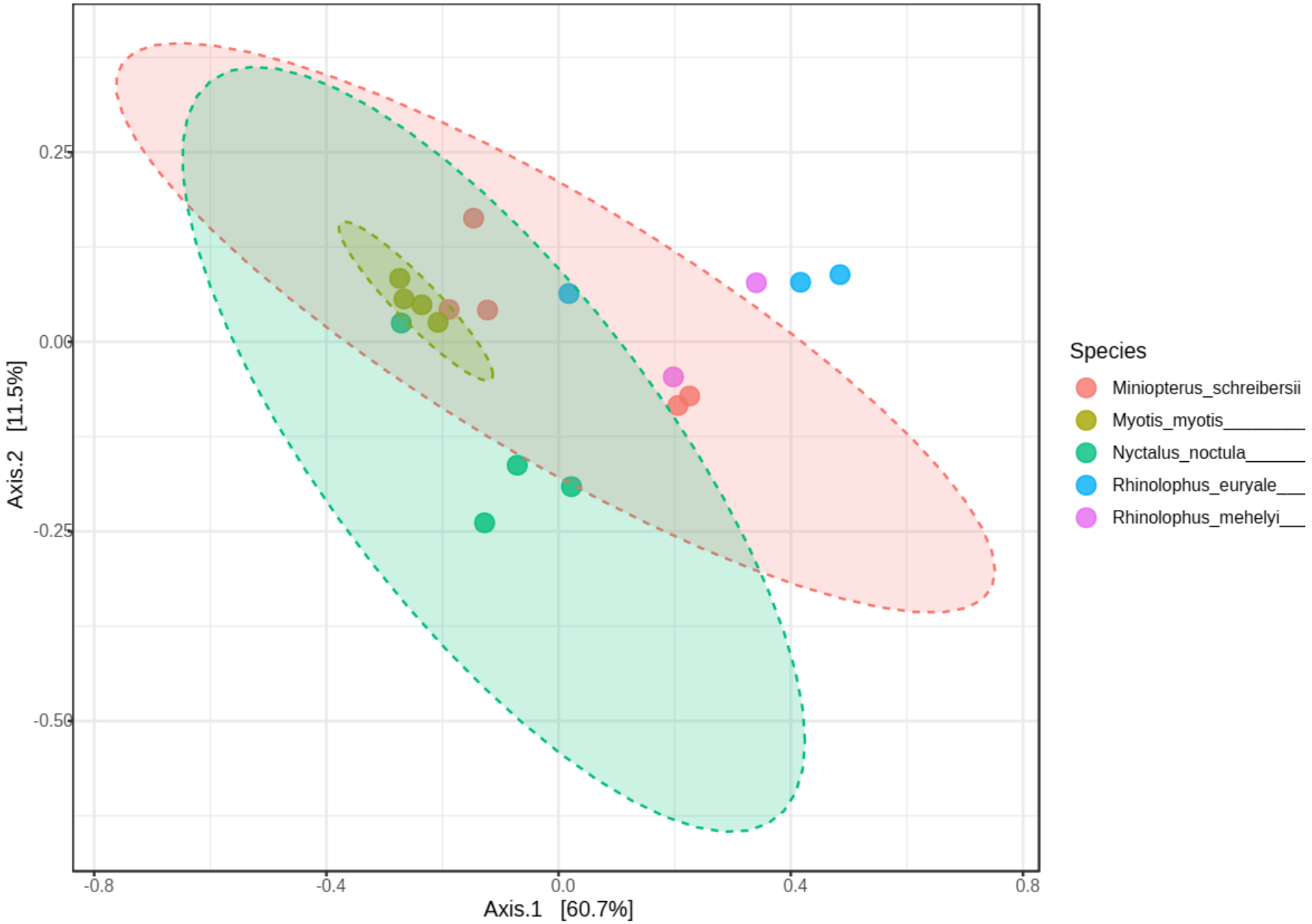
>40 GENERA

- Others
- Streptomyces
- Staphylococcus
- Clostridium
- Bradyrhizobium
- Mycobacterium
- Bacillus
- Bartonella
- Pseudomonas
- Paraburkholderia
- Flavobacterium
- Acinetobacter
- Chryseobacterium
- Mycoplasma
- Campylobacter
- Shewanella
- Klebsiella
- Paenibacillus
- Streptococcus
- Sphingobacterium
- Fusobacterium
- Vibrio
- Polaribacter
- Nocardioides
- Enterococcus
- Corynebacterium
- Spiroplasma
- Aquimarina
- Nostoc
- Bacteroides
- Mycolicibacterium
- Blattabacterium
- Buchnera
- Pseudoalteromonas
- Aliarcobacter
- Burkholderia
- Mycoplasmopsis
- Treponema
- anthomonas
- Microbacterium
- Sphingomonas

