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DIVERSIDADE ECOLÓGICA DE FUNGOS EM SOLOS DE FITOFISIONOMIAS DA CANGA DE CARAJÁS

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RESUMO EXECUTIVO

A Serra dos Carajás possui diferentes fitofisiomomias que se desenvolvem sob a influência de Canga. Os ecossistemas de canga são compostos por comunidades vegetais com um alto grau de endemismo e sua preservação é mandatória. Nestes ambientes, a comunidade de fungos do solo atua como facilitadora no estabelecimento da vegetação, condicionando o solo e aumentando a resistência e a resiliência das plantas. O impacto da atividade antrópica nestes ambientes se reflete na densidade e diversidade de fungos, alterando serviços ecossistêmicos e aumentando a suscetibilidade da vegetação a efeitos adversos no ambiente, dificultando sua regeneração natural. No presente trabalho, foram identificados fungos importantes compondo a comunidade microbiana das cangas nativas de Carajás. Neste trabalho, destacou-se a diversidade encontrada de grupos funcionais de fungos saprotrófos de solo, além dos fungos micorrízicos, endofíticos, patogênicos, parasíticos, além de fungos que se associam a líquens. Muitos dos grupos encontrados possuem função essencial na manutenção de espécies de plantas e do solo sobre a canga. O conhecimento da comunidade fúngica e de sua funcionalidade em cada fitofisionomia poderá contribuir para o monitoramento ambiental além da definição de estratégias para a conservação.

RESUMO

A Serra dos Carajás possui diferentes fitofisionomias que crescem sob influência da Canga, cujas plantas possuem elevado grau de endemismo. Neste ambiente a comunidade de fungos do solo atua como facilitadora do crescimento vegetal, ao prover condições favoráveis no solo, além de incrementar a resistência e resiliência das plantas. Tal comunidade está sujeita a perturbações severas causadas pela mineração que impactam a diversidade de fungos e eventualmente a capacidade de manutenção e recuperação do solo. O conhecimento da diversidade funcional de fungos nas fitofisionomias específicas de canga pode fornecer subsídios para estratégias de sua conservação e recuperação. Tendo em vista os progressos metodológicos em ecologia de fungos, existem novas perspectivas para acessar a diversidade de fungos do solo nos mais diversos ecossistemas, como o *DNA metabarcoding*. Este trabalho objetivou realizar inferências funcionais da comunidade de fungos presentes no solo das diferentes fitofisionomias sobre canga em Carajás. Foram coletadas e analisadas 48 amostras representativas do solo das fitofisionomias capão florestal (CF), campo rupestre arbustivo (CRA), campo graminoso (CG) e campo rupestre de vellozias (CRV). O DNA das amostras foi extraído pelo kit Powersoil, e a região ITS2 amplificada, sequenciada e analisada. A classificação taxonômica teve como base a base de dados UNITE, tendo seu produto correlacionado a dados ecológicos pelo programa FUNGuild. Ao fim das análises foram utilizadas 397707 sequências que geraram 3574 unidades taxonômicas (OTUs), das quais 633 tiveram metadados assinalados e classificados dentro de grupos funcionais. Por meio desta análise foi possível identificar grupos de fungos até então desconhecidos em solos da canga de Carajás. Os táxons de maior abundância encontrados se relacionavam a condições intrísecas das fitofisionomias o que permitiu fazer uma clara distinção entre elas. Foi destaque no CF a ordem Archaeorhizomycetales, no CRA a ordem Hymenochaetales, no CRV a ordem Tremelalles e no CG a ordem Capnodiales. Destacou-se também a abundância de saprotrófos de solo e “saprotrófos gerais” encontradas no CF e CRA, o que provavelmente se deve a maiores profundidades de solo e acúmulo de matéria orgânica nestas fitofisionomias. Quanto aos fungos simbiontes destaca-se a identificação de 4 tipos de fungos micorrízicos, com a presença de fungos possivelmente formadores de micorrizas de orquidea além de um número elevado de endosimbiontes no CG, e fungos formadores de líquens, os quais são responsáveis pela nutrição de plantas, mineralização da matéria orgânica e possívelmente da formação do solo de canga. Esse conhecimento pode basear análises e técnicas adicionais na recuperação e conservação de áreas de interesse da mineração.

Palavras-chave: Fungo. Solo. Amazônia. Ecologia. Metabarcoding.

ABSTRACT

The Serra dos Carajás has different phytophysiognomies that grow under the influence of Canga, which plants have a high degree of endemism. In this environment the soil fungal community acts as a facilitator of plant growth by providing favorable soil conditions and increasing plant resistance and resilience. Soil community is subject to severe mining disruptions that impact fungal diversity and eventually soil maintenance and recovery capacity. In-depth knowledge of the functional diversity of fungi in canga-specific phytophysiognomies can provide insights for conservation and recovery strategies. Given the methodological advances in fungal ecology, there are new perspectives for accessing soil fungal diversity in diverse ecosystems, such as DNA metabarcoding. This work aimed to make functional inferences of the fungi community present in the soil of different phytophysiognomies in Carajás. Forty-eight representative soil samples of the forest capon phytophysiognomies (CF), shrub field (CRA), grassy field (CG) and vellozias rupestrian field (CRV) were collected and analyzed. The DNA of the samples was extracted by the Powersoil kit, and the ITS2 region amplified, sequenced and analyzed. The taxonomic classification was based on the UNITE database, and its product was correlated with ecological and functional data by the FUNGuild program. At the end of the analysis, 397707 sequences were used to generate 3574 taxonomic units (OTUs), of which 633 had metadata marked and classified within functional groups. The most abundant taxa found were related to intrinsic conditions of phytophysiognomies, which allowed a clear distinction between them. The Archaeorhizomycetales order was highlighted in the CF, the Hymenochaetales order in the CRA, the Tremelalles order in the CRV and the Capnodiales order in the CG. Also important was the abundance of soil saprotrophs and "general saprotrophs" found in CF and CRA, which is probably due to greater depths of soil and accumulation of organic matter in these phytophysiognomies. As for symbiont fungi it’s noteworthy the identification of 4 types of mycorrhizal fungi between phytophysiognomies, the presence of fungi possibly forming orchid mycorrhizae, as well as a high number of endosymbionts in the CG, and fungi forming lichens, which are responsible for the nutrition of plants, mineralization of organic matter and possibly the formation of the canga soil. This knowledge can base additional analysis and techniques on the recovery and conservation of areas of interest in mining.

Keywords: Fungi. Soil. Amazon. Ecology. Metabarcoding;

SUMÁRIO

1 INTRODUÇÃO

A mineração é uma atividade importante à economia, mas impacta a diversidade de organismos e os serviços ecossistêmicos do local em que ocorre. Em ecossistemas como o da canga de Carajás, que possui condições adversas como alto teor de metáis e altas temperaturas, é necessário que seja descrita a diversidade que mantém a sustentabilidade de tais condições e que será impactada pela mineração.

Fungos no geral são importantes agentes de serviços ecossistêmicos e indicadores ambientais e desta forma apoiam tanto direta quanto indiretamente o bem-estar humano. Apesar disso, a descrição composicional da sua comunidade era difícil pela dificuldade na identificação morfológica. A chegada de inovações em tecnologias de DNA trouxera novas perspectivas na identificação de fungos, e assim chegou a técnica *DNA Metabarcode*.

O *DNA metabarcode* permite identificar a composição de espécies de determinada amostra de solo a partir de “códigos de barras de DNA”. O código de barra em questão é uma região do DNA cujas diferenças discriminam a taxonomia dos organismos identificados.

O conhecimento taxonômico dos fungos da canga pode nos informar quem está lá, e pelo conhecimento de ecologia de fungos podemos inferir qual o papel ecológico dos táxons fúngicos identificados. As hipóteses trazidas pela descoberta potencializam a sustentabilidade ambiental, pois podem basear técnicas de manejo e intervenção futuras no ambiente que possui fungos como atores e indicadores de mudança.

2 OBJETIVOS

2.1OBJETIVO GERAL

- Identificar a composição da comunidade de fungos no solo e caracterizar a diversidade de funções ecológicas nas quatro fitofisionomias sobre a Canga de Carajás.

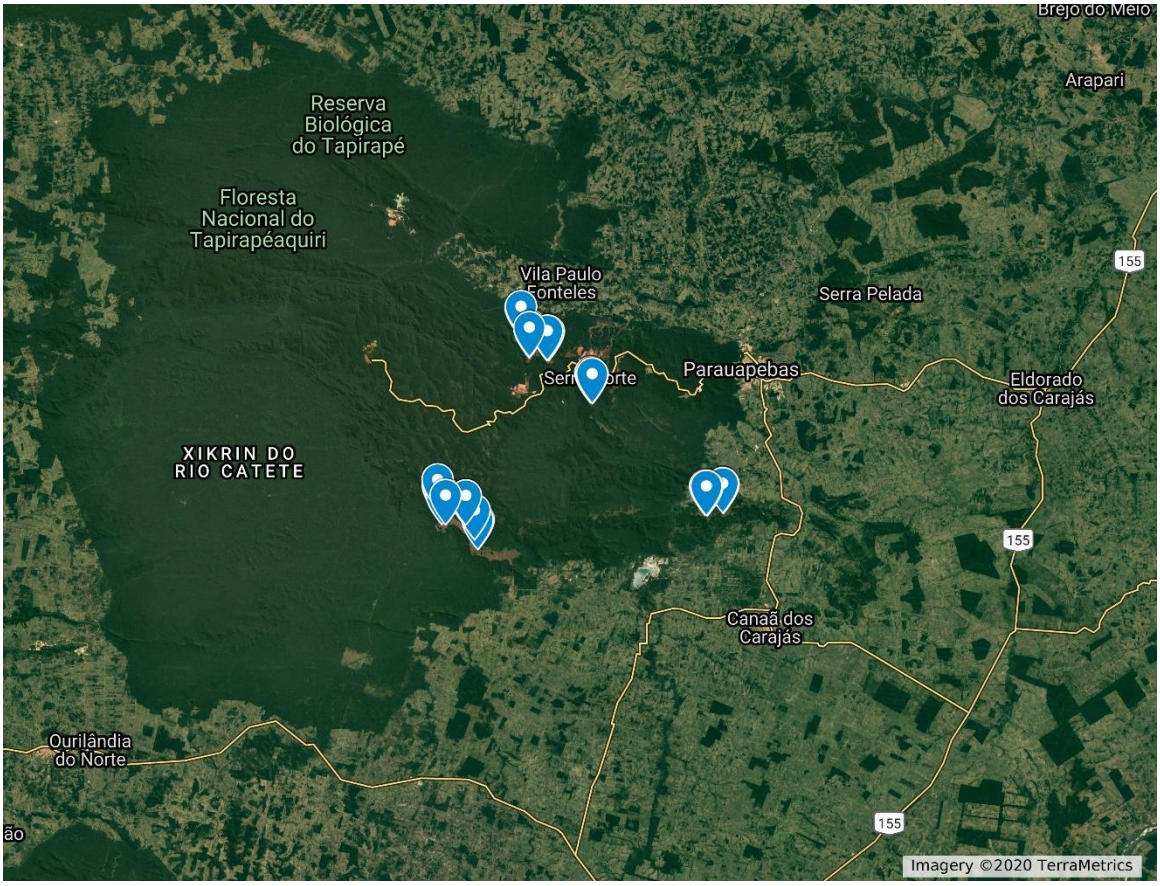
2.2 OBJETIVOS ESPECÍFICOS

- Identificar a composição taxonômica dos fungos presentes no solo das quatro fitofisionomias sobre a canga de Carajás;
- Caracterizar as fitofisionomias com base na abundância de seus principais táxons de fungos;
- Definir quais guildas ecológicas estão presentes e como se distribuem em cada fitofisionomia;
- Comparar a estrutura dos táxons e das guildas ecológicas entre as fitofisionomias e discutir seu papel ecológico, dando destaque aos fungos com maior predominância por grupo funcional;

3 METODOLOGIA

3.1 LOCAL DE ESTUDO

Figura 1 - Mapa do local de coleta.



Fonte: Imagens Google.

O presente estudo foi desenvolvido na Floresta Nacional (FLONA) de Carajás (figura 2). Na amostragem de solo de áreas nativas os 3 geoambientes compartimentalizados por Schaefer (2016) em “encostas e grotas florestadas”, foram tomados como uma única fitofisionomia de matas denominada “capão florestal”. Assim, no presente estudo foram consideradas quatro fitofisionomias, sendo: “Encostas com campo rupestre com *Vellozia* sobre canga ferrífera” (CRV), “Encostas com campo rupestre arbustivo sobre canga ferrífera” (CRA), “Capão florestal” (CF) e “Campo graminoso moderadamente drenado sobre canga nodular” (CG), ou Canga Herbácea.

Foram realizados 12 transectos de aproximadamente 300 metros, e demarcadas parcelas de 20x10m -quatro parcelas por transecto. Cada parcela no transecto

compreende uma fitofisionomia de canga nativa. Em cada parcela foi coletada uma amostra de solo superficial (0–5 cm) composta por cinco pontos de amostragem. As amostras foram posteriormente homogeneizadas com o objetivo de que se formasse uma amostra composta por parcela, totalizando 48 amostras.

Todas as amostras de solo foram embaladas em sacos plásticos, mantidos em gelo e posteriormente armazenadas a -80 ºC até a extração de DNA.

3.2 IDENTIFICAÇÃO MOLECULAR DE FUNGOS

O material coletado foi sequenciado na plataforma Illumina Miseq, onde os passos abaixo seguiram em grande parte as recomendações do fabricante.

O DNA foi extraído a partir de 250mg de solo usando kits comerciais de isolamento de DNA “PowerSoil” Mobio (Mobio Laboratories, USA), seguindo o protocolo em anexo (Anexo 1). Após extraído, o DNA foi quantificado usando o fluorímetro Qubit 3.0 (Thermo Fisher Scientific Inc.). A integridade do DNA extraído do solo foi confirmada por eletroforese em gel de agarose.

O sequenciamento na plataforma MiSeq da Illumina foi realizado na Universidade Federal do Pará - UFPA (Belém, Pará), seguindo o protocolo da fabricante. Neste processo, a região ITS2 do rDNA extraído foi amplificada utilizando os primers “ITS4i” (GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTCCTCCGCTTATTGATATGC) e flTS7i- (TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTGARTCATCGAATCTTTG), com os adaptadores, seguindo os ciclos demonstrados pelo quadro 1.

Quadro 1 - ciclo de PCR utilizado

1 ciclo	35 ciclos			1 ciclo	
Desnaturação inicial	Desnaturação	Anelamento	Extensão	Extensão Final	Resfriamento
1 minuto	30 segundos	30 segundos	1 minuto e 30 segundos	7 minutos	

a 95ºC	a 95°C	a 60°C	a 72°C	a 72°C	4°C

Fonte: próprio autor, 2019.

3.3 ANÁLISE TAXONÔMICA

A análise das leituras foi feita pelo Pipeline padrão do PIPITS (GWEON *et* al. 2015). Os passos da análise incluem a junção das *paired-end reads* pelo VSEARCH (ROGNES, 2016), a filtragem por qualidade – onde no mínimo 80% das bases na sequência deve possuir 30 de escore de qualidade para ser aceita- pelo FASTX (GORDON, 2010), a sua dereplicação. Em seguida, é realizada a extração da região ITS pelo ITSx (BENGTSSON-PALME, 2013), além da remoção de sequências < 100bp, retirada de sequências únicas pelo próprio PIPITS.

É feita então a clusterização em OTUs (Unidades Taxonômicas Operacionais) em 97% de similaridade através do VSEARCH, a remoção de quimeras tendo como base o UCHIME (EDGAR, 2011) também em 97% de similaridade de sequencia pelo VSEARCH.

A classificação taxonômica teve como base o UNITE (NILSSON *et* al., 2019), onde as OTUs foram classificadas em táxons de acordo com a classificação do “RDP Classifier” (LAN *et* al., 2012). Como o último passo na obtenção dos resultados foi feita então a correlação com dados de função ecológica da literatura foi feita pelo programa FUNGuild (NGUYEN, 2016).

3.4 ANÁLISE ESTATÍSTICA

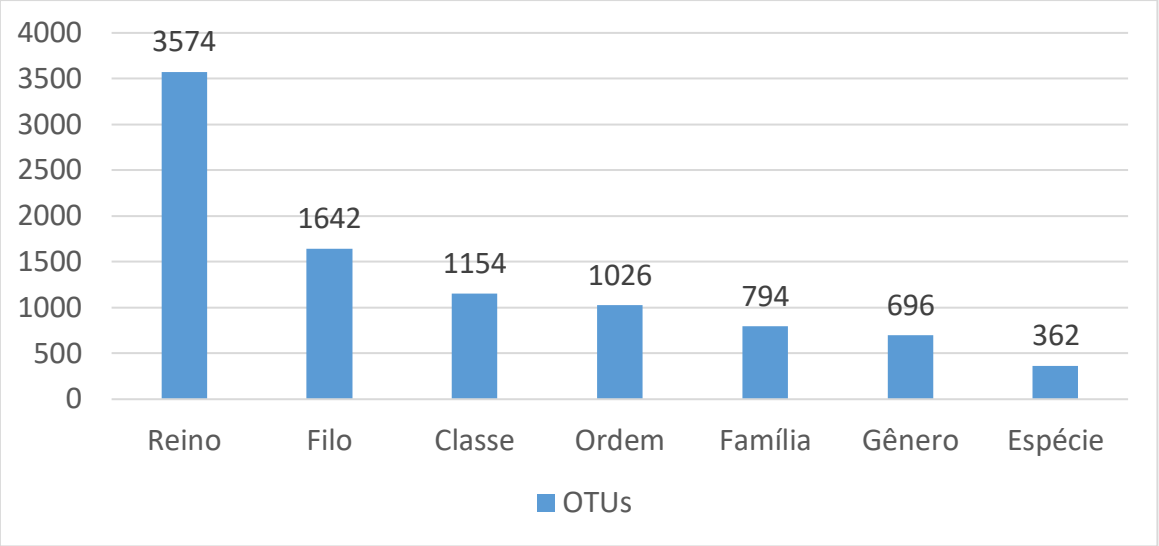
Através do pacote vegan (OKSANEN. et al., 2019) e ggplot2 (WICKHAM, 2016) do software R (R Core Team, 2019, foi feita a análise de NMDS (escalonamento dimensional não métrico). Esta análise teve o objetivo de informar visualmente como se agrupavam os resultados das amostras vindas das fitofisionomias, tendo como base a tabela de abundâncias de OTUs.

As abundâncias das OTUs vindas de amostras de um mesmo táxon ou grupo funcional pertecente a uma mesma fitofisionomia foram somadas para as análises posteriores.

4 RESULTADOS E DISCUSSÃO

Ao fim das análises no PIPITS, foram utilizadas 397707 sequências na obtenção de 3574 OTUs, das quais 633 tiveram metadados assinalados pelo FUNGuild. Na figura 3 está ilustrado o número de OTUs encontradas por nível taxonômico.

Figura 3 - Números de OTUs identificadas por nível taxonômico.