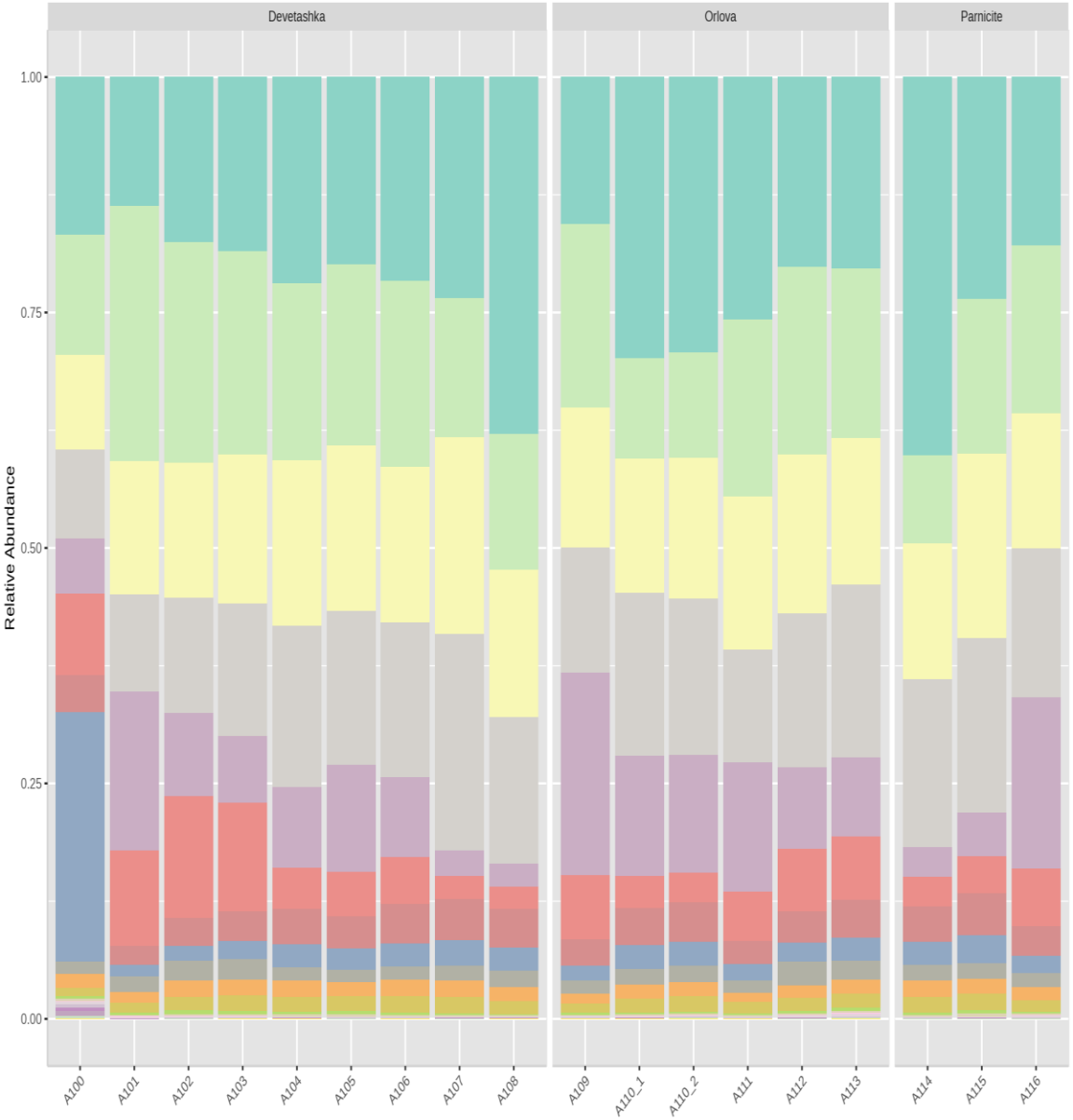
Bacterial alpha biodiversity by bat species – Simpson index



Bacterial beta biodiversity between bat species - Bray-Curtis index



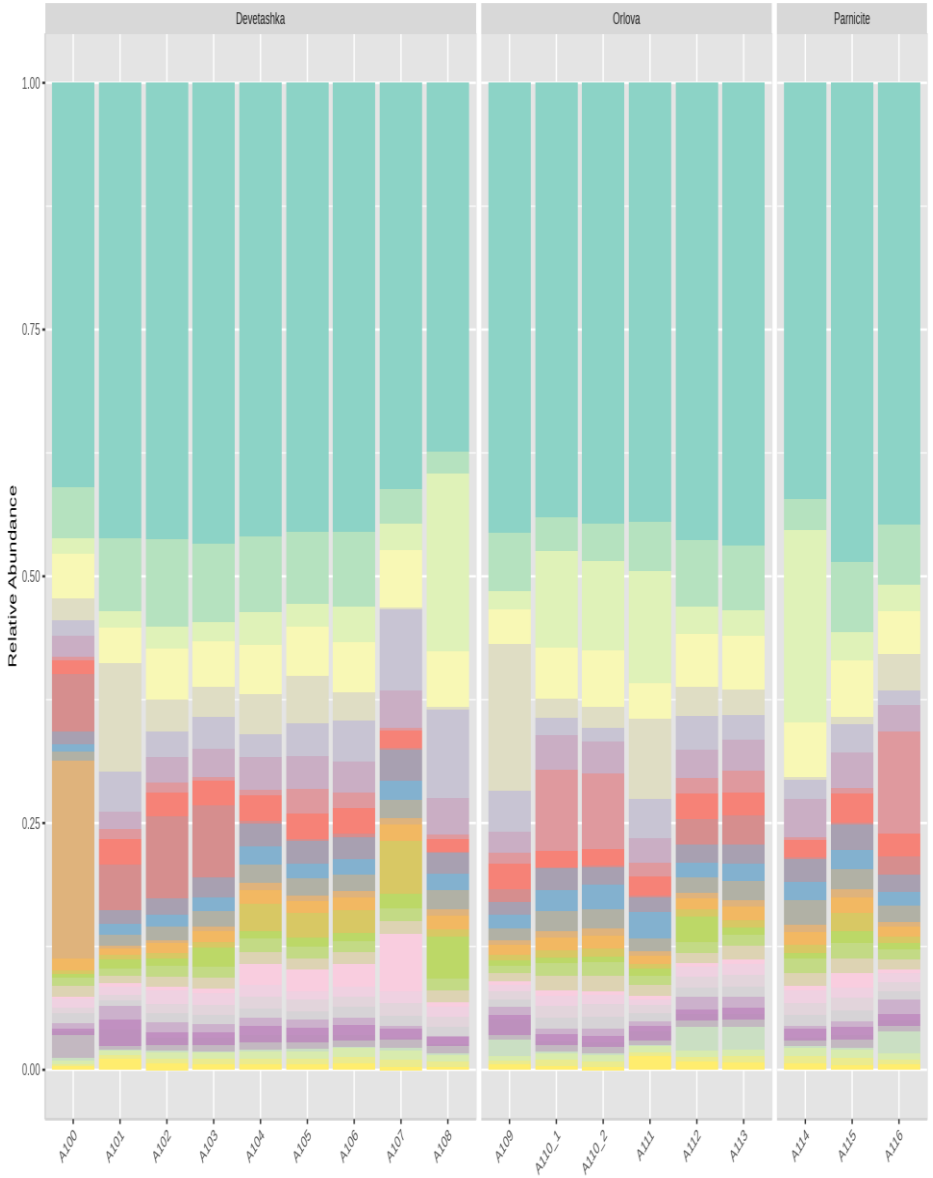
Bacterial phyla/class by cave



PHYLUM/CLASS

- Firmicutes
- Actinobacteria
- Gammaproteobacteria
- Bacteroidetes
- Alphaproteobacteria
- Betaproteobacteria
- delta_epsilonB_subdivisions
- Tenericutes
- Spirochaetia
- Fusobacteriia
- Cyanobacteria_MelainabacteriaB_group
- Deinococcus
- Planctomycetes
- Deltaproteobacteria
- Verrucomicrobia
- Thermodesulfobacteria
- Oligoflexia
- Aquificae
- Endomicrobia
- Cyanobacteria
- Hydrogenophilalia