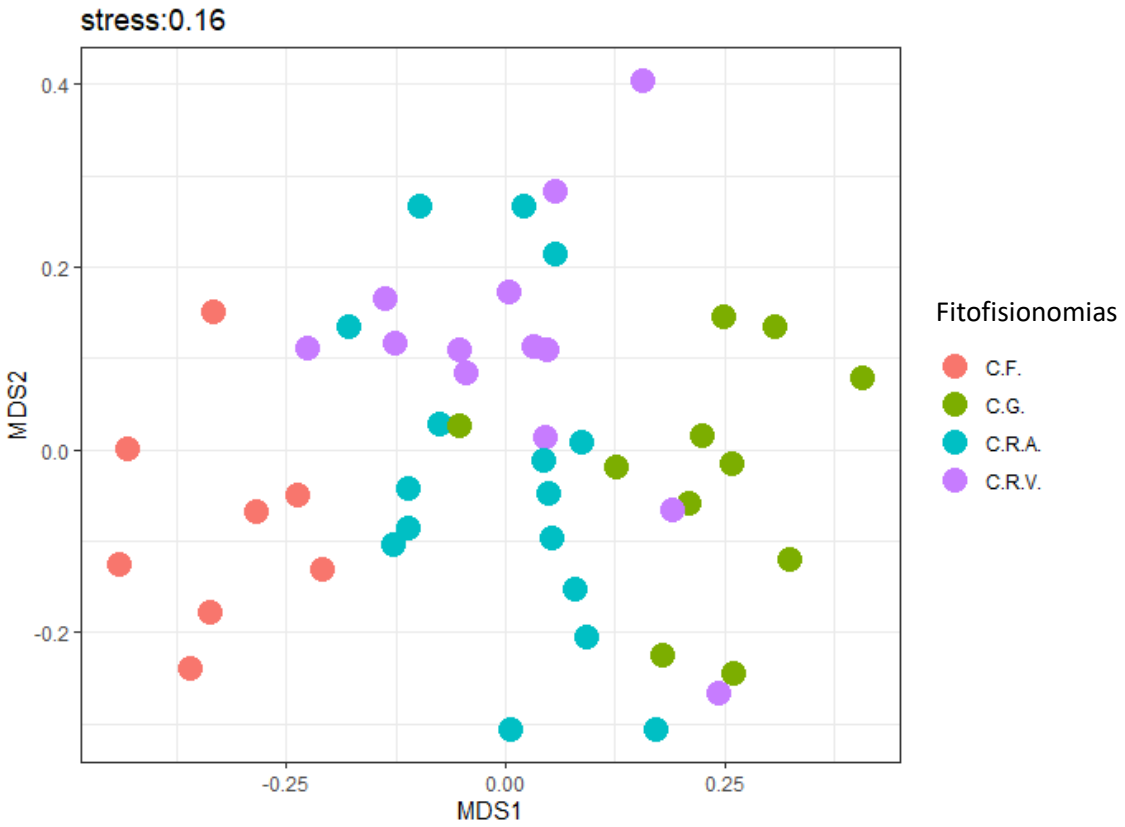
Fonte: próprio autor, 2019.

Com a tabela de OTUs foi possível produzir a análise de NMDS, apresentada na figura 4. Nesta representação é possível notar a separação entre a composição de fungos entre as fitofisionomias, na qual CF e CG se apresentaram com maior separação, assim como a proximidade entre CRA e CRV - ambos Campos Rupestres.

Dentre os filos encontrados os mais abundantes (número de sequências encontradas) foram: Ascomycota (93,82%), seguido pelo Basidiomycota (4,63%), sendo identificados outros 6 cuja abundância de sequências individuais totais foi < 1% (Mucoromycota, Chytridiomycota, Rozellomycota, Mortierellomycota, Monoblepharomycota, Basidiobolomycota).

Dentre as ordens mais abundantes, as principais em número de sequências foram: Archaeorhizomycetales (25,20%), Capnodiales(17,11%), Tremellales (8,13%), Agaricales (8,11%), Chaetothyriales (7,49%), Xylariales (5,52%), Pleosporales (5,43)%, Helotiales (4,44%), Hymenochaetales (1,62%), Archaeosporales (1,51%), entre 56 outras cuja abundância individual foi < 1,5%.

Figura 4 - Representação gráfica do NMDS com 0.16 de stress.



Fonte: próprio autor, 2019.

No gráfico presente na figura 5 está representada graficamente a distribuição por fitofisionomia de todas as ordens identificadas. Tal diversidade de fungos encontrada permite uma melhor caracterização das fitofisionomias levando-se em consideração a distribuição das ordens de fungos nelas encontradas. Determinadas fitofisionomias destacaram-se pela porcentagem presentes de determinadas ordens, sendo as principais:

CF : “Archaeorhizomycetales” e “Agaricales”;

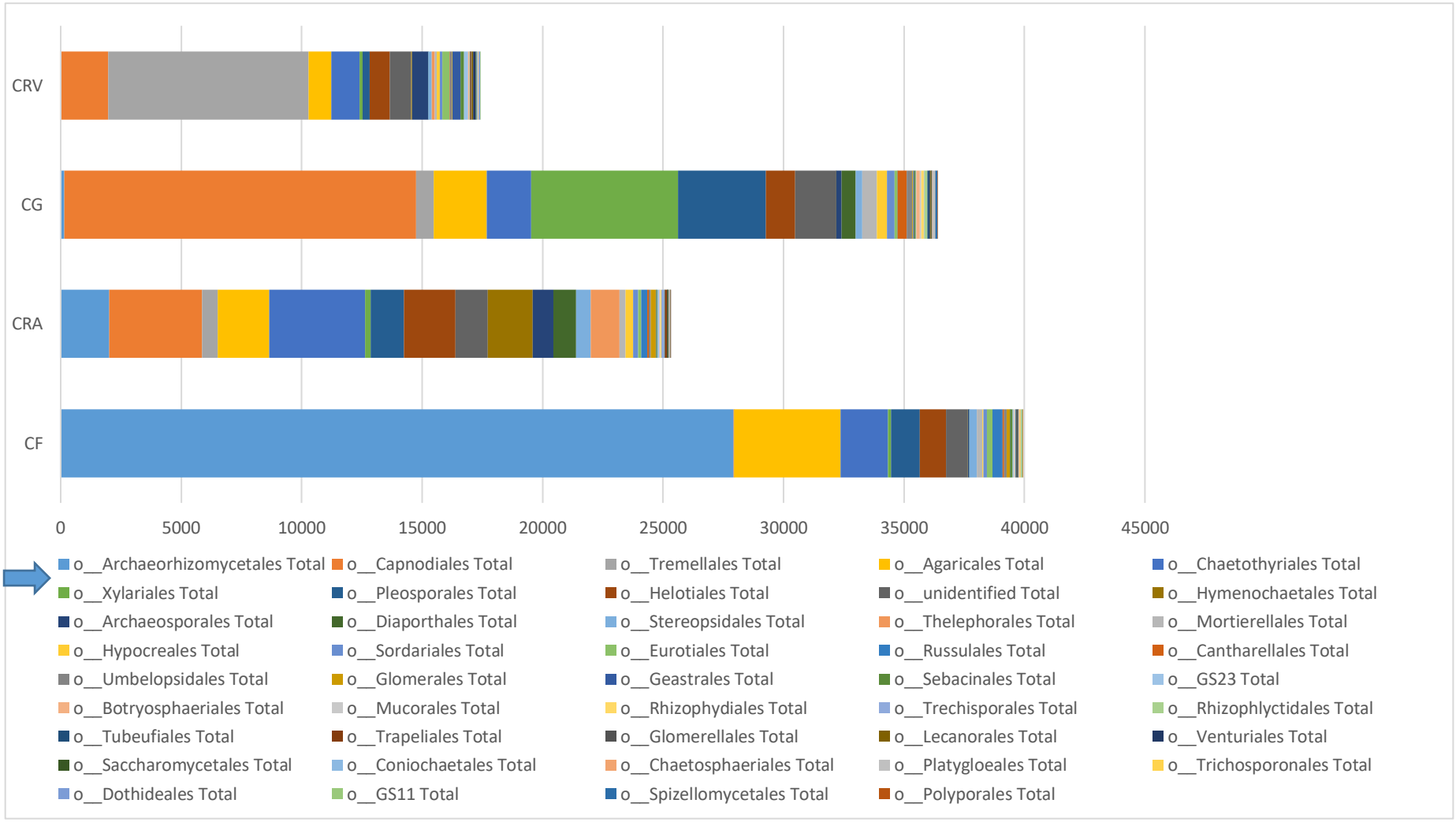
CRA: “Chaetothyriales”, “Helotiales”, “Hymenochaetales” e “Archaeosporales”;

CRV: “Tremellales”, “Archaeosporales”;

CG: “Capnodiales”, “Xylariales” e “Pleosporales

Mais detalhes sobre as abundâncias de cada ordem identificadas podem ser encontrados na Tabela 1.

Figura 2 - Gráfico de barras representando todas as ordens identificadas por fitofisionomia.



Fonte: Autor.

Tabela 1 - Abundância de ordens por fitofisionomia. A tabela abaixo está ordenada da maior para a menor em número de sequências por ordem.

ORDEM	CF	CRA	CRV	CG
o__Archaeorhizomycetales Total	**27936**	2010	15	146
o__Capnodiales Total	12	3864	1962	**14596**
o__Tremellales Total	9	644	**8312**	746
o__Agaricales Total	**4410**	2129	950	2201
o__Chaetothyriales Total	1970	**3978**	1163	1842
o__Xylariales Total	131	249	130	**6083**
o__Pleosporales Total	1192	1367	284	**3646**
o__Helotiales Total	1089	**2146**	853	1219
o__unidentified Total	877	1336	871	1693
o__Hymenochaetales Total	13	**1870**	41	16
o__Archaeosporales Total	64	**848**	**672**	220
o__Diaporthales Total	10	942	3	579
o__Stereopsidales Total	309	614	147	284
o__Thelephorales Total	0	1203	115	0
o__Mortierellales Total	230	252	82	606
o__Hypocreales Total	34	297	133	421
o__Sordariales Total	155	223	110	317
o__Eurotiales Total	239	115	280	115
o__Russulales Total	412	261	0	11
o__Cantharellales Total	71	55	22	372
o__Umbelopsidales Total	89	79	90	235
o__Glomerales Total	150	210	24	35
o__Geastrales Total	7	29	332	47
o__Sebacinales Total	98	51	152	68
o__GS23 Total	37	79	171	3
o__Botryosphaeriales Total	23	27	33	157
o__Mucorales Total	63	12	29	52
o__Rhizophydiales Total	0	39	0	117
o__Trechisporales Total	2	128	0	26
o__Rhizophlyctidales Total	6	5	16	110
o__Tubeufiales Total	23	22	5	86
o__Trapeliales Total	0	86	35	9
o__Glomerellales Total	58	7	29	31
o__Lecanorales Total	0	11	63	42
o__Venturiales Total	8	2	97	5
o__Saccharomycetales Total	12	27	35	29
o__Coniochaetales Total	22	18	59	1

o__Chaetosphaeriales Total	36	31	10	20
o__Platygloeales Total	0	5	0	90
o__Trichosporonales Total	57	5	13	18
o__Dothideales Total	7	7	65	13
o__GS11 Total	62	24	2	0
o__Spizellomycetales Total	0	1	0	77
o__Polyporales Total	28	5	0	18
o__Diversisporales Total	6	0	17	21
o__Geminibasidiales Total	0	0	0	42
o__Ustilaginales Total	0	0	0	32
o__Ostropales Total	28	1	0	0
o__Cystobasidiomycetes_ord_Incertae_sedis Total	0	0	0	26
o__Gigasporales Total	7	2	0	11
o__Orbiliales Total	0	2	5	11
o__Sporidiobolales Total	0	16	2	0
o__Auriculariales Total	0	7	0	8
o__Leucosporidiales Total	14	0	0	0
o__Pezizales Total	3	9	0	0
o__Basidiobolales Total	4	2	2	3
o__Corticiales Total	0	11	0	0
o__GS21 Total	3	0	7	1
o__Phaeomoniellales Total	6	4	0	0
o__GS33 Total	0	8	0	0
o__Onygenales Total	0	0	7	0
o__Erythrobasidiales Total	0	0	0	6
o__Microascales Total	6	0	0	0
o__Lichenostigmatales Total	0	0	0	5
o__Branch07 Total	3	1	0	0
o__Entorrhizales Total	1	2	0	0
o__Pucciniales Total	0	1	2	0

Fonte: Autor.

Archaeorhizomycetales são caracterizados pelo seu crescimento em altas temperaturas e num ambiente rico em carbono (ROSLING et al., 2011), o que pode explicar sua maior presença no CF (92,78%), assim como no CRA (6,67%). Em relação ao CF, sua abundância pode estar relacionada a uma maior profundidade de solo, característica da fitofisionomia, que permite um maior acúmulo de matéria orgânica (SCHAEFER *et* al., 2016).

Archaeorhizomycetales possui grande relação com raízes de plantas (ROSLING et al., 2011), sendo considerado um táxon fundamental ao solo e que costuma estar relacionado a fungos micorrízicos arbusculares da ordem Glomerales (ZENG *et* al., 2019), da qual foram encontradas 50,11% e 35,79% de suas sequências no CRA e CF, respectivamente.

A ordem Archaeosporales também forma associações micorrízicas (BILLS, 2015), mas diferiu na distribuição de sequências entre as fitofisionomias. Esta ordem teve a maior parte de suas sequências encontradas nos Campos Rupestres (CRA 47% e CRV 37.25%), seguido pelo CG (12.2%) com somente 3,55% de suas sequências encontradas no CF.

A ordem agaricales foi predominante no CF (45,51%). Esta ordem é considerada cosmopolitana pela grande diversidade de habitats que podem colonizar (KIRK *et* al., 2008). A maior parte dos fungos desta ordem são saprotróficos, terrestres, com algumas espécies sendo parasitas de plantas e outras micorrízicas (AHMADJIAN *et* al., 2020)

Brinkmann e colaboradores (2019) observaram uma diminuição da abundância de Agaricales entre uma floresta tropical e uma plantação de dendêzeiros. Visto que a distribuição de tal ordem entre as fitofisionomias foi maior no CF, sendo moderada no CRA e CG, e menor no CRV, é sugestiva à similaridade entre o CF e a floresta tropical, e CRV e a plantação de dendêzeiros citadas no estudo.

A ordem Tremellales foi predominante no CRV (85,59%). Esta ordem contem espécies que tipicamente parasitam outros fungos ou líquens crescendo em madeira morta de arbustos ou árvores (INGOLD, 1959). Além disso, seu corpo gelatinoso (VERMA, 2019) está possivelmente relacionado a uma adaptação (INGOLD, 1959) desta ordem a condição de aridez presente CRV (SCHAEFER, 2016;).

A espécie com maior presença nos Tremellales foi a *Cryptococcus sp*, contando com 98.8% (9595) das sequências de Tremellales encontradas. Segundo Vishniac (2006), a predominância de *Cryptococcus* em ambientes áridos se deve a suas cápsulas de polissacarídeos, o que lhes garante uma vantagem na competição com bactérias. Além disso, esta ordem sob condições de pH baixo e alta condutividade

elétrica possui vantagens de crescimento em relação a outras ordens de fungos (VISHNIAC, 2006).

Os Chaetothyriales foram encontrados principalmente no CRA (44,43%), porém assim como os Agaricales, possuiram uma moderada distribuição entre as outras fitofisionomias (CF 22.00%, CG 20.57%, CRV 13.00%), com uma diminuição na CRV. Fungos desta ordem podem ser encontrados em diversos ambientes com condições dadas como adversas, incluindo superfícies rochosas, climas áridos, e sob alto teor de metáis tóxicos (HOOG, 2014).

Os Chaetothyriales caracterizados por serem geralmente epifitas e produzirem um micelium rico em melanina, que garante que tais fungos sejam chamados de fungos negros. Tal característica não é vista como a fonte de sua patogenicidade, mas tanto sua virulência quanto sua cor podem estar relacionadas a uma adaptação para a sobrevivência em ambientes extremos (SCHNITZLER *et* al., 1999; FENG *et* al., 2001; LIU *et* al., 2004).

A presença de Hymenochaetales foi maior no CRA (96,39%). Esta ordem está relacionada à maior presença de material ligninico em contato com o solo, visto que a maior parte destes fungos são dados como saprotrófos causadores da podridão branca (WAGNER, FISCHER, 2001; KOROTKIN *et* al., 2018.), podendo também ser capaz de formar micorrízas (TEDERSOO, SMITH, 2013). A maior presença de Hymenochaetales no CRA pode ser devido a características intrínsecas da variedade de plantas presente nesta fitofisionomia, fato que leva a uma maior deposição de madeira neste ambiente arbustivo (SCHAEFER, 2016; FERNANDES, 2016; GÓES-NETO, 2000).

Foram encontradas 40,43% das sequências de Helotiales no CRA, seguida de 22,97% no CG, 20,52% no CF e 16,07% no CRV. Os fungos desta ordem são caracterizados pela diversidade de associações que podem exercer num ambiente como patogenia, saprotrofia, endofitismo, além de associações micorrízicas (micorriza ericóide e ecto micorriza) (VRÅLSTAD *et* al., 2002).

Apesar do número de sequências identificadas a nível de ordem como sendo Helotiales (5307), em muitas delas não foi possível identificar a família (3729). Muitas destas sequências não identificadas pertencem ao CRA (1905), o que dificultou a

discussão da ecologia desta ordem tão diversa. A presença de famílias não identificadas pode ter relação com a presença de táxons ainda desconhecidos e possivelmente endêmicos da ordem Helotiales nesta fitofisionomia.

Os táxons de Helotiales cuja definição foi além da ordem, abrangeram espécies de fungos com semelhantes ericoides (como os do gênero *Oidiodentron*) (KOHOUT, 2017; VOHNÍK *et* al., 2013) e micorrízicos de orquídeas, como os do gênero *Meliniomyces* (MARTIN, 2016) -*encontrado com grande abundância no CG*.

Oliveira e colaboradores (2014) relataram uma co-ocorrência na rizosfera de orquídeas, das ordem Helotiales com a ordem de Basidiomicetos “Cantharellales” (71,53%), outro potencial fungo micorrízico, além das ordens de Ascomycetos “Capnodiales”, “Sordariales” e “Xylariales”, cuja presença também foi mais acentuada no CG.

A presença de Capnodiales majoritariamente no CG (71,42%) pode ser atribuída à sua capacidade de associação a raízes de orquídeas (OLIVEIRA *et* al., 2014). O CG possui grande incidência de dendrobiums (orquídeas) da espécie *Sobralia liliastrum*, caracterizada como uma planta endêmica nesta área (KOCH *et* al., 2018; FERNANDES, 2016).

Os Canopdiales assim como os Pleosporales (56,18% de suas sequências no CG) são considerados moduladores da diversidade de fungos na rizosfera de orquídeas. A regulação é atribuída a sua predominância durante a florescência, cujo crescimento da população fungica é correlacionado ao da planta (WANG *et* al., 2017). Em tais ordens de fungos potencialmente endofíticos (OLIVEIRA *et* al., 2014), a variedade de funções que podem ser exercidas (de relações patogênicas até relações mutualísticas), torna difícil a definição do papel ecológico na orquídea (ARNOLD 2007, VENDRAMIN et al. 2010).

Os Xylariales (92,26% de suas sequencias encontradas no CG) são considerados fungos endofíticos comumente associados a hospedeiros tropicais, juntamente com os Sordariales (39,27% de suas sequencias encontradas no CG), onde a afinidade com o hospedeiro e com o ambiente define o desfecho da função ecológica do fungo (ARNOLD *et* al., 2003). Eles são considerados microrganismos comuns e até dominantes na composição da rizosfera de orquídeas (BAYMAN et al.,

1997), possuindo como endofíticos um papel importante na regulação da produção de metabólitos secundários nas plantas hospedeiras (CHEN, GUO, 2005).

Um estudo por Vaz e colaboradores (2014) relacionou os Xylariales ao aumento da precipitação no local e a diminuição da temperatura. Tais características, ao se assemelharem às condições do campo graminoso (um ambiente mal drenado que por vezes é encotrado proximo de lagos, com orquídeas endêmicas), justificam a presença de tais fungos na fitofisionomia (FERNANDES, 2016; YUAN *et* al., 2009; BAYMAN, OTERO, 2006).

Os filos e Ordens identificados como "unidentified" foram incluídos na contagem por terem suas espécies e/ou funcionalidade ecológica identificadas de acordo com o banco do UNITE. Contudo, existe uma dificuldade em realizar uma discussão a nível de espécie, o que inclui falta de dados sobre as espécies identificadas por nomes genéricos (Ex: *s__Ascomycota_sp_* SH11983328FU) e pela grande quantidade de espécies identificadas dessa forma.

Por meio destes dados foram encontrados, baseados na versão 1.1 do banco de dados da FUNGuild grupos funcionais de fungos no solo como: ectomicorrízicos, micorrízicos arbusculares, micorrízicos ericóides, micorrízicos de orquídeas, patógenos de plantas, parasitas de líquens, fungos e plantas, assim como fungos liquenizados ou formadores de líquens, e saprotrófos comumente encontrados em folhas, plantas, madeira, assim como no solo e em fezes.

Uma visão geral de todos os grupos funcionais encontrados segundo o FUNGuild, assim como o seu respectivo número de leituras em cada fitofisionomia é demonstrada pela figura 6. Por meio desta figura, assim como da figura 7, que ilustra a distribuição de leituras de fungos por fitofisionomia por sua estratégia trófica, é possível notar a predominância de alguns grupos funcionais e de trofismos em determinadas fitofisionomias, demonstrando certas "preferências" por elas. Ilustrações de predominâncias nas fitofisionomias, dessa vez sobre classes de fungos existentes em alguns dos grupos funcionais com importancia ecológica, podem ser encontradas nos Apêndices de "A" a "J".

Através da identificação de táxons de fungos foi possível inferir a existência de uma distribuição de guildas funcionais de fungos no solo de canga, até então

desconhecida. Nos parágrafos abaixo é feita uma breve descrição de cada um dos grupos funcionais encontrados e de suas possíveis relações com a manutenção dos ecossistemas de canga.

4.1 SAPROTRÓFOS

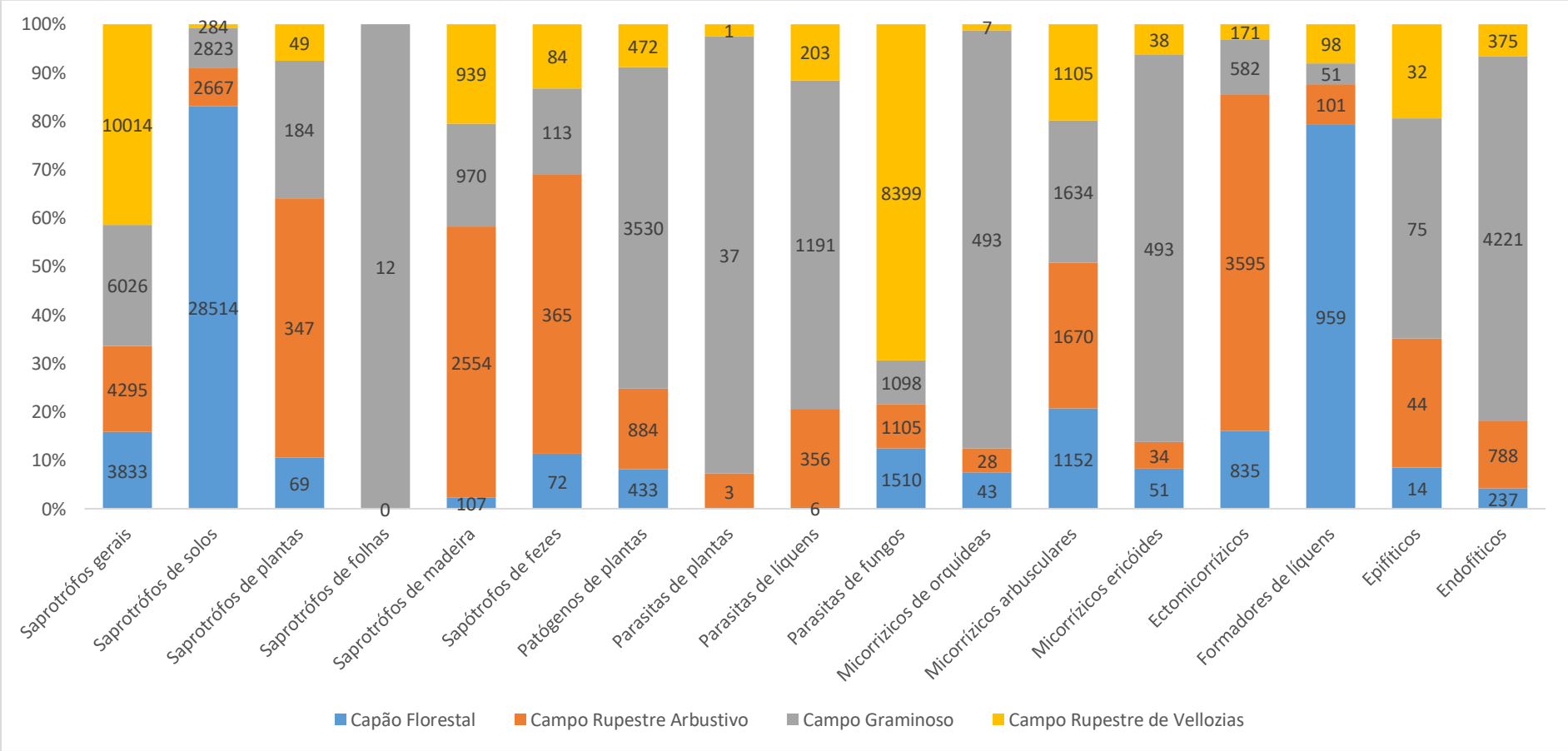
4.1.1 Saprotrófos de madeira

Em nosso resultado tais fungos foram responsáveis por 107, 2554, 970, 939 sequências nas fitofisionomias do CF, CRA, CG, CRV, respectivamente. As ordens de maior abundância nos saprotrófos de madeira foram Hymenochaetales no CRA, Agaricales no CRV, Helotiales nos CG e CF, respectivamente, pertencentes a classe dos Leotiomycetos e Agaricomycetos. Um gráfico referente à predominância de ordens por fitofisionomia neste grupo funcional está disposto no **Apêndice A**.

Os fungos saprotrófos de madeira por vezes também englobam saprotrófos de plantas (69, 347, 184, 49 sequências no CF, CRA, CG e CV) e os saprotrófos de folhas (12 sequências no CRA). Isso provavelmente se deve à similaridade da fonte de nutrientes, e a menor dificuldade de adquirir nutrientes visto ser composto de material menos recalcitrante que a madeira. Tal capacidade os faz desempenhar papéis centrais na decomposição orgânica e ciclagem de nutrientes (HOBBIE, HORTON, 2007), em locais com menor acúmulo de matéria [orgânica no qual é necessária alta rotatividade do ciclo do carbono, como CRA e CRV.

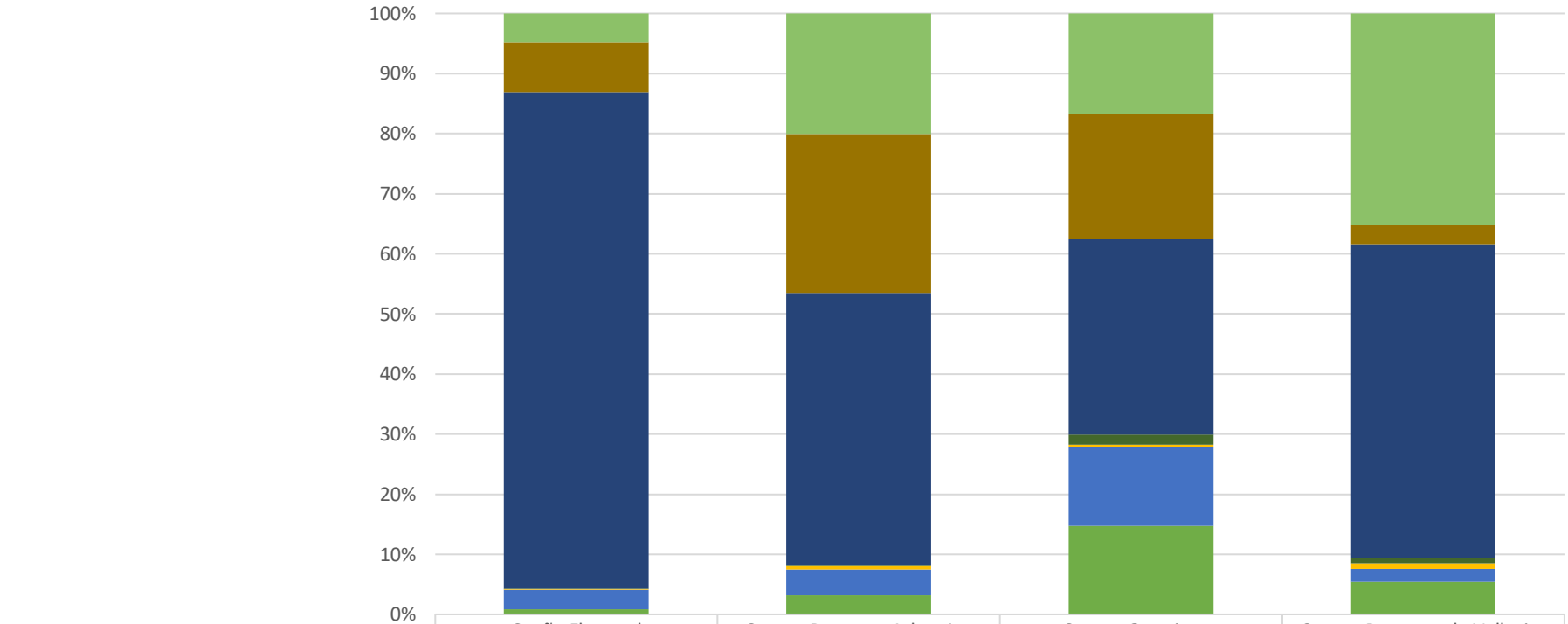
O gênero mais abundante encontrado no solo de saprotrofos de folhas foi a *Mycena*, somente encontrada no CG. Já a ordem de maior abundância nos saprotrofos de plantas, foi a Pleosporales, o que se refletiu em todas as fitofisionomias, ordem essa pertencente a classe dos Dothideomycetos. Um gráfico referente à predominância de ordens por fitofisionomia no grupo de saprotrófos de plantas está disposto no **Apêndice B**.

Figura 6 - Quantidade de leituras por fitofisionomia (subdivisão das colunas), em cada uma das funções ecológicas (colunas). Nas colunas o 100% é considerado a soma dos valores de todas as leituras encontradas em dada função nas 4 fitofisionomias. As leituras de uma funçao ecológica são as leituras de todas OTUs que tiveram a respectiva função ecológica a elas assinalada.



Fonte: Autor.

Figura 7 - Distribuição de estratégias tróficas assinaladas pelo FUNGuild a fungos no solo das 4 fitofisinomias da Serra dos Carajás.



	Capão Florestal	Campo Rupestre Arbustivo	Campo Graminoso	Campo Rupestre de Vellozias
Simbiotróficos Total	1683	2317	1737	1259
Saprotróficos-Simbiotróficos Total	2858	3059	2150	119
Saprotróficos Total	28767	5244	3373	1868
Patotróficos-Simbiotróficos Total	38	14	178	34
Patotróficos-Saprotróficos-Simbiotróficos Total	10	62	36	31
Patotróficos-Saprotróficos Total	1135	500	1365	79
Patotróficos Total	312	364	1526	193

Fonte: Autor.

4.1.2 Saprotrófos gerais

Tal grupo foi uma das mais abundantes em nosso estudo, contando com 3833, 4295, 6026, e 10.014 sequências no CF, CRA, CG e CRV. Assim como neste estudo, esse grupo funcional costuma ter alta % de OTUs encontradas (WEI *et* al., 2018; XU *et* al., 2018). Tal fato, provavelmente se deve tanto a diversidade ainda desconhecida de fungos, quanto a dificuldade em serem classificados funcionalmente os que já são conhecidos.

As ordens de maior abundância nos saprótrofos gerais foram Agaricales no CF, CRA e CG, sendo no CG a predominância dividida entre Agaricales e Geastrales*,* pertencentes a classe dos Agaricomycetos. Um gráfico referente à predominância de ordens por fitofisionomia neste grupo funcional está disposto no **Apêndice C**.

4.1.3 Saprotrófos de solos

Em nosso estudo, foram responsáveis por 28514, 2667, 2823, e 284 sequências no CF, CRA, CG, e CRV, respectivamente. Tendo a sua maior incidência no CF provavelmente justificada pela maior profundidade de solos, assim como sua menor incidência no CRV pelo motivo contrário.

As ordens de maior abundância entre saprótrofos de solos foram Archaeorhizomycetales (gênero *Archaeorhizomyces)* no CF, CRA e minoritariamente no CRV, e no CG a predominância foi de Xylariales (espécie *Bartalinia robillardoides*)*,* essas ordens pertencentes a classe dos Archaeorhizomycetes e Sordariomycetos*,* respectivamente. Um gráfico referente à predominância de ordens por fitofisionomia neste grupo funcional está disposto no **Apêndice D**.

4.1.4 Saprotrófos de fezes

Destes fungos, no solo das fitofisionomias CF, CRA, CG, e CRV foram encontrados, respectivamente, 72, 365, 113, 84 sequências. As ordens de maior abundância entre os saprótrofos de fezes foram Pleosporales no CRA, CF e minoritariamente no CV, sendo no CG a predominância dividida entre Pleosporales e Sordariales*,* pertencentes a classe dos Dothideomycetes e Sordariomycetos*,* respectivamente. Um gráfico referente à predominância de ordens por fitofisionomia neste grupo funcional está disposto no **Apêndice E.**

4.2 PARASITAS E PATÓGENOS

4.2.1 Parasitas de fungos

Os fungos que parasitam outros fungos foram responsáveis por 1510, 1105, 1098, 8399 sequencias nas fitofisionomias CF, CRA, CG, e CRV, respectivamente. Neste grupo, a ordem predominante em todas as fitofisionomias foi a dos Chaetothyriales*,* pertencentes a classe dos Eurotiomycetes*.* Um gráfico referente à predominância de ordens por fitofisionomia neste grupo funcional está disposto no **Apêndice F**.

Neste estudo, parasitas de plantas e líquens foram responsáveis no CF, CRA, CG, e CRV por “0, 3, 37, 1” e “0, 356, 1191, e 203”, respectivamente.

No grupo de parasitas de plantas a ordem Ustilaginales foi predominante no CG, assim como a Agaricales como sendo a única ordem encontrada no CRA e CRV, pertencentes a classe dos Ustilaginomycetos e Agaricomycetos. No grupo de parasitas de fungos a predominância no CG e CF foi da ordem Pleosporales *,* sendo desta ordem a única sequencia deste grupo encontrada no CF, e Hypocreales nos CRA e CRV, pertencentes a classe dos Sordariomycetes e Dothideomycetes.

É possível que a presença preferencial de fungos patogênicos e parasíticos seja devida a diversidade de funções necessária para conseguir nutrientes em determinadas condições ambientais presentes no “CRV” e “CG”.

4.2.2 Patógenos de plantas

Neste estudo os patógenos de plantas compuseram 433, 884, 3530, 472, nas fitofisionomias CF, CRA, CG e CRV, respectivamente. A ordem de maior abundância nos patógenos de plantas foi a de Pleosporales em todas as fitofisionomias, pertencente a classe dos Dothideomycetes. Um gráfico referente à predominância de ordens por fitofisionomia neste grupo funcional está disposto no **Apêndice G**.

4.3 FUNGOS MICORRÍZICOS

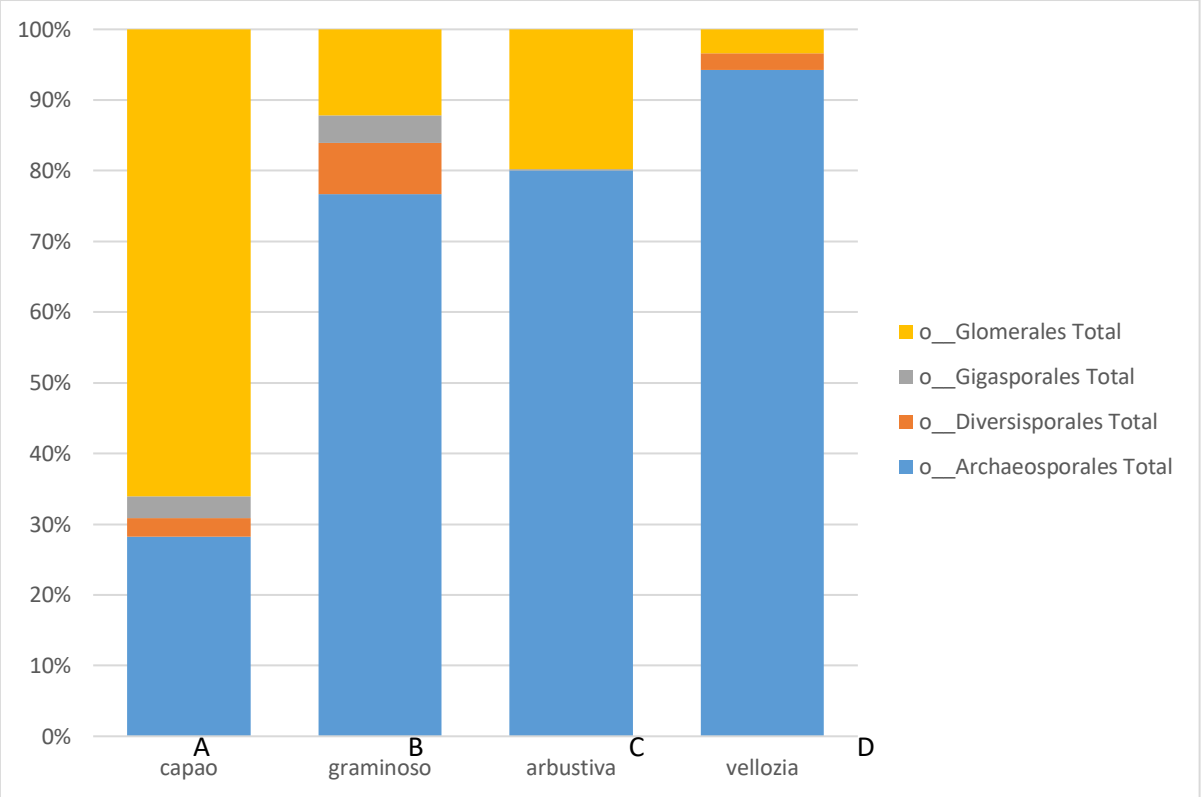
Os fungos micorrízicos encontrados no estudo no solo das fitofisionomias CF, CRA, CG e CRV foram, respectivamente: micorrízicos arbusculares (1152, 1670, 1634, 1105), ectomicorrízicos (835, 3595, 582, 171), micorriza ericóide (51, 34, 493, 38), micorriza de orquídeas (43, 28, 493, 7).

4.3.1 Micorrízicos arbusculares

As ordens de fungos micorrízicos de maior abundância no presente estudo foram de Archaeosporales nos CRA, CRV e CG, e de *Glomerales* no CF, respectivamente, pertencentes as classes Archaeosporomycetes e Glomeromycetes. Um gráfico referente à predominância de ordens por fitofisionomia neste grupo funcional está disposto na figura 8. Na figura é observado que Glomeromycetes só são mais presentes que Archaeosporomycetes no CF, o que pode ser devido a maior porção de solo lá existente, o que pode amenizar efeitos adversos do ambiente -como influência de metais- na seletividade da população de fungos prevalente.

A presença de fungos micorrízicos arbusculares pode ser fundamental na sobrevivência de plantas no ambiente de canga. O conhecimento de qual fungo micorrízico é mais adaptado a determinado tipo de fitofisionomia e suas relações de especificidade com as plantas da canga pode ser um forte aliado para a reintrodução de espécies e reabilitação do ambiente.

Figura 8 - Predominâncias de ordens de fungos micorrízicos arbusculares encontrados. Na figura é possível observar como diferem as ”%” de abundância das ordens encontradas em cada uma das 4 fitofisionomias estudadas, divididas em barras “A” (capão florestal), “B” (campo graminoso), “C” (campo rupestre arbustivo) e “D” (campo rupestre de *Vellozias*).



Fonte: Autor.

4.3.2 Fungos ectomicorrízicos

Neste grupo funcional as ordens de maior abundância foram Hymenochaetales *e* Thelephorales no CRA, Helotiales no CG, Russulales no CF, e Thelephorales no CRV, pertencentes as classes Agaricomycetos e Leotiomycetos. Gráficos de barra referentes às diferenças; nas predominâncias das ordens presentes nos fungos ectomicorrízicos por fitofisionomia, estão dispostos no **Apêndice H**.

4.3.3 Fungos micorrízicos de orquídea

Neste grupo funcional os gêneros predominantes encontrados foram *Meliniomyces* no CG, CRA e CRV o único gênero desse grupo funcional encontrado nessas fitofisionomias, além do gênero *Serendipita* no CF (ordem Sebacinales). Tais gêneros pertencentes as classes Agaricomycetos e Leotiomycetos.

4.3.4 Micorrrizas ericóides

Os gêneros de maior abundância neste grupo funcional foram os *Meliniomyces* no CG e CRA, e *Oidiodendron* no CF e CRV, ambos pertencentes a classe dos Leotiomycetes (ordem Helotiales).