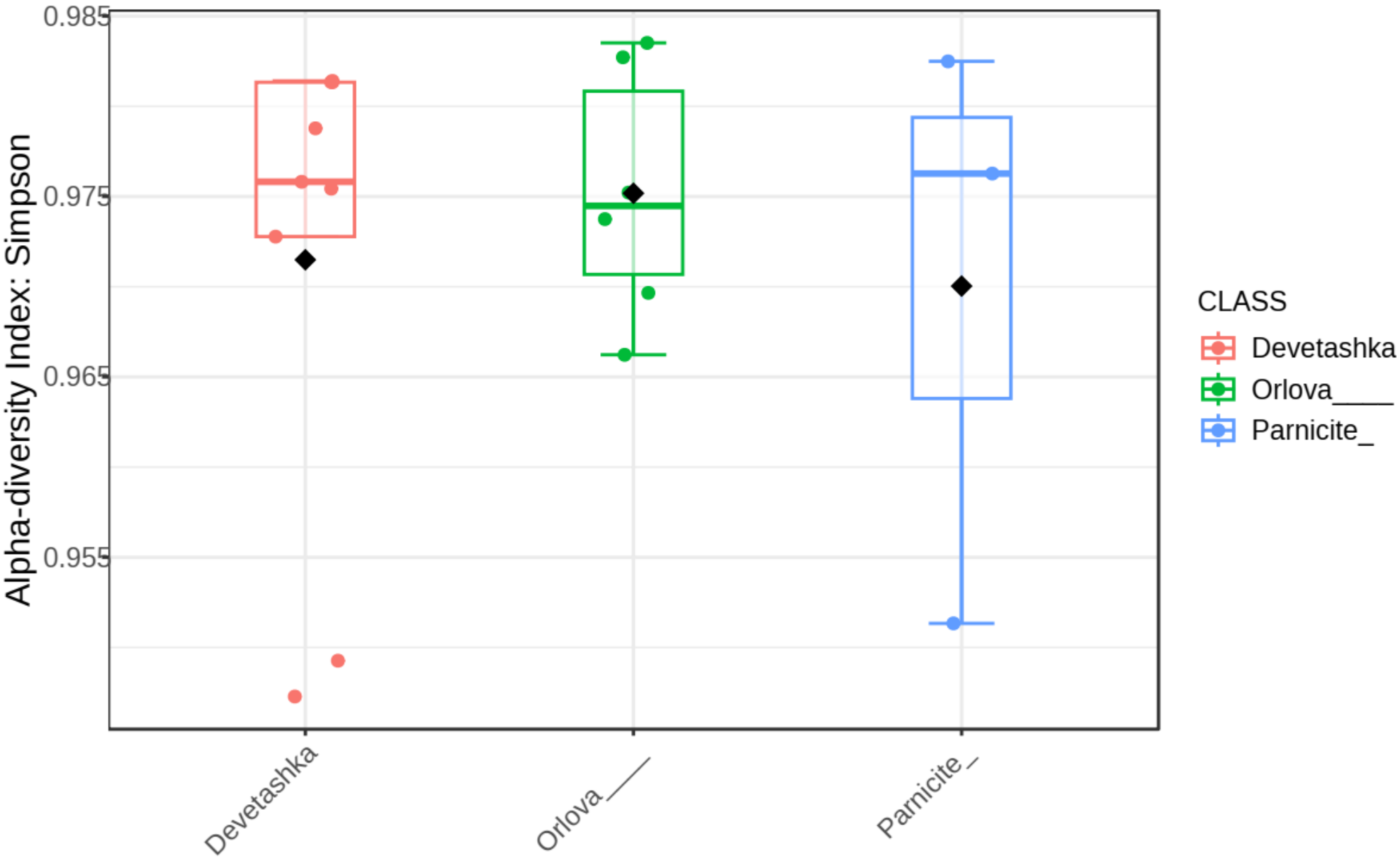
Bacterial genera by cave



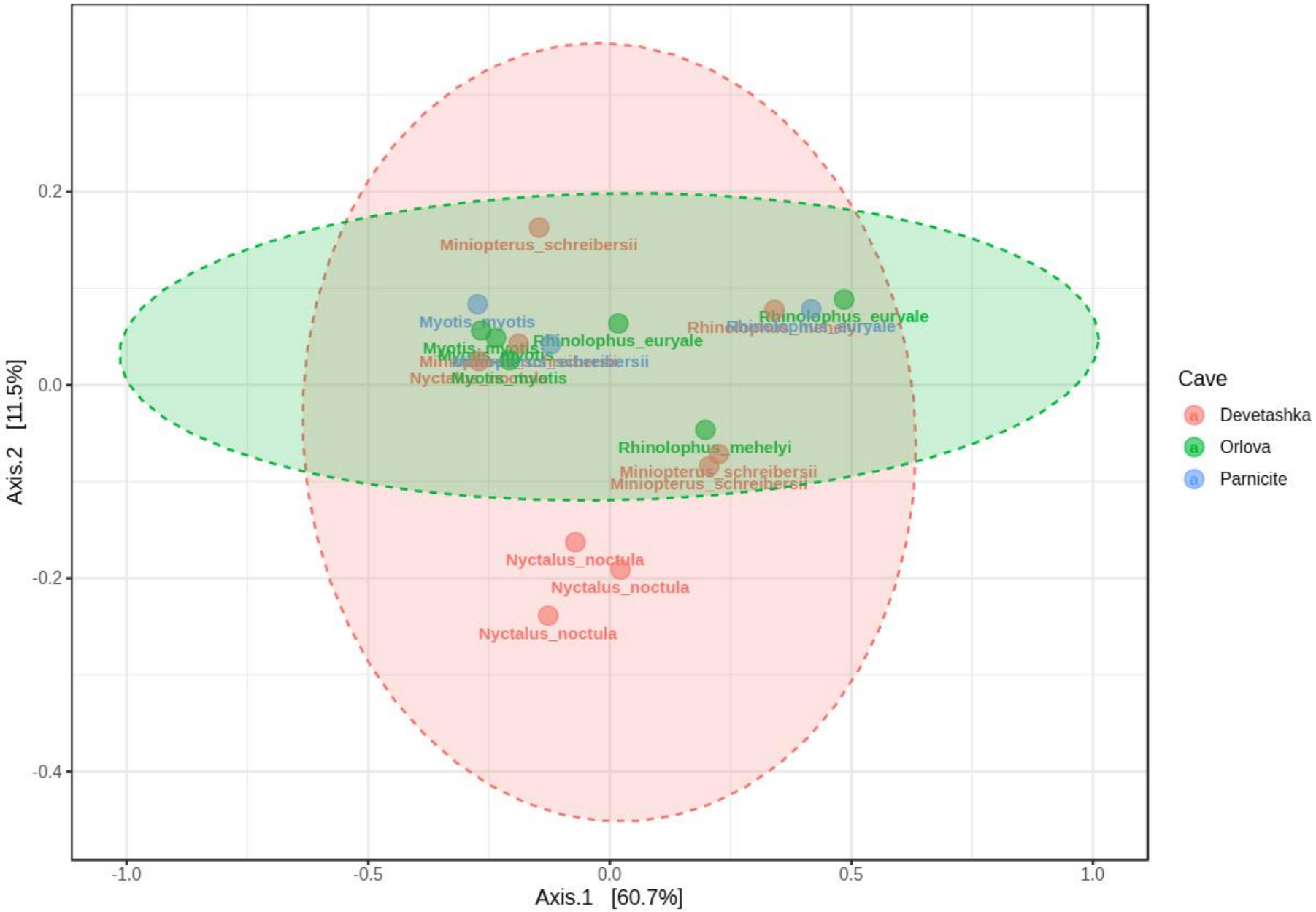
GENUS

- Others
- Streptomyces
- Staphylococcus
- Clostridium
- Bradyrhizobium
- Mycobacterium
- Bacillus
- Bartonella
- Pseudomonas
- Paraburkholderia
- Flavobacterium
- Acinetobacter
- Chryseobacterium
- Mycoplasma
- Campylobacter
- Shewanella
- Klebsiella
- Paenibacillus
- Streptococcus
- Sphingobacterium
- Fusobacterium
- Vibrio
- Polaribacter
- Nocardioides
- Enterococcus
- Corynebacterium
- Spiroplasma
- Aquimarina
- Nostoc
- Bacteroides
- Mycolicibacterium