4.4 FUNGOS EPIFÍTICOS

Os fungos epifíticos foram responsáveis por 14, 44, 75, e 32 sequências, respectivamente, nas as fitofisionomias CF, CRA, CG e CRV. Neste grupo o único gênero presente foi o fungo "dark-septado" *Veronaeopsis* da classe dos *Dothideomycetos.*

4.5 FUNGOS ENDOFÍTICOS

Em nosso estudo tais fungos tiveram 237, 788, 4221, 375 sequências encontradas, respectivamente nas fitofisionomias CF, CRA, CG e CRV. As ordens de maior abundância neste grupo funcional foram Xylariales no CG e CRA, Agaricales no CRV e Chaetosphaeriales no CF, pertencentes as classes Sordariomycetes e Agaricomycetes. Um gráfico referente à predominância de ordens por fitofisionomia neste grupo funcional está disposto no **Apêndice I**.

4.6 LÍQUENS

Em nosso estudo, foram encontrados 959, 101, 91, e 98 sequências de fungos formadores de líquens os CF, CRA, CG e CRV.

As ordens de maior abundância neste grupo funcional foram Agaricales no CF, Trapeliales no CRA, e Lecanorales no CRV e CG, pertencentes as classes Agaricomycetes e Lecanoromycetes. Um gráfico referente à predominância de ordens por fitofisionomia neste grupo funcional está disposto no **Apêndice J**.

5 CONCLUSÃO

Por meio desta análise foi possível identificar grupos de fungos até então desconhecidos em solos da canga de Carajás. Segundo a literatura disponível, muitos dos fungos encontrados apresentam resistência a condições extremas, o que justifica sua presença no ambiente de canga. Além disso, os táxons de maior abundância encontrados se relacionavam a condições intrísecas das fitofisionomias o que permitiu fazer uma clara distinção entre elas.

Destacou-se no Capão Florestal a maior presença de fungos saprotrófos de solo, Archaeorhizomycetales, associados às maiores porções de solo presentes nessa fitofisionomia. Os fungos decompositores encontrados nas outras fitofisionomias estão possivelmente associados as condições ambientais e a necessidades específicas para a aquisição de nutrientes.

O Campo Rupestre Arbustivo teve destaque na ordem Hymenochaetales, possivelmente relacionada à maior presença de material ligninico em contato com o solo. O Campo Rupestre de Vellozias se destacou pela presença da ordem Tremelalles possivelmente relacionado à aridez mais severa. O Campo Graminoso se destacou pela identificação de muitas ordens relacionadas a presença de orquídeas como “Capnodiales”, “Xylariales” e “Pleosporales”, assim como fungos micorrízicos de orquídeas.

Vários grupos de fungos micorrízicos arbusculares também foram identificados. Tais fungos podem ser essenciais para o estabelecimento e desenvolvimento de plantas na canga. Nos FMA a ordem Glomerales se apresentou mais abundante nos solos do CF enquanto que Archaeosporales foi dominantes nas outras três fitofisionomias.

Este trabalho traz perspectivas quanto ao estudo de fungos para a conservação de solos de canga e sua biota associada. Espera-se que no prosseguimento da pesquisa possam ser relacionados os diferentes grupos de fungos com variáveis ambientais e propriedades do solo das diferentes fitofisionomias. Seria então possível explicar a distribuição, correlacionar grupos de fungos com espécies de plantas e vislumbrar possíveis aplicações na conservação de acordo com as especificidades de cada ambiente.

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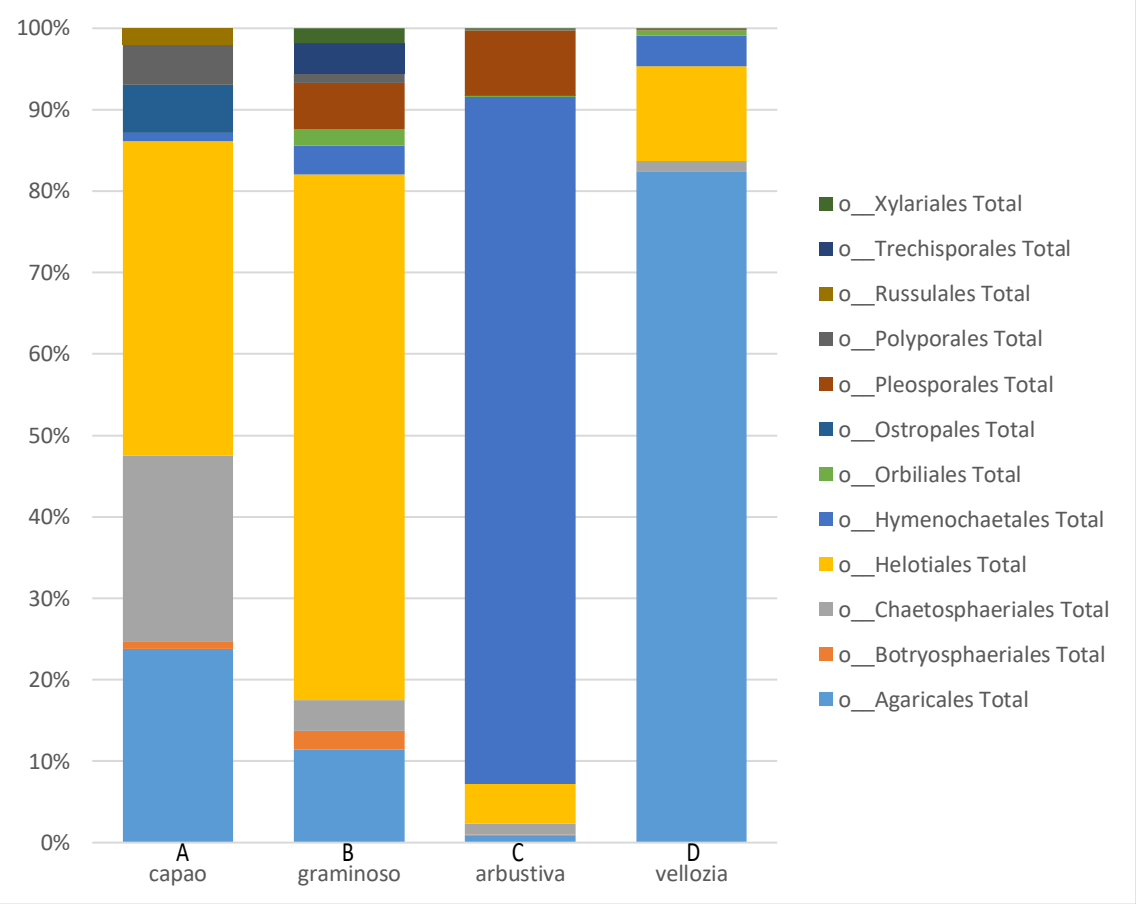
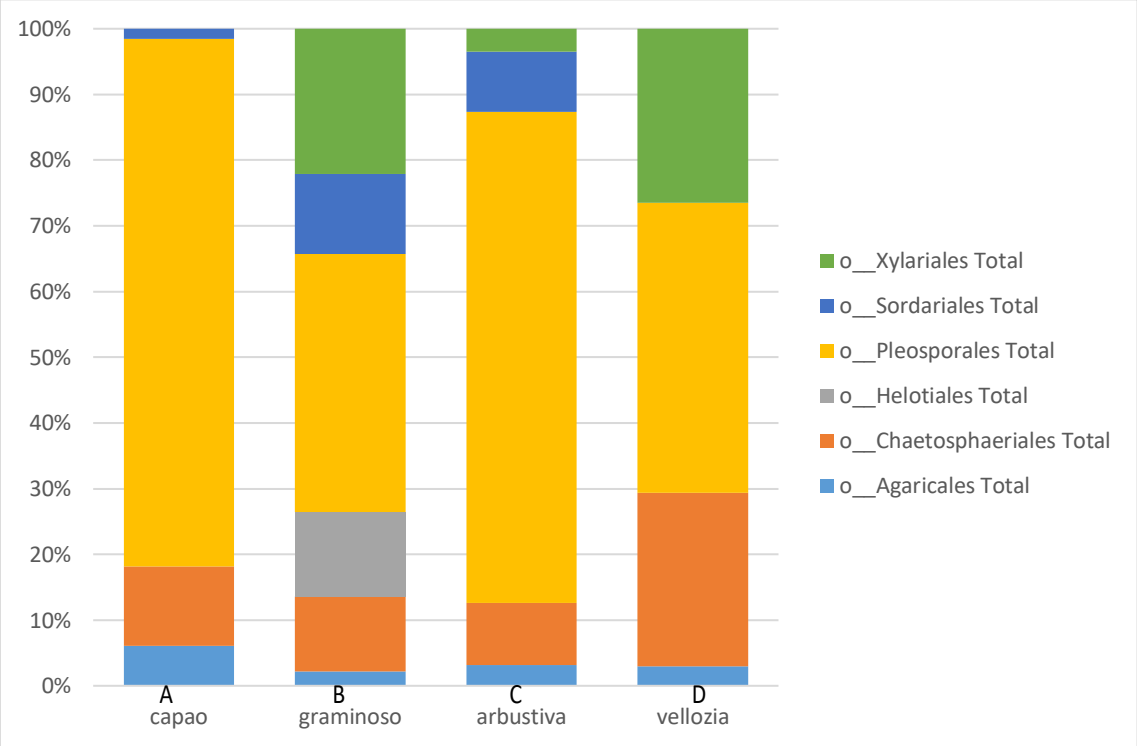
APÊNDICES

APÊNDICE A - Predominâncias de ordens de fungos saprotrófos de madeira encontrados. Na figura é possível observar como diferem as ”%” de abundância das ordens encontradas em cada uma das 4 fitofisionomias estudadas divididas em barras “A” (capão florestal), “B” (campo graminoso), “C” (campo rupestre arbustivo) e “D” (campo rupestre de Vellozias).



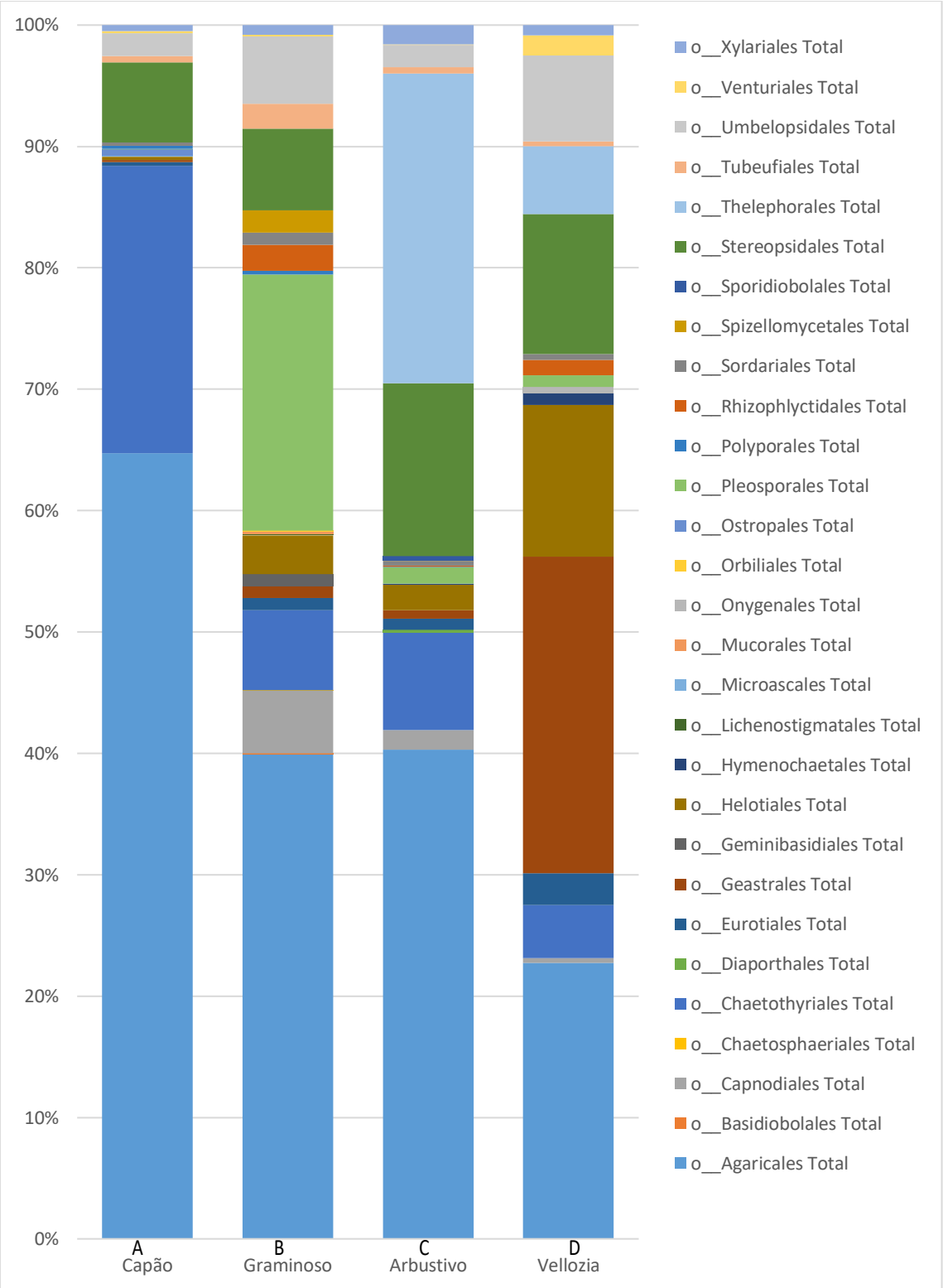
Fonte: Autor.

APÊNDICE B – Predominâncias de ordens de fungos saprotróf os de plantas encontrados. Na figura é possível observar como diferem as ”%” de abundância das ordens encontradas em cada uma das 4 fitofisionomias estudadas divididas em barras “A” (capão florestal), “B” (campo graminoso), “C” (campo rupestre arbustivo) e “D” (campo rupestre de Vellozias).



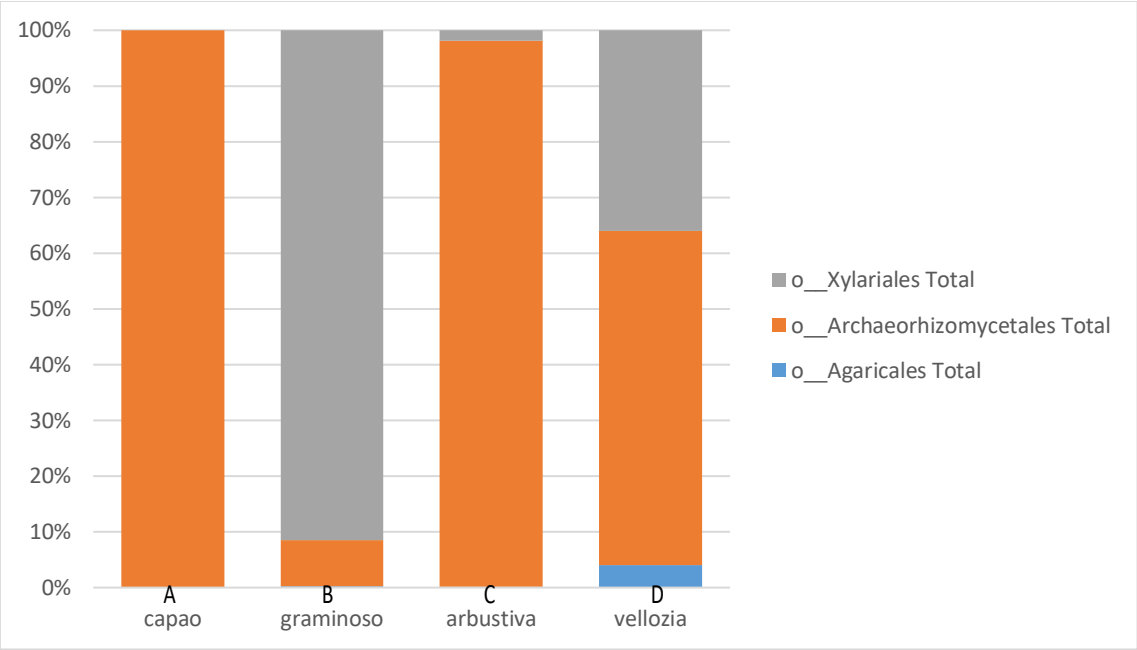
Fonte: Autor.

APÊNDICE C – Predominâncias de ordens de fungos saprotrófos gerais encontrados. Na figura é possível observar como diferem as ”%” de abundância das ordens encontradas em cada uma das 4 fitofisionomias estudadas divididas em barras “A” (capão florestal), “B” (campo graminoso), “C” (campo rupestre arbustivo) e “D” (campo rupestre de Vellozias).



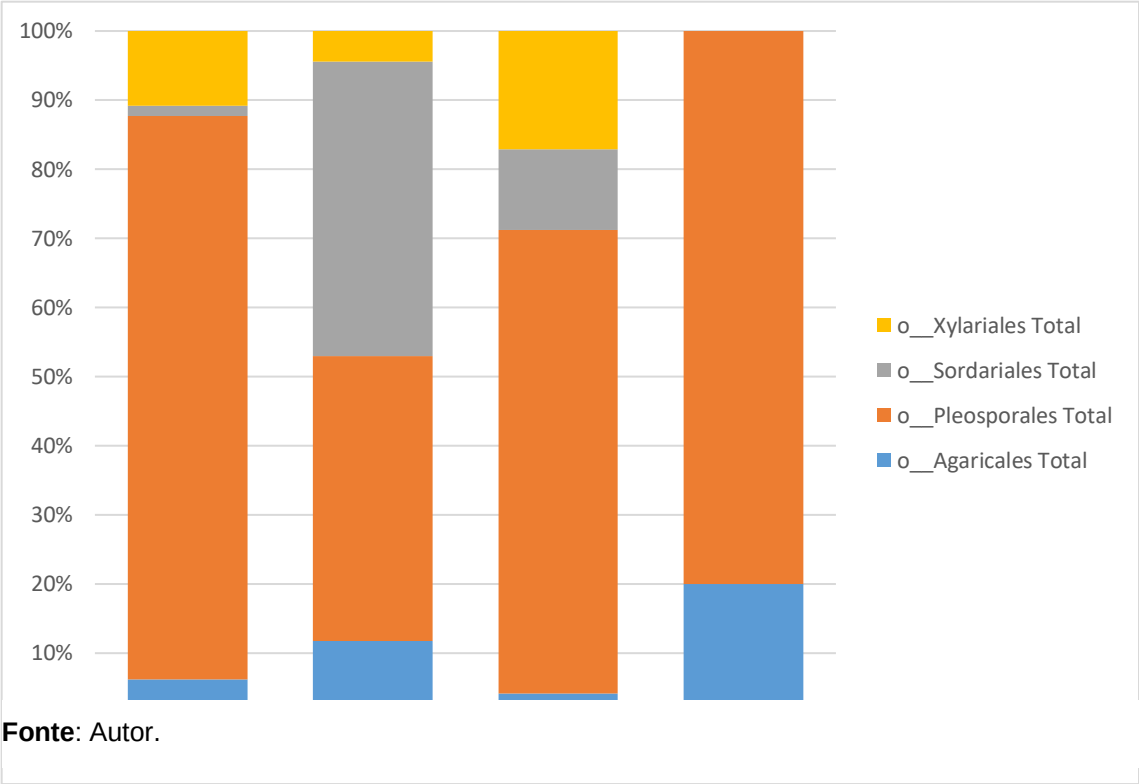
Fonte: Autor.

APÊNDICE D – Predominâncias de ordens de fungos saprotrófos do solo encontrados. Na figura é possível observar como diferem as ”%” de abundância das ordens encontradas em cada uma das 4 fitofisionomias estudadas divididas em barras “A” (capão florestal), “B” (campo graminoso), “C” (campo rupestre arbustivo) e “D” (campo rupestre de Vellozias).



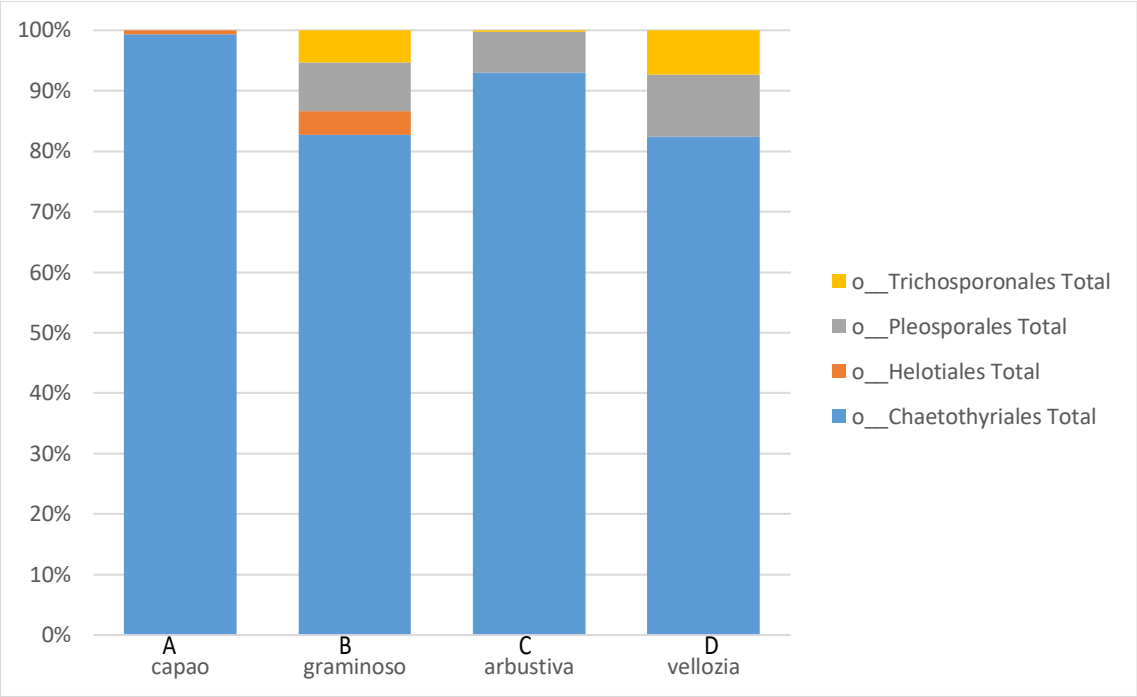
Fonte: Autor.

APÊNDICE E – Predominâncias de ordens de fungos saprotrófos de fezes encontrados. Na figura é possível observar como diferem as ”%” de abundância das ordens encontradas em cada uma das 4 fitofisionomias estudadas divididas em barras “A” (capão florestal), “B” (campo graminoso), “C” (campo rupestre arbustivo) e “D” (campo rupestre de Vellozias).



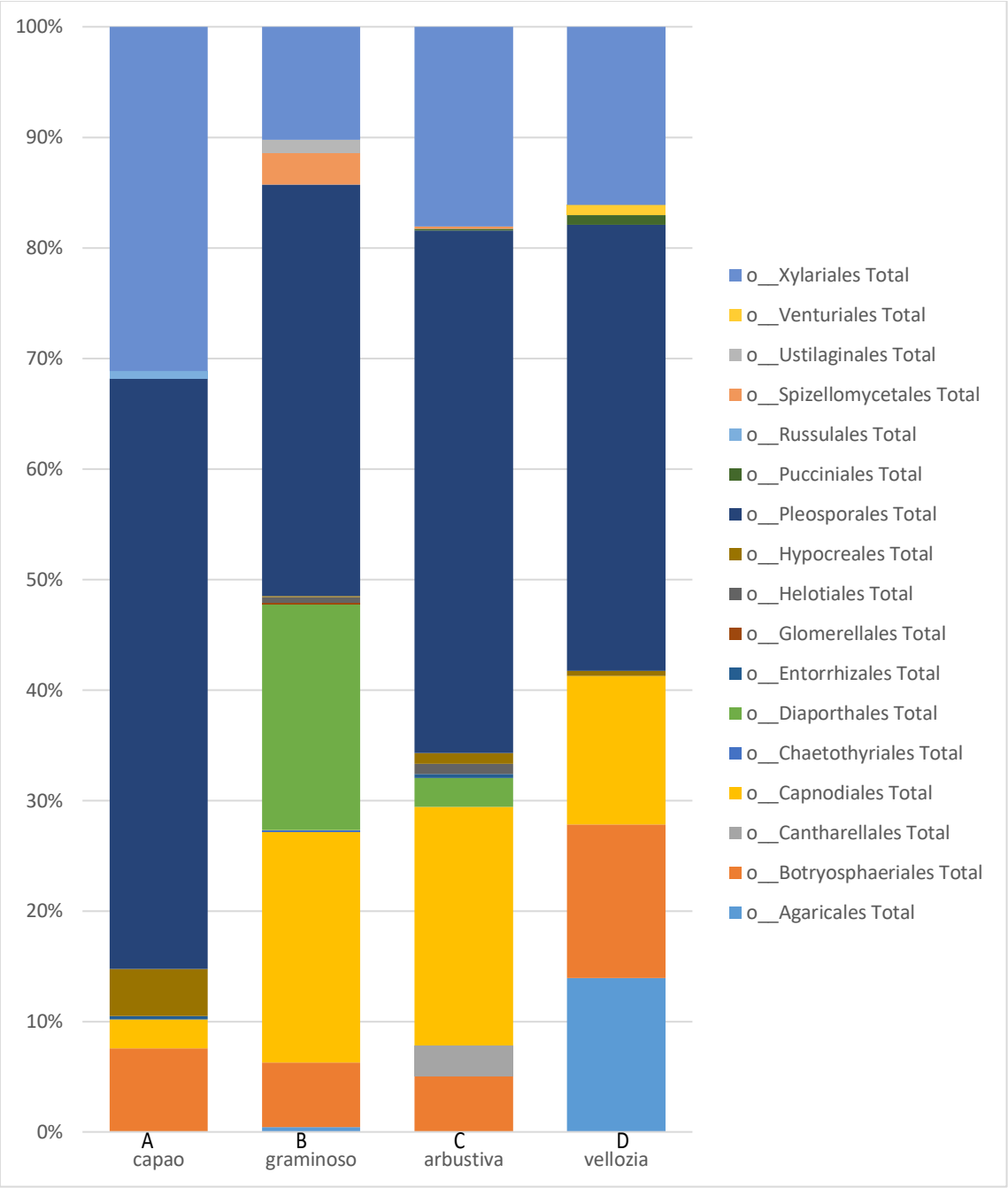
Fonte: Autor.

APÊNDICE F– Predominâncias de ordens de fungos que parasitam outros fungos, encontrados. Na figura é possível observar como diferem as ”%” de abundância das ordens encontradas em cada uma das 4 fitofisionomias estudadas divididas em barras “A” (capão florestal), “B” (campo graminoso), “C” (campo rupestre arbustivo) e “D” (campo rupestre de Vellozias).



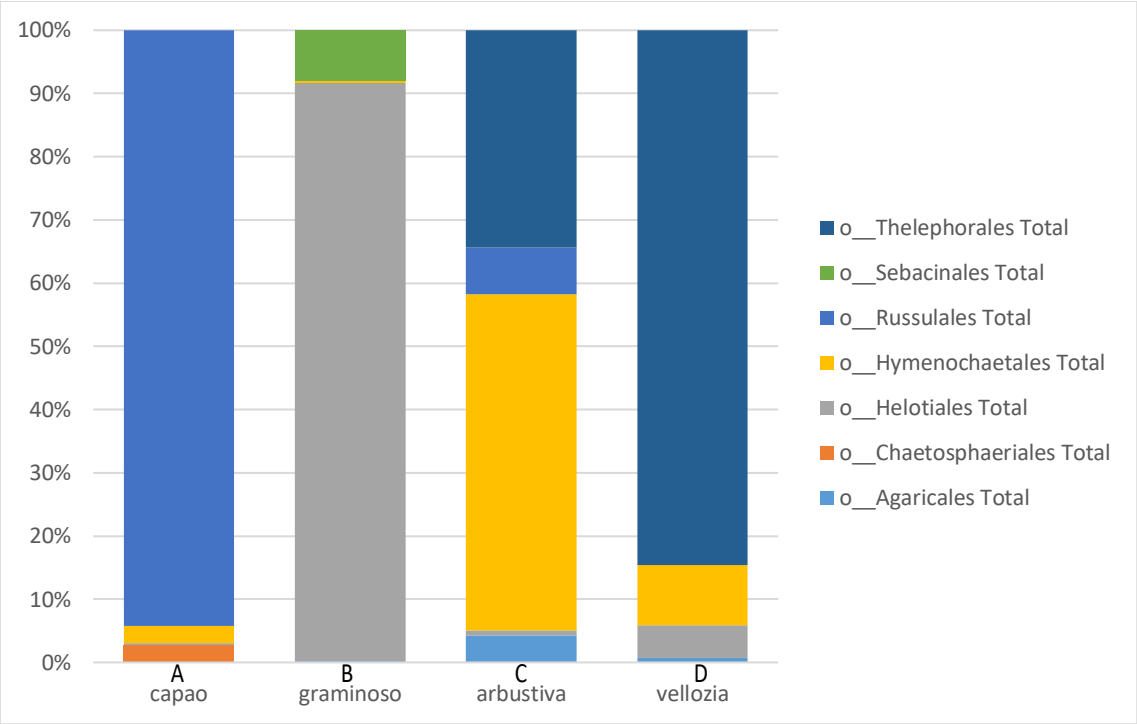
Fonte: Autor.

APÊNDICE G – Predominâncias de ordens de fungos patógenos de plantas encontrados. Na figura é possível observar como diferem as ”%” de abundância das ordens encontradas em cada uma das 4 fitofisionomias estudadas divididas em barras “A” (capão florestal), “B” (campo graminoso), “C” (campo rupestre arbustivo) e “D” (campo rupestre de Vellozias).



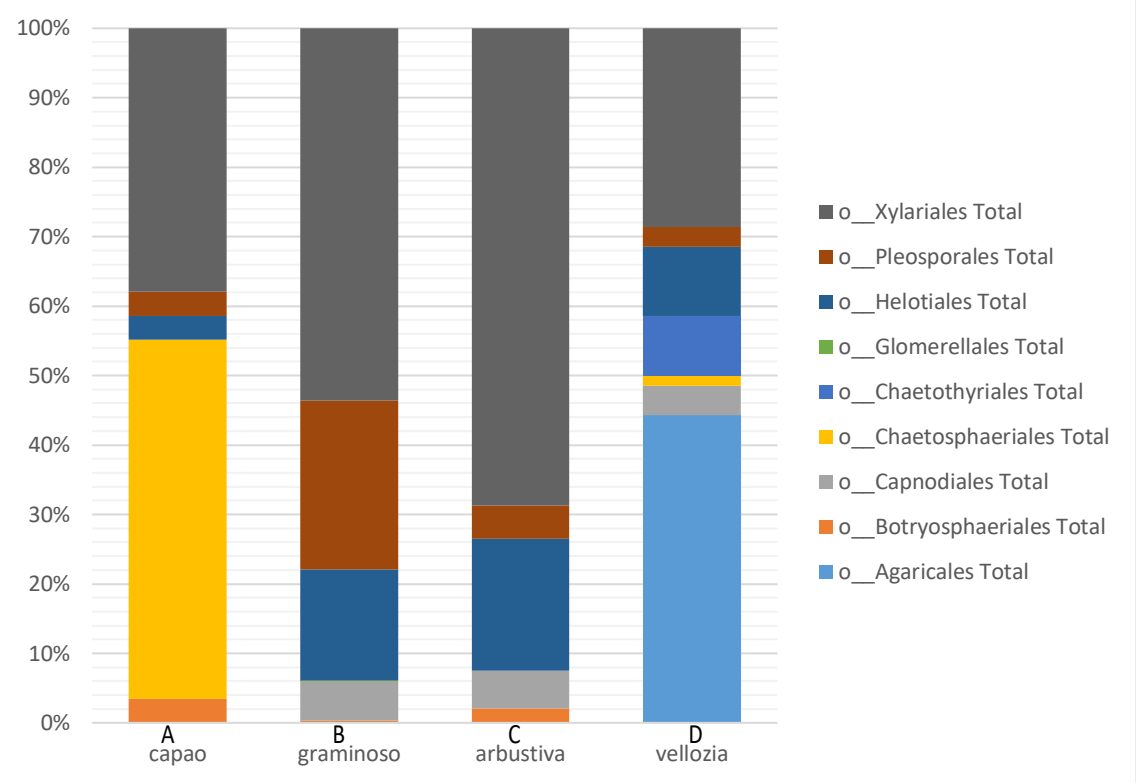
Fonte: Autor.

APÊNDICE H – Predominâncias de ordens de fungos ectomicorrízicos encontrados. Na figura é possível observar como diferem as ”%” de abundância das ordens encontradas em cada uma das 4 fitofisionomias estudadas divididas em barras “A” (capão florestal), “B” (campo graminoso), “C” (campo rupestre arbustivo) e “D” (campo rupestre de Vellozias).



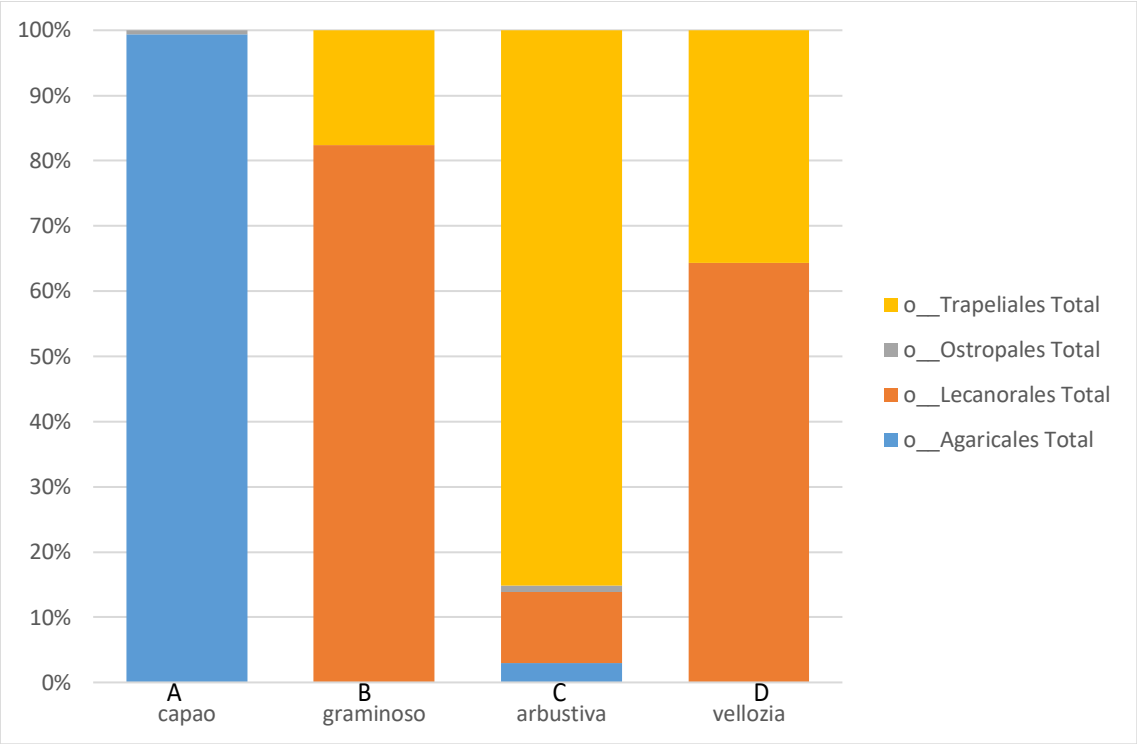
Fonte: Autor.

APÊNDICE I – Predominâncias de ordens de fungos endofíticos encontrados. Na figura é possível observar como diferem as ”%” de abundância das ordens encontradas em cada uma das 4 fitofisionomias estudadas divididas em barras “A” (capão florestal), “B” (campo graminoso), “C” (campo rupestre arbustivo) e “D” (campo rupestre de Vellozias).



Fonte: Autor.

APÊNDICE J – Predominâncias de ordens de fungos formadores de líquens, encontrados. Na figura é possível observar como diferem as ”%” de abundância das ordens encontradas em cada uma das 4 fitofisionomias estudadas divididas em barras “A” (capão florestal), “B” (campo graminoso), “C” (campo rupestre arbustivo) e “D” (campo rupestre de Vellozias).



Fonte: Autor.

ANEXO A

MO BIO Laboratories, Inc.

Saving You Time For Life

PowerSoil® DNA Isolation Kit

Catalog No. 12888-50

Quantity: 50 preps

Catalog No. 12888-100

Quantity: 100 preps

INSTRUCTION MANUAL

Version 07272016

Please recycle

www.mobio.com | T: 800-606-6246 | T: 760-929-9911 | technical@mobio.com

TABLE OF CONTENTS

INTRODUCTION

The PowerSoil® DNA Isolation Kit is comprised of a novel and proprietary method for isolating genomic DNA from environmental samples utilizing our patented Inhibitor Removal Technology® (IRT). The kit is intended for use with environmental samples containing a high humic acid content including difficult soil types such as compost, sediment, and manure. Other more common soil types have also been used successfully with this kit. The isolated DNA has a high level of purity allowing for more successful PCR amplification of organisms from the sample. PCR analysis has been performed to detect a variety of organisms including bacteria (e.g. *Bacillus subtilis, Bacillus anthracis*), fungi (e.g. *yeasts, molds*), algae and Actinomycetes (e.g. *Streptomyces*).

PROTOCOL OVERVIEW

The PowerSoil® DNA Isolation Kit is effective at removing PCR inhibitors from even the most difficult soil types. Environmental samples are added to a bead beating tube for rapid and thorough homogenization. Cell lysis occurs by mechanical and chemical methods. Total genomic DNA is captured on a silica membrane in a spin column format. DNA is then washed and eluted from the membrane. DNA is then ready for PCR analysis and other downstream applications.

BEAD BEATING OPTIONS

The PowerSoil® DNA Isolation Kit does not require homogenization using a high velocity bead beater. However, if the microorganism of interest requires stronger homogenization than provided by a vortex, or if using a bead beater is desired, the PowerSoil® DNA Isolation Kit may be used in conjunction with the PowerLyzer® 24 homogenizer. MO BIO now offers the PowerLyzer® PowerSoil® DNA Isolation Kit (cat# 12855-50) with a Bead Tube suitable for high powered bead beating of soil. For more information about these products, or for references using the PowerSoil® DNA Isolation Kit with a FastPrep® instrument, please contact Technical Service at 1-800-606-6246 or technical@mobio.com.

Additional information can be found at www.mobio.com/blog in the following articles:

https://mobio.com/blog/cat/technical-tips/post/Molecular_Biology_of_Soil_an_introduction/

https://mobio.com/blog/cat/technical-tips/post/Molecular_Biology_of_Soil_DNA_Isolation_Part_I/

PowerLyzer® 24 Bench Top Bead-Based Homogenizer
The PowerLyzer® 24 Bench Top Bead-Based Homogenizer is a bead beating instrument uniquely designed for the most efficient and complete lysis and homogenization of any biological sample. In as little as 30 seconds, the PowerLyzer® 24 homogenizer is capable of processing up to 24 samples in 2 ml tubes. With true "hands-free" operation, the downtime associated with manipulating samples through multiple cycles is eliminated. Even the toughest and most difficult samples such as pine needles, seeds, spores, fungal mats, and clay soils are easily and effectively lysed. For more information and protocols, call technical service.