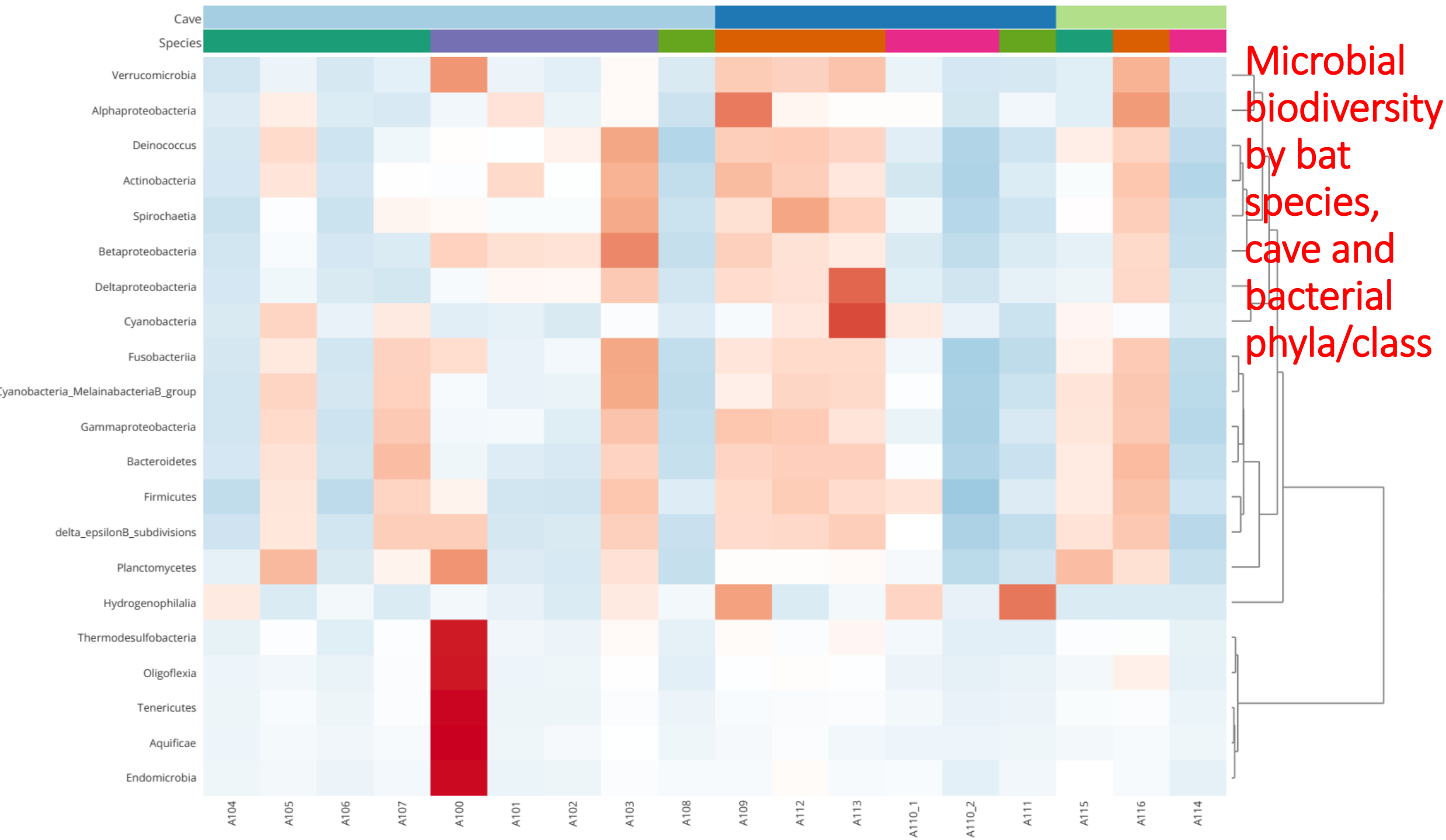
Bacterial alpha diversity by cave – Simpson index