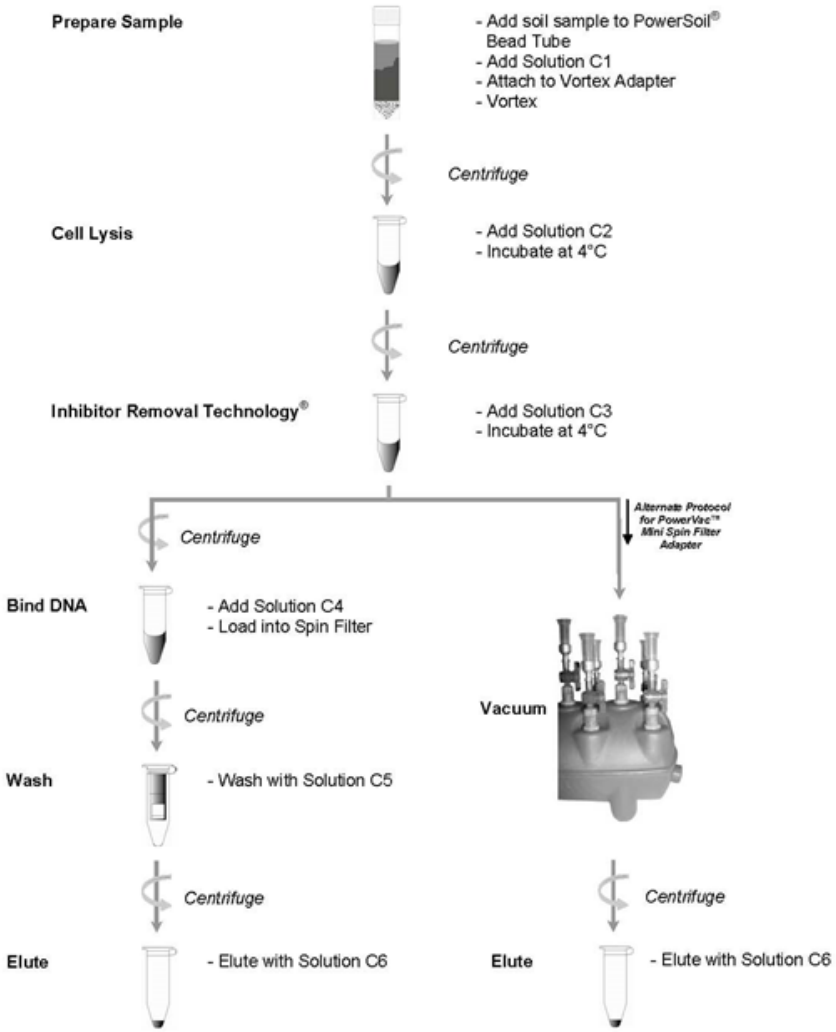
PowerLyzer® 24
Bench Top Bead-Based Homogenizer
Catalog#13155
www.mobio.com/powerlyzer

High Throughput Options
MO BIO offers a vacuum based protocol for faster processing without centrifugation for the DNA binding and column washing steps for Spin Filters. The MO BIO PowerVac™ Manifold allows for processing of up to 20 spin filter preps at a time using the PowerVac™ Mini Spin Filter Adapters. For additional high throughput options MO BIO offers the PowerSoil®-htp 96 Well Soil DNA Isolation Kit for processing up to 2 x 96 samples using a centrifuge capable of spinning two 96 Well Blocks stacked (13 cm x 8 cm x 5.5 cm) at 2500 x g. For 96 well homogenization of soil, MO BIO offers the 96 Well Plate Shaker and Plate Adapter Set (MO BIO Catalog# 11996 & 11990, respectively.)

This kit is for research purposes only. Not for diagnostic use.

Other Related Products	Catalog #	Quantity
PowerMax® Soil DNA Isolation Kit	12988-10	10 preps
PowerSoil®-htp 96 Well Soil DNA Isolation Kit	12955-4	4 x 96 preps

PowerSoil® DNA Isolation Kit



EQUIPMENT REQUIRED

- ☐ Microcentrifuge (10,000 x g)
- ☐ Pipettors (50 µl - 500 µl)
- ☐ Vortex-Genie® 2 Vortex (MO BIO Catalog# 13111-V or 13111-V-220)
- ☐ Vortex Adapter (MO BIO Catalog # 13000-V1-24)

REAGENTS REQUIRED BUT NOT INCLUDED

- ☐ 100% ethanol (for the PowerVac™ Manifold protocol only)

KIT CONTENTS

	Kit Catalog #		Kit Catalog #	
Component	**Catalog #**	**Amount**	**Catalog #**	**Amount**
PowerBead Tubes (contain 750 µl solution)	12888-50-PBT	50	12888-100-PBT	100
PowerSoil® Solution C1	12888-50-1	3.3 ml	12888-100-1	6.6 ml
PowerSoil® Solution C2	12888-50-2	14 ml	12888-100-2	28 ml
PowerSoil® Solution C3	12888-50-3	11 ml	12888-100-3	22 ml
PowerSoil® Solution C4	12888-50-4	72 ml	12888-100-4	144 ml
PowerSoil® Solution C5	12888-50-5	30 ml	12888-100-5	60 ml
PowerSoil® Solution C6	12888-50-6	6 ml	12888-100-6	12 ml
PowerSoil® Spin Filters (units in 2 ml tubes)	12888-50-SF	50	12888-100-SF	100
PowerSoil® 2 ml Collection Tubes	12888-50-T	200	12888-100-T	400

KIT STORAGE

Kit reagents and components should be stored at room temperature (15-30°C).

PRECAUTIONS

Please wear gloves when using this product. Avoid all skin contact with reagents in this kit. In case of contact, wash thoroughly with water. Do not ingest. See Material Safety Data Sheets for emergency procedures in case of accidental ingestion or contact. All SDS information is available upon request (760-929-9911) or on our web site at www.mobio.com. Reagents labeled flammable should be kept away from open flames and sparks.

WARNING: Solution C5 contains ethanol. It is flammable. Do not use bleach to clean the inside of the PowerVac™ Manifold or to rinse the PowerVac™ Mini Spin Filter Adapters when attached to the manifold.

IMPORTANT NOTE FOR USE: Make sure the 2 ml PowerBead Tubes rotate freely in your centrifuge without rubbing. Shake to mix Solution C4 before use.

EXPERIENCED USER PROTOCOL
PowerSoil® DNA Isolation Kit
Catalog No. 12888-50 & 12888-100

Please wear gloves at all times

1. To the **PowerBead Tubes** provided, add 0.25 grams of soil sample.
2. Gently vortex to mix.
3. **Check Solution C1**. If **Solution C1** is precipitated, heat solution to 60°C until dissolved before use.
4. Add 60 µl of **Solution C1** and invert several times or vortex briefly.
5. Secure **PowerBead Tubes** horizontally using the MO BIO Vortex Adapter tube holder for the vortex (MO BIO Catalog# 13000-V1-24) or secure tubes horizontally on a flat-bed vortex pad with tape. Vortex at maximum speed for 10 minutes.

Note

If you are using the 24 place Vortex Adapter for more than 12 preps, increase the vortex time by 5-10 minutes.

6. Make sure the **PowerBead Tubes** rotate freely in your centrifuge without rubbing. Centrifuge tubes at 10,000 x g for 30 seconds at room temperature. **CAUTION**: Be sure not to exceed 10,000 x g or tubes may break.
7. Transfer the supernatant to a clean **2 ml Collection Tube** (provided).

Note

Expect between 400 to 500 µl of supernatant. Supernatant may still contain some soil particles.

8. Add 250 µl of **Solution C2** and vortex for 5 seconds. Incubate at 4°C for 5 minutes.
9. Centrifuge the tubes at room temperature for 1 minute at 10,000 x g.
10. Avoiding the pellet, transfer up to, but no more than, 600 µl of supernatant to a clean **2 ml Collection Tube** (provided).
11. Add 200 µl of **Solution C3** and vortex briefly. Incubate at 4°C for 5 minutes.
12. Centrifuge the tubes at room temperature for 1 minute at 10,000 x g.
13. Avoiding the pellet, transfer up to, but no more than, 750 µl of supernatant into a clean **2 ml Collection Tube** (provided).
14. Shake to mix **Solution C4** before use. Add 1200 µl of **Solution C4** to the supernatant and vortex for 5 seconds.

15. Load approximately 675 µl onto a **Spin Filter** and centrifuge at 10,000 x g for 1 minute at room temperature. Discard the flow through and add an additional 675 µl of supernatant to the **Spin Filter** and centrifuge at 10,000 x g for 1 minute at room temperature. Load the remaining supernatant onto the **Spin Filter** and centrifuge at 10,000 x g for 1 minute at room temperature.

Note

A total of three loads for each sample processed are required.

16. Add 500 µl of **Solution C5** and centrifuge at room temperature for 30 seconds at 10,000 x g.

17. Discard the flow through.

18. Centrifuge again at room temperature for 1 minute at 10,000 x g.

19. Carefully place spin filter in a clean **2 ml Collection Tube** (provided). Avoid splashing any **Solution C5** onto the **Spin Filter**.

20. Add 100 µl of Solution C6 to the center of the white filter membrane. Alternatively, sterile DNA-Free PCR Grade Water may be used for elution from the silica Spin Filter membrane at this step (MO BIO Catalog# 17000-10).

21. Centrifuge at room temperature for 30 seconds at 10,000 x g.

22. Discard the **Spin Filter**. The DNA in the tube is now ready for any downstream application. No further steps are required.

We recommend storing DNA frozen (-20° to -80°C). **Solution C6** contains no EDTA. To concentrate the DNA see the Hints & Troubleshooting Guide.

Thank you for choosing the PowerSoil® DNA Isolation Kit!

DETAILED USER PROTOCOL *(DESCRIBES WHAT IS HAPPENING AT EACH STEP)*

PowerSoil® DNA Isolation Kit

Catalog No. 12888-50 & 12888-100

Please wear gloves at all times

1. To the **PowerBead Tubes** provided, add 0.25 grams of soil sample.

   What's happening: After your sample has been loaded into the PowerBead Tube, the next step is a homogenization and lysis procedure. The PowerBead Tube contains a buffer that will (a) help disperse the soil particles, (b) begin to dissolve humic acids and (c) protect nucleic acids from degradation.

2. Gently vortex to mix.

   What's happening: Gentle vortexing mixes the components in the PowerBead Tube and begins to disperse the sample in the PowerBead Solution.

3. **Check Solution C1**. If **Solution C1** is precipitated, heat solution to 60°C until the precipitate has dissolved before use.

   What's happening: Solution C1 contains SDS and other disruption agents required for complete cell lysis. In addition to aiding in cell lysis, SDS is an anionic detergent that breaks down fatty acids and lipids associated with the cell membrane of several organisms. If it gets cold, it will form a white precipitate in the bottle. Heating to 60°C will dissolve the SDS and will not harm the SDS or the other disruption agents. Solution C1 can be used while it is still warm.

4. Add 60 µl of **Solution C1** and invert several times or vortex briefly.

5. Secure **PowerBead Tubes horizontally** using the MO BIO Vortex Adapter tube holder for the vortex (MO BIO Catalog# 13000-V1-24) or secure tubes horizontally on a flat-bed vortex pad with tape. Vortex at maximum speed for 10 minutes.

Note

Note: If you are using the 24 place Vortex Adapter for more than 12 preps, increase the vortex time by 5-10 minutes.

Note

The vortexing step is critical for complete homogenization and cell lysis. Cells are lysed by a combination of chemical agents from steps 1-4 and mechanical shaking introduced at this step. By randomly shaking the beads in the presence of disruption agents, collision of the beads with microbial cells will cause the cells to break open.

What's happening: The MO BIO Vortex Adapter is designed to be a simple platform to facilitate keeping the tubes tightly attached to the vortex. It should be noted that although you can attach tubes with tape, often the tape becomes loose and not all tubes will shake evenly or efficiently. This may lead to inconsistent results or lower yields. Therefore, the use of the MO BIO Vortex Adapter is a highly recommended and cost effective way to obtain maximum DNA yields.

6. Make sure the **PowerBead Tubes** rotate freely in your centrifuge without rubbing. Centrifuge tubes at 10,000 x g for 30 seconds at room temperature. **CAUTION**: Be sure not to exceed 10,000 x g or tubes may break.

7. Transfer the supernatant to a clean **2 ml Collection Tube** (provided).

Note

Expect between 400 to 500 µl of supernatant at this step. The exact recovered volume depends on the absorbency of your starting material and is not critical for the procedure to be effective. The supernatant may be dark in appearance and still contain some soil particles. The presence of carry over soil or a dark color in the mixture is expected in many soil types at this step. Subsequent steps in the protocol will remove both carry over soil and coloration of the mixture.

8. Add 250 µl of **Solution C2** and vortex for 5 seconds. Incubate at 4°C for 5 minutes.

What's happening: Solution C2 is patented Inhibitor Removal Technology® (IRT). It contains a reagent to precipitate non-DNA organic and inorganic material including humic substances, cell debris, and proteins. It is important to remove contaminating organic and inorganic matter that may reduce DNA purity and inhibit downstream DNA applications.

9. Centrifuge the tubes at room temperature for 1 minute at 10,000 x g.

10. Avoiding the pellet, transfer up to 600 µl of supernatant to a clean **2 ml Collection Tube** (provided).

What's happening: The pellet at this point contains non-DNA organic and inorganic material including humic acid, cell debris, and proteins. For the best DNA yields, and quality, avoid transferring any of the pellet.

11. Add 200 µl of **Solution C3** and vortex briefly. Incubate at 4°C for 5 minutes.

What's happening: Solution C3 is patented Inhibitor Removal Technology® (IRT) and is a second reagent to precipitate additional non-DNA organic and inorganic material including humic acid, cell debris, and proteins. It is important to remove contaminating organic and inorganic matter that may reduce DNA purity and inhibit downstream DNA applications.

12. Centrifuge the tubes at room temperature for 1 minute at 10,000 x g.

13. Transfer up to 750 µl of supernatant to a clean **2 ml Collection Tube** (provided).

What's happening: The pellet at this point contains additional non-DNA organic and inorganic material including humic acid, cell debris, and proteins. For the best DNA yields, and quality, avoid transferring any of the pellet.

14. Shake to mix **Solution C4** before use. Add 1.2 ml of **Solution C4** to the supernatant (be careful solution doesn't exceed rim of tube) and vortex for 5 seconds.

What's happening: Solution C4 is a high concentration salt solution. Since DNA binds tightly to silica at high salt concentrations, this will adjust the DNA solution salt concentrations to allow binding of DNA, but not non-DNA organic and inorganic material that may still be present at low levels, to the Spin Filters.

15. Load approximately 675 µl onto a **Spin Filter** and centrifuge at 10,000 x g for 1 minute at room temperature. Discard the flow through and add an additional 675 µl of supernatant to the **Spin Filter** and centrifuge at 10,000 x g for 1 minute at room temperature. Load the remaining supernatant onto the **Spin Filter** and centrifuge at 10,000 x g for 1 minute at room temperature.

Note

A total of three loads for each sample processed are required.

What's happening: DNA is selectively bound to the silica membrane in the Spin Filter device in the high salt solution. Contaminants pass through the filter membrane, leaving only DNA bound to the membrane.

16. Add 500 µl of **Solution C5** and centrifuge at room temperature for 30 seconds at 10,000 x g.

What's happening: Solution C5 is an ethanol based wash solution used to further clean the DNA that is bound to the silica filter membrane in the Spin Filter. This wash solution removes residual salt, humic acid, and other contaminants while allowing the DNA to stay bound to the silica membrane.

17. Discard the flow through from the **2 ml Collection Tube.**

What's happening: This flow through fraction is just non-DNA organic and inorganic waste removed from the silica Spin Filter membrane by the ethanol wash solution.

18. Centrifuge at room temperature for 1 minute at 10,000 x g.

What's happening: This second spin removes residual Solution C5 (ethanol wash solution). It is critical to remove all traces of wash solution because the ethanol in Solution C5 can interfere with many downstream DNA applications such as PCR, restriction digests, and gel electrophoresis.

19. Carefully place **Spin Filter** in a clean **2 ml Collection Tube** (provided). Avoid splashing any **Solution C5** onto the **Spin Filter**.

Note

It is important to avoid any traces of the ethanol based wash solution.

20. Add 100 µl of **Solution C6** to the center of the white filter membrane.

Note

Placing the Solution C6 (sterile elution buffer) in the center of the small white membrane will make sure the entire membrane is wetted. This will result in a more efficient and complete release of the DNA from the silica Spin Filter membrane. As Solution C6 (elution buffer) passes through the silica membrane, DNA that was bound in the presence of high salt is selectively released by Solution C6 (10 mM Tris) which lacks salt.

Alternatively, sterile DNA-Free PCR Grade Water may be used for DNA elution from the silica Spin Filter membrane at this step (MO BIO Catalog# 17000-10). Solution C6 contains no EDTA. If DNA degradation is a concern, Sterile TE may also be used instead of Solution C6 for elution of DNA from the Spin Filter.

21. Centrifuge at room temperature for 30 seconds at 10,000 x g.

22. Discard the **Spin Filter**. The DNA in the tube is now ready for any downstream application. No further steps are required.

We recommend storing DNA frozen (-20° to -80°C). **Solution C6** does not contain any EDTA. To concentrate DNA see the Hints & Troubleshooting Guide.

Thank you for choosing the PowerSoil® DNA Isolation Kit!

VACUUM PROTOCOL USING THE POWERVAC™ MANIFOLD

Please wear gloves at all times

For each sample lysate, use one Spin Filter column. Keep the Spin Filter in the attached 2 ml Collection Tube and continue using the Collection Tube as a Spin Filter holder until needed for the Vacuum Manifold Protocol. Label each Collection Tube top and Spin Filter column to maintain sample identity. If the Spin Filter becomes clogged during the vacuum procedure, you can switch to the procedure for purification of the DNA by centrifugation.

You will need to provide 100% ethanol for step 4 of this protocol.

1. For each prep, attach one aluminum **PowerVac™ Mini Spin Filter Adapter** (MO BIO Catalog# 11992-20) into the Luer-Lok® fitting of one port in the manifold. Gently press a **Spin Filter column** into the PowerVac™ Mini Spin Filter Adapter until snugly in place. Ensure that all unused ports of the vacuum manifold are closed.

Note

Aluminum PowerVac™ Mini Spin Filter Adapters are reusable.

2. Transfer 650 µl of prepared sample lysate (from step 14) to the **Spin Filter column**.

3. Turn on the vacuum source and open the stopcock of the port. Hold the tube in place when opening the stopcock to keep the spin filter steady. Allow the lysate to pass through the **Spin Filter column**. After the lysate has passed through the column completely, load again with the next 650 µl of lysate. Continue until all of the lysate has been loaded onto the **Spin Filter column**. Close the one-way Luer-Lok® stopcock of that port.

Note

If Spin Filter Columns are filtering slowly, close the ports to samples that have completed filtering to increase the pressure to the other columns.

4. Load 800 µl of 100% ethanol into the **Spin Filter** so that it completely fills the column. Open the stopcock while holding the column steady. Allow the ethanol to pass through the column completely. Close the stopcock.

5. Add 500 µl of **Solution C5** to each Spin Filter. Open the Luer-Lok® stopcock and apply a vacuum until **Solution C5** has passed through the Spin Filter completely. Continue to pull a vacuum for another minute to dry the membrane. Close each port.

6. Turn off the vacuum source and open an unused port to vent the manifold. If all 20 ports are in use, break the vacuum at the source. Make certain that all vacuum pressure is released before performing the next step. It is important to turn off the vacuum at the source to prevent backflow into the columns.

7. Remove the **Spin Filter column** and place in the original labeled **2 ml Collection Tube**. Place into the centrifuge and spin at 13,000 × g for 1 minute to completely dry the membrane.

8. Transfer the S**pin Filter column** to a new **2 ml Collection Tube** and add 100 µl of **Solution C6** to the center of the white filter membrane. Alternatively, sterile DNA-Free PCR Grade Water may be used for elution from the silica **Spin Filter** membrane at this step (MO BIO Catalog # 17000-10).

9. Centrifuge at room temperature for 30 seconds at 10,000 x g.

10. Discard the **Spin Filter column**. The DNA in the tube is now ready for any downstream application. No further steps are required.

We recommend storing DNA frozen (-20° to -80°C). **Solution C6** contains no EDTA. To concentrate the DNA see the Hints & Troubleshooting Guide.

Thank you for choosing the PowerSoil® DNA Isolation Kit!

HINTS AND TROUBLESHOOTING GUIDE

Amount of Soil to Process
This kit is designed to process 0.25 grams of soil. For inquiries regarding the use of larger sample amounts, please contact technical support for suggestions. For wet soils, see information under "Wet Soil Sample" below.

Wet Soil Sample
If soil sample is high in water content, remove contents from PowerBead Tube (beads and solution) and transfer into another sterile microcentrifuge tube (not provided). Add soil sample to PowerBead Tube and centrifuge at room temperature for 30 seconds at 10,000 x g. Remove as much liquid as possible with a pipet tip. Add beads and bead solution back to PowerBead Tube and follow protocol starting at step 2.

If DNA Does Not Amplify
- Make sure to check DNA yields by gel electrophoresis or spectrophotometer reading. An excess amount of DNA will inhibit a PCR reaction.
- Diluting the template DNA should not be necessary with DNA isolated with the PowerSoil® DNA Isolation Kit; however, it should still be attempted.
- If DNA will still not amplify after trying the steps above, then PCR optimization (changing reaction conditions and primer choice) may be needed.

Eluted DNA Sample Is Brown
We have not observed any coloration in DNA isolated using the PowerSoil® DNA Isolation Kit. If you observe coloration in your samples, please contact technical support for suggestions.

Alternative Lysis Methods
- After adding Solution C1, vortex 3-4 seconds, then heat to 70°C for 5 minutes. Vortex 3-4 seconds. Heat another 5 minutes. Vortex 3-4 seconds. This alternative procedure will reduce shearing but may also reduce yield.
- If cells are difficult to lyse, a 10 minute incubation at 70°C, after adding Solution C1, can be performed. Follow by continuing with protocol step 5.

Concentrating the DNA
The final volume of eluted DNA will be 100 µl. The DNA may be concentrated by adding 4 µl of 5 M NaCl and inverting 3-5 times to mix. Next, add 200 µl of 100% cold ethanol and invert 3-5 times to mix. Centrifuge at 10,000 x g for 5 minutes at room temperature. Decant all liquid. Remove residual ethanol in a speed vac, dessicator, or air dry. Resuspend precipitated DNA in sterile water or sterile 10 mM Tris.

DNA Floats Out of Well When Loaded on a Gel
This usually occurs because residual Solution C5 remains in the final sample. Prevent this by being careful in step 19 not to transfer liquid onto the bottom of the spin filter basket. Ethanol precipitation (described in "Concentrating the DNA") is the best way to remove residual Solution C5.

Storing DNA
DNA is eluted in Solution C6 (10 mM Tris) and must be stored at -20° to -80°C to prevent degradation. DNA can be eluted in TE without loss, but the EDTA may inhibit downstream reactions such as PCR and automated sequencing. DNA may also be eluted with sterile DNA-Free PCR Grade Water (MO BIO Catalog# 17000-10).

Cleaning of the PowerVac™ Mini Spin Filter Adapters
It is recommended to rinse the PowerVac™ Mini Spin Filter Adapters promptly after use to avoid salt build up. To clean the PowerVac™ Mini Spin Filter Adapters, rinse each adapter with DI water followed by 70% ethanol and flush into the manifold base. Alternatively, remove the adapters and wash in laboratory detergent and DI water. PowerVac™ Mini Spin Filter Adapters may be autoclaved.

Do not use bleach to clean the PowerVac™ Mini Spin Filter Adapters while attached to the PowerVac™ Manifold. Bleach should never be mixed with solutions containing guanidine and should not be used to clean the PowerVac™ Manifold. For more information on cleaning the PowerVac™ Manifold, please refer to the PowerVac™ Manifold manual.

TECHNICAL SUPPORT

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Committed to resolving your technical questions promptly, our technical support team is trained to work with you to rapidly and effectively trouble shoots any issues. We commit to providing you with relevant online support resources that help you complete your research projects.

Frequently Asked Questions:
https://mobio.com/faq

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ANEXO B

16S Metagenomic Sequencing Library Preparation

Preparing 16S Ribosomal RNA Gene Amplicons for the Illumina MiSeq System

Introduction

Metagenomic studies are commonly performed by analyzing the prokaryotic 16S ribosomal RNA gene (16S rRNA), which is approximately 1,500 bp long and contains nine variable regions interspersed between conserved regions. Variable regions of 16S rRNA are frequently used in phylogenetic classifications such as genus or species in diverse microbial populations.

Which 16S rRNA region to sequence is an area of debate, and your region of interest might vary depending on things such as experimental objectives, design, and sample type. This protocol describes a method for preparing samples for sequencing the variable V3 and V4 regions of the 16S rRNA gene. This protocol can also be used for sequencing other regions with different region-specific primers. This protocol combined with a benchtop sequencing system, on-board primary analysis, and secondary analysis using MiSeq Reporter or BaseSpace, provides a comprehensive workflow for 16S rRNA amplicon sequencing.

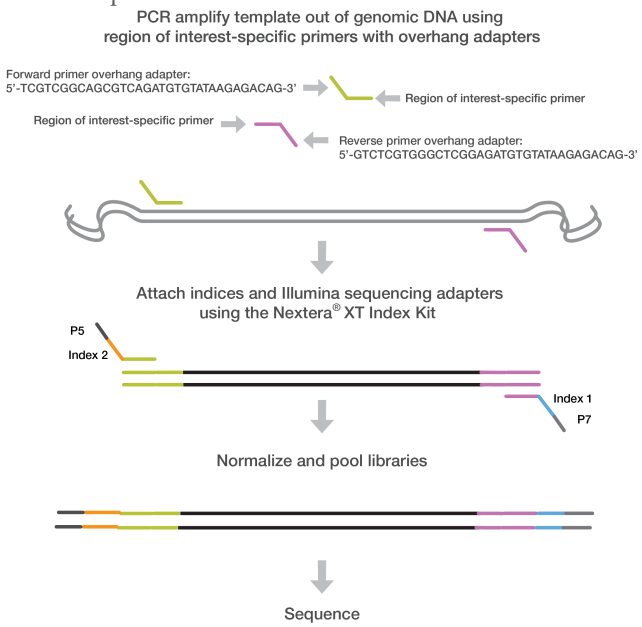
Workflow Summary:

1. Order amplicon primers–The protocol includes the primer pair sequences for the V3 and V4 region that create a single amplicon of approximately ~460 bp. The protocol also includes overhang adapter sequences that must be appended to the primer pair sequences for compatibility with Illumina index and sequencing adapters. Illumina does not sell these primers. They must be ordered from a third party. See Amplicon Primers, on page 3 for more information on amplicon primers.
2. Prepare library–The protocol describes the steps to amplify the V3 and V4 region and using a limited cycle PCR, add Illumina sequencing adapters and dual-index barcodes to the amplicon target. Using the full complement of Nextera XT indices, up to 96 libraries can be pooled together for sequencing.
3. Sequence on MiSeq–Using paired 300-bp reads, and MiSeq v3 reagents, the ends of each read are overlapped to generate high-quality, full-length reads of the V3 and V4 region in a single 65-hour run. The MiSeq run output is approximately > 20 million reads and, assuming 96 indexed samples, can generate > 100,000 reads per sample, commonly recognized as sufficient for metagenomic surveys.
4. Analyze on MSR or BaseSpace–The Metagenomics workflow is a secondary analysis option built into the MiSeq Reporter (on-system software) or available on BaseSpace (cloud-based software). The Metagenomics Workflow performs a taxonomic classification using the Greengenes database showing genus or species level classification in a graphical format.

This protocol can be used to sequence alternative regions of the 16S rRNA gene and for other targeted amplicon sequences of interest. When using this protocol for amplicon sequencing other than 16S rRNA, use the Generate FASTQ Workflow (secondary analysis option). For more information, see MiSeq Reporter Metagenomics Workflow, on page 20.

DISCLAIMER
The information in this Illumina Demonstrated Protocol is being provided as a courtesy; in some cases reagents are required to be purchased from non-authorized third-party suppliers. Illumina does not guarantee nor promises technical support for the performance of our products used with reagents purchased from a non-authorized third-party supplier.

Figure 1 16S V3 and V4 Amplicon Workflow



User-defined forward and reverse primers that are complementary upstream and downstream of the region of interest are designed with overhang adapters, and used to amplify templates from genomic DNA. A subsequent limited-cycle amplification step is performed to add multiplexing indices and Illumina sequencing adapters. Libraries are normalized and pooled, and sequenced on the MiSeq system using v3 reagents.

Amplicon Primers

- The gene-specific sequences used in this protocol target the 16S V3 and V4 region. They are selected from the Klindworth et al. publication (Klindworth A, Pruesse E, Schweer T, Peplles J, Quast C, et al. (2013) Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. Nucleic Acids Res 41(1).) as the most promising bacterial primer pair. Illumina adapter overhang nucleotide sequences are added to the gene-specific sequences. The full length primer sequences, using standard IUPAC nucleotide nomenclature, to follow the protocol targeting this region are:
  16S Amplicon PCR Forward Primer = 5'
  TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG
  16S Amplicon PCR Reverse Primer = 5'
  GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC
- This method can also be utilized to target other regions on the genome (either for 16S with other sets of primer pairs, or non-16S regions throughout the genome; ie any amplicon). The overhang adapter sequence must be added to the locus-specific primer for the region to be targeted (Figure 1). The Illumina overhang adapter sequences to be added to locus-specific sequences are:
  Forward overhang: 5′ TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-[locus-specific sequence]
  Reverse overhang: 5′ GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-[locus-specific sequence]

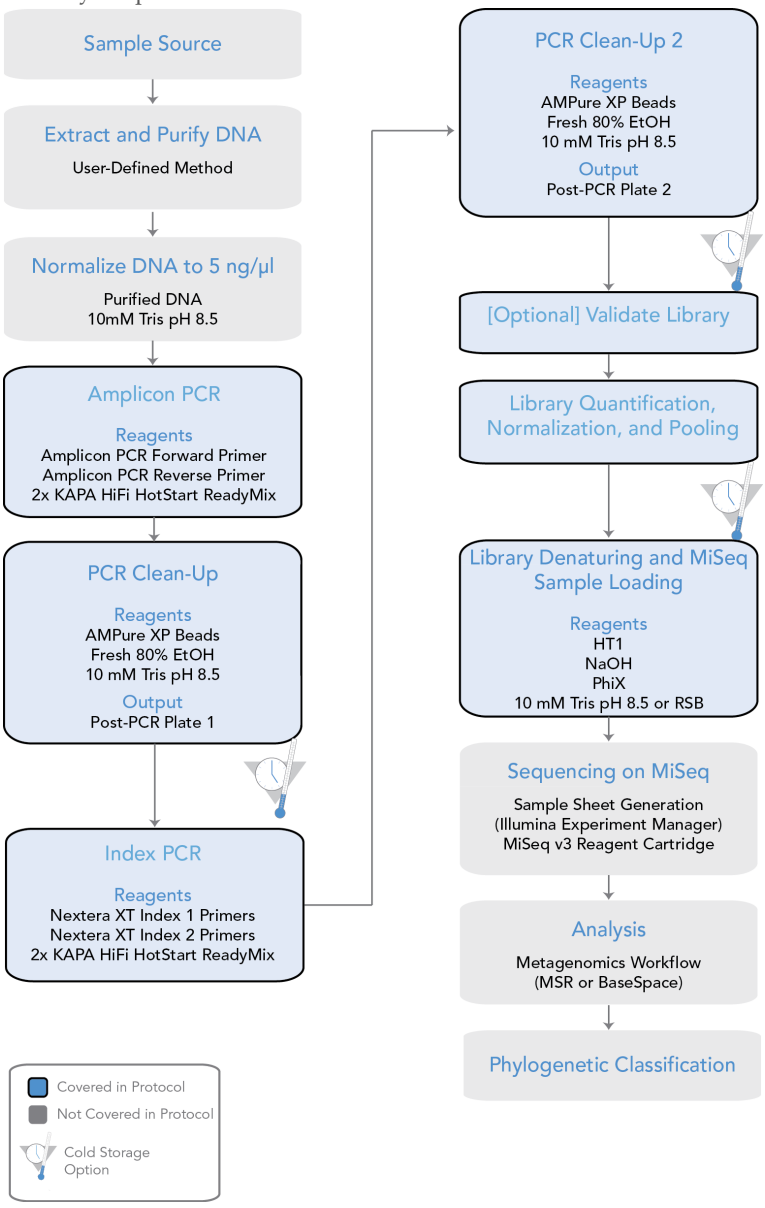
- The following considerations are recommended for designing other locus-specific primers:
  - **a** Illumina recommends targeting regions that result in an amplicon that when sequenced with paired-end reads has at least ~50 bp of overlapping sequence in the middle. For example, if running 2x300 bp paired-end reads Illumina recommends having an insert size of 550 bp or smaller so that the bases sequenced at the end of each read overlap.
  - **b** The locus-specific portion of primer (not including overhang sequence) must have a melting temperature (Tm) of 60°–65°C. You can use online PCR primer sequence analysis tools (e.g. http://www.idtdna.com/analyzer/Applications/OligoAnalyzer/) to check the properties of primer designs. For the Tm calculation only, the gene-specific portion must be used in calculation. For hairpin and dimer calculations, the fully-assembled primer sequence (including the overhang) should be used.
  - **c** Illumina recommends using standard desalting purification when ordering oligo primer sets.

NOTE
For more information on reagents used in the protocol, see Consumables and Equipment, on page 21.

16S Library Preparation Workflow

The following diagram illustrates the workflow using the 16S Library Preparation Protocol. Safe stopping points are marked between steps.

Figure 2 16S Library Preparation Workflow



Amplicon PCR

This step uses PCR to amplify template out of a DNA sample using region of interest-specific primers with overhang adapters attached. For more information on primer sequences, see Amplicon Primers, on page 3.

Consumables

NOTE
For more information on consumables and equipment for this protocol see Consumables and Equipment, on page 21.

Item	Quantity	Storage
Microbial Genomic DNA (5 ng/µl in 10 mM Tris pH 8.5)	2.5 µl per sample	-15° to -25°C
Amplicon PCR Reverse Primer (1 µM)	5 µl per sample	-15° to -25°C
Amplicon PCR Forward Primer (1 µM)	5 µl per sample	-15° to -25°C
2x KAPA HiFi HotStart ReadyMix	12.5 µl per sample	-15° to -25°C
Microseal 'A' film		
96-well 0.2 ml PCR plate	1 plate	
[Optional] Bioanalyzer chip (Agilent DNA 1000 kit catalog # 5067-1504)		

Procedure

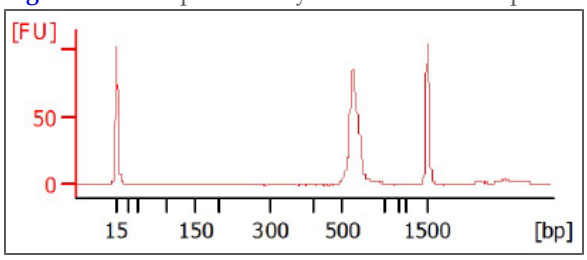
1 Set up the following reaction of DNA, 2x KAPA HiFi HotStart ReadyMix, and primers:

	Volume
Microbial DNA (5 ng/µl)	2.5 µl
Amplicon PCR Forward Primer 1 µM	5 µl
Amplicon PCR Reverse Primer 1 µM	5 µl
2x KAPA HiFi HotStart ReadyMix	12.5 µl
Total	**25 µl**

2 Seal plate and perform PCR in a thermal cycler using the following program:
- 95°C for 3 minutes
- 25 cycles of:
  - 95°C for 30 seconds
  - 55°C for 30 seconds
  - 72°C for 30 seconds
- 72°C for 5 minutes
- Hold at 4°C

3 **[Optional]** Run 1 µl of the PCR product on a Bioanalyzer DNA 1000 chip to verify the size. Using the V3 and V4 primer pairs in the protocol, the expected size on a Bioanalyzer trace after the Amplicon PCR step is ~550 bp.

Figure 3 Example Bioanalyzer Trace after Amplicon PCR Step



PCR Clean-Up

This step uses AMPure XP beads to purify the 16S V3 and V4 amplicon away from free primers and primer dimer species.

Consumables

Item	Quantity	Storage
10 mM Tris pH 8.5	52.5 µl per sample	-15° to -25°C
AMPure XP beads	20 µl per sample	2° to 8°C
Freshly Prepared 80% Ethanol (EtOH)	400 µl per sample	
96-well 0.2 ml PCR plate	1 plate	
[Optional] Microseal 'B' film		
[Optional] 96-well MIDI plate	1 plate	

Preparation

- Bring the AMPure XP beads to room temperature.

Procedure

1. Centrifuge the Amplicon PCR plate at 1,000 × g at 20°C for 1 minute to collect condensation, carefully remove seal.
2. **[Optional - for use with shaker for mixing]** Using a multichannel pipette set to 25 µl, transfer the entire Amplicon PCR product from the PCR plate to the MIDI plate. Change tips between samples.

   NOTE
   Transfer the sample to a 96-well MIDI plate if planning to use a shaker for mixing. If mixing by pipette, the sample can remain in the 96-well PCR plate.
3. Vortex the AMPure XP beads for 30 seconds to make sure that the beads are evenly dispersed. Add an appropriate volume of beads to a trough depending on the number of samples processing.
4. Using a multichannel pipette, add 20 µl of AMPure XP beads to each well of the Amplicon PCR plate. Change tips between columns.
5. Gently pipette entire volume up and down 10 times if using a 96-well PCR plate or seal plate and shake at 1800 rpm for 2 minutes if using a MIDI plate.
6. Incubate at room temperature without shaking for 5 minutes.
7. Place the plate on a magnetic stand for 2 minutes or until the supernatant has cleared.
8. With the Amplicon PCR plate on the magnetic stand, use a multichannel pipette to remove and discard the supernatant. Change tips between samples.

9 With the Amplicon PCR plate on the magnetic stand, wash the beads with freshly prepared 80% ethanol as follows:

- **a** Using a multichannel pipette, add 200 µl of freshly prepared 80% ethanol to each sample well.
- **b** Incubate the plate on the magnetic stand for 30 seconds.
- **c** Carefully remove and discard the supernatant.

10 With the Amplicon PCR plate on the magnetic stand, perform a second ethanol wash as follows:

- **a** Using a multichannel pipette, add 200 µl of freshly prepared 80% ethanol to each sample well.
- **b** Incubate the plate on the magnetic stand for 30 seconds.
- **c** Carefully remove and discard the supernatant.
- **d** Use a P20 multichannel pipette with fine pipette tips to remove excess ethanol.

11 With the Amplicon PCR plate still on the magnetic stand, allow the beads to air-dry for 10 minutes.

12 Remove the Amplicon PCR plate from the magnetic stand. Using a multichannel pipette, add 52.5 µl of 10 mM Tris pH 8.5 to each well of the Amplicon PCR plate.

13 Gently pipette mix up and down 10 times, changing tips after each column (or seal plate and shake at 1800 rpm for 2 minutes). Make sure that beads are fully resuspended.

14 Incubate at room temperature for 2 minutes.

15 Place the plate on the magnetic stand for 2 minutes or until the supernatant has cleared.

16 Using a multichannel pipette, carefully transfer 50 µl of the supernatant from the Amplicon PCR plate to a new 96-well PCR plate. Change tips between samples to avoid cross-contamination.

SAFE STOPPING POINT
If you do not immediately proceed to *Index PCR*, seal plate with Microseal "B" adhesive seal and store it at -15° to -25°C for up to a week.

Index PCR

This step attaches dual indices and Illumina sequencing adapters using the Nextera XT Index Kit.

Consumables

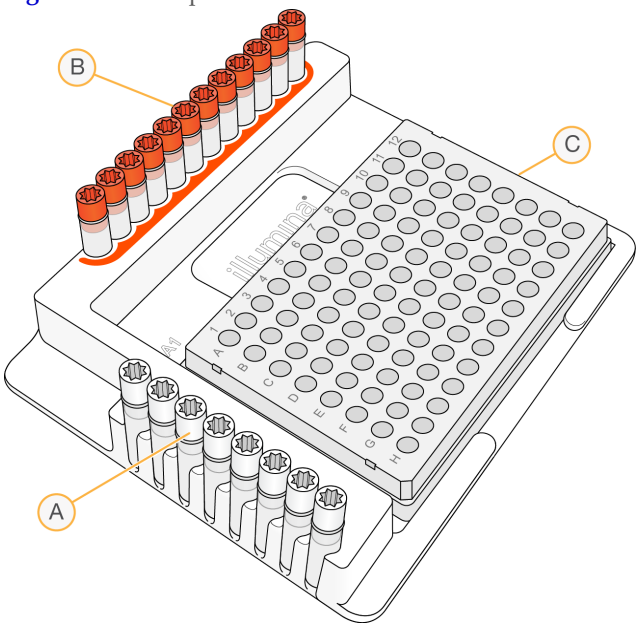
Item	Quantity	Storage
2x KAPA HiFi HotStart ReadyMix	25 µl per sample	-15° to -25°C
Nextera XT Index 1 Primers (N7XX) from the Nextera XT Index kit (FC-131-1001 or FC-131-1002)	5 µl per sample	-15° to -25°C
Nextera XT Index 2 Primers (S5XX) from the Nextera XT Index kit (FC-131-1001 or FC-131-1002)	5 µl per sample	-15° to -25°C
PCR Grade Water	10 µl per sample	
TruSeq Index Plate Fixture (FC-130-1005)	1	
96-well 0.2 ml PCR plate	1 plate	
Microseal 'A' film	1	

Procedure

1. Using a multichannel pipette, transfer 5 µl from each well to a new 96-well plate. The remaining 45 µl is not used in the protocol and can be stored for other uses.
2. Arrange the Index 1 and 2 primers in a rack (i.e. the TruSeq Index Plate Fixture) using the following arrangements as needed:
   a. Arrange Index 2 primer tubes (white caps, clear solution) vertically, aligned with rows A through H.
   b. Arrange Index 1 primer tubes (orange caps, yellow solution) horizontally, aligned with columns 1 through 12.