Bacterial beta diversity by cave – Bray-Curtis index

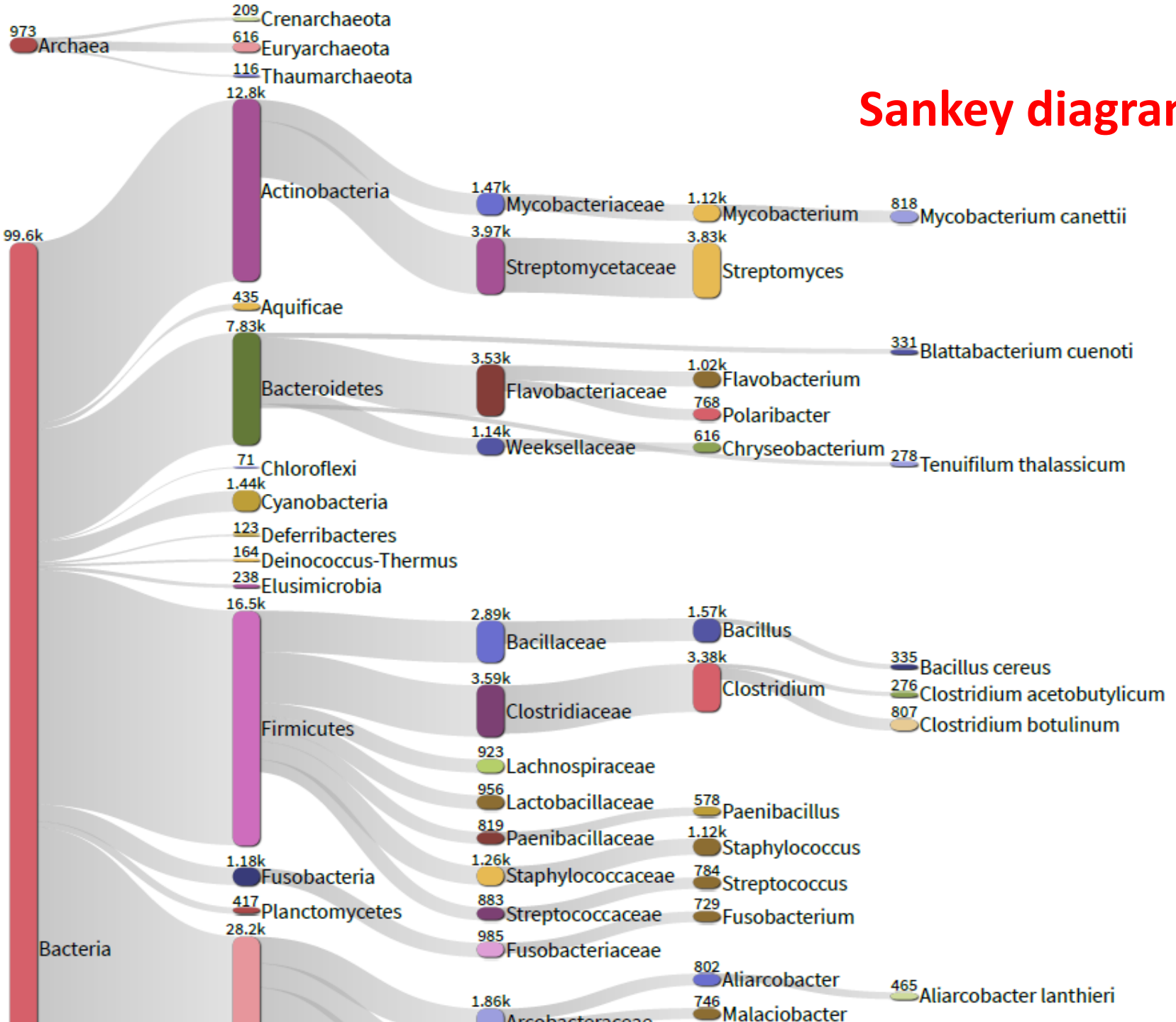


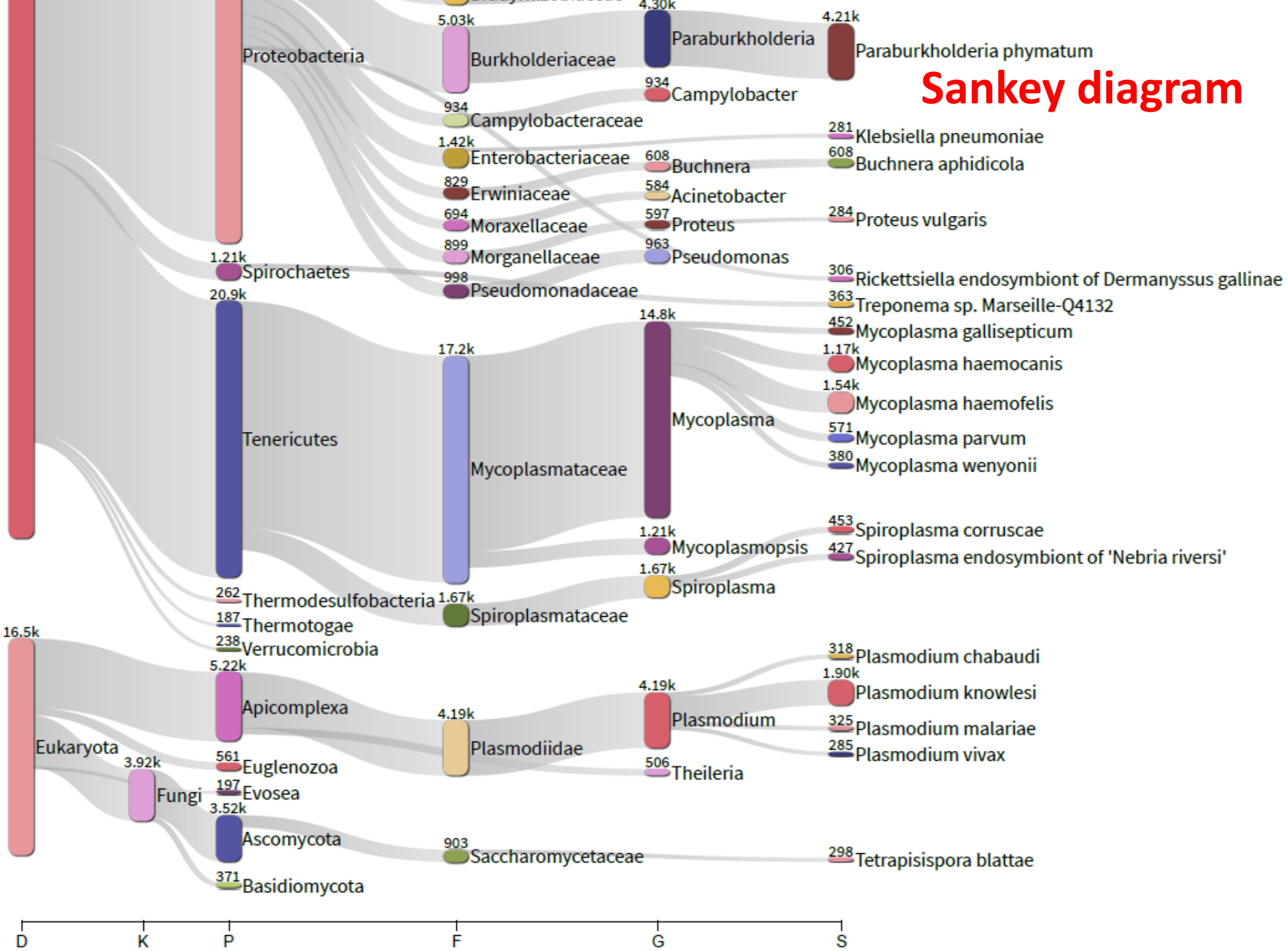
Cave
- Devetashka
- Orlova
- Parnicite



Microbial biodiversity by bat species, cave and bacterial phyla/class

Sankey diagram




Sankey diagram
Proteobacteria
Spirochaetes
Tenericutes
Thermodesulfobacteria
Thermotogae
Verrucomicrobia
Eukaryota
Fungi
Apicomplexa
Euglenozoa
Evosea
Ascomycota
Basidiomycota
Burkholderiaceae
Campylobacteraceae
Enterobacteriaceae
Erwiniaceae
Moraxellaceae
Morganellaceae
Pseudomonadaceae
Mycoplasmataceae
Spiroplasmataceae
Plasmodiidae
Saccharomycetaceae
Paraburkholderia
Campylobacter
Buchnera
Acinetobacter
Proteus
Pseudomonas
Mycoplasma
Mycoplasmopsis
Spiroplasma
Plasmodium
Theileria
Paraburkholderia phymatum
Klebsiella pneumoniae
Buchnera aphidicola
Proteus vulgaris
Rickettsiella endosymbiont of Dermanyssus gallinae
Treponema sp. Marseille-Q4132
Mycoplasma gallisepticum
Mycoplasma haemocanis
Mycoplasma haemofelis
Mycoplasma parvum
Mycoplasma wenyonii
Spiroplasma corruscae
Spiroplasma endosymbiont of 'Nebria riversi'
Plasmodium chabaudi
Plasmodium knowlesi
Plasmodium malariae
Plasmodium vivax
Tetrapisispora blattae
D
K
P
F
G
S

Conclusion

A significant number of microbial pathogenic species were identified in the bats‘ blood. Sequencing reads exceeding 10K were identified for Mycobacteria, Bartonella, Plasmodium and Leishmania species. Soil, water and guano samples demonstrated high abundancy of mycobacterial presence. Other pathogenic species such as Toxoplasma have also been identified but less evidently according to the number of reads. While tuberculosis, bartonelosis and leishmania are rare diseases, malaria is almost absent in Bulgaria. Our results demonstrated that bats in Bulgaria are a hidden or potential reservoir for malaria and atypical mycobacterial infections.

Thank you for your attention

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