   For more information on index selection, see Dual Indexing Principle, on page 23.

Figure 4 TruSeq Index Plate Fixture



A Index 2 primers (white caps)
B Index 1 primers (orange caps)
C 96-well plate

3 Place the 96-well PCR plate with the 5 µl of resuspended PCR product DNA in the TruSeq Index Plate Fixture.

4 Set up the following reaction of DNA, Index 1 and 2 primers, 2x KAPA HiFi HotStart ReadyMix, and PCR Grade water:

	Volume
DNA	5 µl
Nextera XT Index Primer 1 (N7xx)	5 µl
Nextera XT Index Primer 2 (S5xx)	5 µl
2x KAPA HiFi HotStart ReadyMix	25 µl
PCR Grade water	10 µl
Total	**50 µl**

5 Gently pipette up and down 10 times to mix.

6 Cover the plate with Microseal 'A'.

7 Centrifuge the plate at 1,000 × g at 20°C for 1 minute.

8 Perform PCR on a thermal cycler using the following program:

- 95°C for 3 minutes
- 8 cycles of:
  - 95°C for 30 seconds
  - 55°C for 30 seconds
  - 72°C for 30 seconds
- 72°C for 5 minutes
- Hold at 4°C

PCR Clean-Up 2

This step uses AMPure XP beads to clean up the final library before quantification.

Consumables

Item	Quantity	Storage
10 mM Tris pH 8.5	27.5 µl per sample	-15° to -25°C
AMPure XP beads	56 µl per sample	2° to 8°C
Freshly Prepared 80% Ethanol (EtOH)	400 µl per sample	
96-well 0.2 ml PCR plate	1 plate	
[Optional] Microseal 'B' film		
[Optional] 96-well MIDI plate	1 plate	

Procedure

1. Centrifuge the Index PCR plate at 280 × g at 20°C for 1 minute to collect condensation.
2. **[Optional - for use with shaker for mixing]** Using a multichannel pipette set to 50 µl, transfer the entire Index PCR product from the PCR plate to the MIDI plate. Change tips between samples.

   NOTE
   Transfer the sample to a 96-well MIDI plate if planning to use a shaker for mixing. If mixing by pipette, the sample can remain in the 96-well PCR plate.
3. Vortex the AMPure XP beads for 30 seconds to make sure that the beads are evenly dispersed. Add an appropriate volume of beads to a trough.
4. Using a multichannel pipette, add 56 µl of AMPure XP beads to each well of the Index PCR plate.
5. Gently pipette mix up and down 10 times if using a 96-well PCR plate or seal plate and shake at 1800 rpm for 2 minutes if using a MIDI plate.
6. Incubate at room temperature without shaking for 5 minutes.
7. Place the plate on a magnetic stand for 2 minutes or until the supernatant has cleared.
8. With the Index PCR plate on the magnetic stand, use a multichannel pipette to remove and discard the supernatant. Change tips between samples.
9. With the Index PCR plate on the magnetic stand, wash the beads with freshly prepared 80% ethanol as follows:
   a. Using a multichannel pipette, add 200 µl of freshly prepared 80% ethanol to each sample well.
   b. Incubate the plate on the magnetic stand for 30 seconds.
   c. Carefully remove and discard the supernatant.

10 With the Index PCR plate on the magnetic stand, perform a second ethanol wash as follows:

- **a** Using a multichannel pipette, add 200 µl of freshly prepared 80% ethanol to each sample well.
- **b** Incubate the plate on the magnetic stand for 30 seconds.
- **c** Carefully remove and discard the supernatant.
- **d** Use a P20 multichannel pipette with fine pipette tips to remove excess ethanol.

11 With the Index PCR plate still on the magnetic stand, allow the beads to air-dry for 10 minutes.

12 Remove the Index PCR plate from the magnetic stand. Using a multichannel pipette, add 27.5 µl of 10 mM Tris pH 8.5 to each well of the Index PCR plate.

13 If using a 96-well PCR plate, gently pipette mix up and down 10 times until beads are fully resuspended, changing tips after each column. If using a MIDI plate, seal plate and shake at 1800 rpm for 2 minutes.

14 Incubate at room temperature for 2 minutes.

15 Place the plate on the magnetic stand for 2 minutes or until the supernatant has cleared.

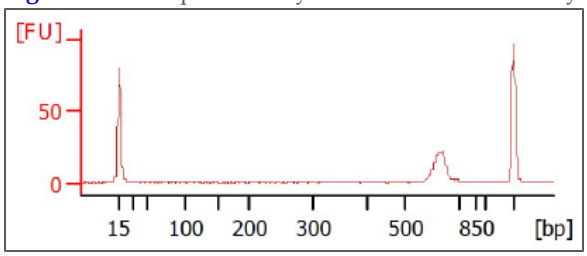
16 Using a multichannel pipette, carefully transfer 25 µl of the supernatant from the Index PCR plate to a new 96-well PCR plate. Change tips between samples to avoid cross-contamination.

SAFE STOPPING POINT
If you do not plan to proceed to *Library Quantification, Normalization, and Pooling, on page 16*, seal the plate with Microseal "B" adhesive seal. Store the plate at -15° to -25°C for up to a week.

[Optional] Validate Library

Run 1 µl of a 1:50 dilution of the final library on a Bioanalyzer DNA 1000 chip to verify the size. Using the V3 and V4 primer pairs in the protocol, the expected size on a Bioanalyzer trace of the final library is ~630 bp.

Figure 5 Example Bioanalyzer Trace of Final Library



Library Quantification, Normalization, and Pooling

Illumina recommends quantifying your libraries using a fluorometric quantification method that uses dsDNA binding dyes.

Calculate DNA concentration in nM, based on the size of DNA amplicons as determined by an Agilent Technologies 2100 Bioanalyzer trace:

$$\frac{(\text{concentration in ng/µl})}{(660\ \text{g/mol} \times \text{average library size})} \times 10^6 = \text{concentration in nM}$$

For example:

$$\frac{15\ \text{ng/µl}}{(660\ \text{g/mol} \times 500)} \times 10^6 = 45\ \text{nM}$$

Dilute concentrated final library using Resuspension Buffer (RSB) or 10 mM Tris pH 8.5 to 4 nM. Aliquot 5 µl of diluted DNA from each library and mix aliquots for pooling libraries with unique indices. Depending on coverage needs, up to 96 libraries can be pooled for one MiSeq run.

For metagenomics samples, >100,000 reads per sample is sufficient to fully survey the bacterial composition. This number of reads allows for sample pooling to the maximum level of 96 libraries, given the MiSeq output of > 20 million reads.

Library Denaturing and MiSeq Sample Loading

In preparation for cluster generation and sequencing, pooled libraries are denatured with NaOH, diluted with hybridization buffer, and then heat denatured before MiSeq sequencing. Each run must include a minimum of 5% PhiX to serve as an internal control for these low-diversity libraries. Illumina recommends using MiSeq v3 reagent kits for improved run metrics.

Consumables

Item	Quantity	Storage
10 mM Tris pH 8.5 or RSB (Resuspension Buffer)	6 µl	-15° to -25°C
HT1 (Hybridization Buffer)	1540 µl	-15° to -25°C
0.2 N NaOH (less than a week old)	10 µl	-15° to -25°C
PhiX Control Kit v3 (FC-110-3001)	4 µl	-15° to -25°C
MiSeq reagent cartridge	1 cartridge	-15° to -25°C
1.7 ml microcentrifuge tubes (screw cap recommended)	3 tubes	
2.5 L ice bucket		

Preparation

1. Set a heat block suitable for 1.7 ml microcentrifuge tubes to 96°C
2. Remove a MiSeq reagent cartridge from -15°C to -25°C storage and thaw at room temperature.
3. In an ice bucket, prepare an ice-water bath by combining 3 parts ice and 1 part water.

Denature DNA

1. Combine the following volumes of pooled final DNA library and freshly diluted 0.2 N NaOH in a microcentrifuge tube:
   - 4 nM pooled library (5 µl)
   - 0.2 N NaOH (5 µl)
2. Set aside the remaining dilution of 0.2 N NaOH to prepare a PhiX control within the next 12 hours.
3. Vortex briefly to mix the sample solution, and then centrifuge the sample solution at 280 × g at 20°C for 1 minute.
4. Incubate for 5 minutes at room temperature to denature the DNA into single strands.
5. Add the following volume of pre-chilled HT1 to the tube containing denatured DNA:
   - Denatured DNA (10 µl)

- Pre-chilled HT1 (990 µl)

Adding the HT1 results in a 20 pM denatured library in 1 mM NaOH.

6 Place the denatured DNA on ice until you are ready to proceed to final dilution.

Dilute Denatured DNA

1 Dilute the denatured DNA to the desired concentration using the following example:

NOTE
Illumina recommends targeting 800–1000 K/mm² raw cluster densities using MiSeq v3 reagents. It is suggested to start your first run using a 4 pM loading concentration and adjust subsequent runs appropriately.

Final Concentration	2 pM	4 pM	6 pM	8 pM	10 pM
20 pM denatured library	60 µl	120 µl	180 µl	240 µl	300 µl
Pre-chilled HT1	540 µl	480 µl	420 µl	360 µl	300 µl

2 Invert several times to mix and then pulse centrifuge the DNA solution.

3 Place the denatured and diluted DNA on ice.

Denature and Dilution of PhiX Control

Use the following instructions to denature and dilute the 10 nM PhiX library to the same loading concentration as the Amplicon library. The final library mixture must contain at least 5% PhiX.

1 Combine the following volumes to dilute the PhiX library to 4 nM:
- 10 nM PhiX library (2 µl)
- 10 mM Tris pH 8.5 (3 µl)

2 Combine the following volumes of 4 nM PhiX and 0.2 N NaOH in a microcentrifuge tube:
- 4 nM PhiX library (5 µl)
- 0.2 N NaOH (5 µl)

3 Vortex briefly to mix the 2 nM PhiX library solution.

4 Incubate for 5 minutes at room temperature to denature the PhiX library into single strands.

5 Add the following volumes of pre-chilled HT1 to the tube containing denatured PhiX library to result in a 20 pM PhiX library:
- Denatured PhiX library (10 µl)
- Pre-chilled HT1 (990 µl)

6 Dilute the denatured 20 pM PhiX library to the same loading concentration as the Amplicon library as follows:

Final Concentration	2 pM	4 pM	6 pM	8 pM	10 pM
20 pM denatured library	60 µl	120 µl	180 µl	240 µl	300 µl
Pre-chilled HT1	540 µl	480 µl	420 µl	360 µl	300 µl

7 Invert several times to mix and then pulse centrifuge the DNA solution.

8 Place the denatured and diluted PhiX on ice.

Combine Amplicon Library and PhiX Control

NOTE
The recommended PhiX control spike-in of ≥ 5% for low diversity libraries is possible with RTA v1.17.28 or later, which is bundled with MCS v2.2. For optimal performance, update to v3 software (MCS 2.3). If you are using an older version of the MiSeq software or sequencing these libraries on the GA or HiSeq, Illumina recommends using ≥ 25% PhiX control spike-in.

1 Combine the following volumes of denatured PhiX control library and your denatured amplicon library in a microcentrifuge tube:
- Denatured and diluted PhiX control (30 µl)
- Denatured and diluted amplicon library (570 µl)

2 Set the combined sample library and PhiX control aside on ice until you are ready to heat denature the mixture immediately before loading it onto the MiSeq v3 reagent cartridge.

3 Using a heat block, incubate the combined library and PhiX control tube at 96°C for 2 minutes.

4 After the incubation, invert the tube 1–2 times to mix and immediately place in the ice-water bath.

5 Keep the tube in the ice-water bath for 5 minutes.

NOTE
Perform the heat denaturation step immediately before loading the library into the MiSeq reagent cartridge to ensure efficient template loading on the MiSeq flow cell.

MiSeq Reporter Metagenomics Workflow

After samples are loaded, the MiSeq system provides on-instrument secondary analysis using the MiSeq Reporter software (MSR). MSR provides several options for analyzing MiSeq sequencing data. For this demonstrated 16S protocol, select the Metagenomics workflow.

By following this 16S Metagenomics protocol, the Metagenomics workflow classifies organisms from your V3 and V4 amplicon using a database of 16S rRNA data. The classification is based on the Greengenes database (http://greengenes.lbl.gov/). The output of this workflow is a classification of reads at several taxonomic levels: kingdom, phylum, class, order, family, genus, and species. The analysis output includes:

- Clusters Graph – shows numbers of raw cluster, clusters passing filter, clusters that did not align, clusters not associated with an index, and duplicates.
- Sample Table – summarizes the sequencing results for each sample.
- Cluster Pie Chart – a graphical representation of the classification breakdown for each sample.

See the *MiSeq Reporter Metagenomics Workflow – Reference Guide* (Part # 15042317) for detailed instructions and guidance.

The method described in this 16S Metagenomics protocol can be used for any targeted amplicon sequencing, relevant to virus research, mutation detection, or other microbiology-related studies. If you use the protocol for other targeted amplicon sequencing studies, select the MiSeq Reporter Generate FASTQ Workflow for on-instrument generation of FASTQ files for downstream analysis. For specific guidance on the Generate FASTQ Workflow, see the *MiSeq Reporter Generate FASTQ Workflow – Reference Guide* (Part # 15042322).

Supporting Information

The protocols described in this guide assume that you are familiar with the contents of this section and have obtained all of the requisite equipment and consumables.

Acronyms

Table 1 Acronyms

Acronym	Definition
HT1	Hybridization Buffer
IEM	Illumina Experiment Manager
MSR	MiSeq Reporter
PCR	Polymerase Chain Reaction
rRNA	Ribosomal RNA
RSB	Resuspension Buffer

Consumables and Equipment

Check to make sure that you have all of the necessary user-supplied consumables and equipment before proceeding to sample preparation.

Table 2 User-Supplied Consumables

Consumable	Supplier
1.7 ml microcentrifuge tubes	General lab supplier
10 µl barrier pipette tips	General lab supplier
10 µl multichannel pipettes	General lab supplier
10 µl single channel pipettes	General lab supplier
20 µl barrier pipette tips	General lab supplier
20 µl multichannel pipettes	General lab supplier
20 µl single channel pipettes	General lab supplier
200 µl barrier pipette tips	General lab supplier
200 µl multichannel pipettes	General lab supplier
200 µl single channel pipettes	General lab supplier
1000 µl barrier pipette tips	General lab supplier

Consumable	Supplier
1000 µl multichannel pipettes	General lab supplier
1000 µl single channel pipettes	General lab supplier
96-well 0.2 ml skirtless PCR plates or Twin.Tec 96-well PCR plates	Bio-Rad, part # MSP-9601
Agencourt AMPure XP 60 ml kit	Beckman Coulter Genomics, part # A63881
Ethanol 200 proof (absolute) for molecular biology (500 ml)	Sigma-Aldrich, part # E7023
Amplicon PCR Forward Primer (HPLC Purified)	
Amplicon PCR Reverse Primer (HPLC Purified)	
KAPA HiFi HotStart ReadyMix (2X)	KAPA Biosystems, part # KK2601
Microseal 'A' adhesive seals	Bio-Rad, part # MSA-5001
Microseal 'B' adhesive seals	Bio-Rad, part # MSB-1001
MiSeq Reagent Kit v2 (500 cycle)	Illumina, catalog # MS-102-2003
Nextera XT Index Kit	Illumina, catalog # FC-131-1001 or Illumina, catalog # FC-131-1002
PhiX Control Kit v3	Illumina, catalog # FC-110-3001
PCR grade water	General lab supplier
Fluorometric quantitation with dsDNA binding dye reagents	General lab supplier
RNase/DNase-free 8-well PCR strip tubes and caps	General lab supplier
RNase/DNase-free multichannel reagent reservoirs, disposable	VWR, part # 89094-658
Tris-HCl 10 mM, pH 8.5	General lab supplier
[Optional] 96-well storage plates, round well, 0.8 ml ("MIDI" plate)	Fisher Scientific, part # AB-0859

Table 3 User-Supplied Equipment

Equipment	Supplier
2.5 L ice bucket	General lab supplier
96-well thermal cycler (with heated lid)	General lab supplier

Equipment	Supplier
Fluorometer for quantitation with dsDNA binding dyes	General lab supplier
Magnetic stand-96	Life Technologies, catalog # AM10027
Microplate centrifuge	General lab supplier
TruSeq Index Plate Fixture Kit (reusable)	Illumina, catalog # FC-130-1005
[Optional] 2100 Bioanalyzer Desktop System	Agilent, part # G2940CA
[Optional] Agilent DNA 1000 Kit	Agilent, part # 5067-1504
[Optional] High Speed Micro Plate Shaker	VWR, catalog # 13500-890 (110V/120V) or VWR, catalog # 14216-214 (230V)

Dual Indexing Principle

The dual indexing strategy uses two 8 base indices, Index 1 (i7) adjacent to the P7 sequence, and Index 2 (i5) adjacent to the P5 sequence. Dual indexing is enabled by adding a unique Index 1 (i7) and Index 2 (i5) to each sample. The 96 sample Nextera XT Index Kit (FC-131–1002) use 12 different Index 1 (i7) adapters (N701–N712) and 8 different Index 2 (i5) adapters (S501–S508). The 24 sample Nextera XT Index Kit (FC-131–1001) uses 6 different Index 1 (i7) adapters (N701–N706) and 4 different Index 2 (i5) adapters (S501–S504). In the Index adapter name, the N or S refers to Nextera XT sample preparation, 7 or 5 refers to Index 1 (i7) or Index 2 (i5), respectively. The 01–12 refers to the Index number. A list of index sequences is provided for generating sample sheets to demultiplex the samples:

Index 1 (i7)	Sequence	Index 2 (i5)	Sequence
N701	TAAGGCGA	S501	TAGATCGC
N702	CGTACTAG	S502	CTCTCTAT
N703	AGGCAGAA	S503	TATCCTCT
N704	TCCTGAGC	S504	AGAGTAGA
N705	GGACTCCT	S505	GTAAGGAG
N706	TAGGCATG	S506	ACTGCATA
N707	CTCTCTAC	S507	AAGGAGTA
N708	CAGAGAGG	S508	CTAAGCCT
N709	GCTACGCT		
N710	CGAGGCTG		
N711	AAGAGGCA		
N712	GTAGAGGA		

Low Plexity Pooling Guidelines

Illumina uses a green laser or LED to sequence G/T and a red laser or LED to sequence A/C. At each cycle, at least one of two nucleotides for each color channel are read to ensure proper registration. It is important to maintain color balance for each base of the index read being sequenced, otherwise index read sequencing could fail due to registration failure. If you choose the dual-indexed sequencing workflow, always use at least two unique and

compatible barcodes for each index (index 1 and index 2). The following tables illustrate possible pooling strategies:

Table 4 Libraries Pooled: 6 or fewer; Sequencing Workflow: Single Index

Plex	Index 1 (i7) Selection	Index 2 (i5) Selection
1-plex (no pooling)	Any Index 1 adapter	Any Index 2 adapter
2-plex	• [option 1] N702 and N701 • [option 2] N702 and N704	
3-plex	• [option 1] N701, N702, and N704 • [option 2] N703, N705, and N706	
4- or 5-plex	• [option 1] N701, N702, N704, and any other Index 1 adapter • [option 2] N703, N705, N706, and any other Index 1 adapter	
6-plex	N701, N702, N703, N704, N705, and N706	

Table 5 Sequencing Workflow: Single or Dual Index

Plex	Index 1 (i7) Selection	Index 2 (i5) Selection
7–12 plex, Dual Index	• [option 1] N701, N702, N704, and any other Index 1 adapter (as needed) • [option 2] N703, N705, N706, and any other Index 1 adapter (as needed)	• [option 1] S501 and S502 • [option 2] S503 and S504 • [option 3] S505 and S506
7–12 plex, Single Index (96 sample Nextera Index adapter kit)	• N701–N706 and any other Index 1 adapter (as needed)	• Any Index 2 (i5) adapter
Greater than 12-plex	N701, N702, N703, N704, N705, N706, and any other Index 1 adapter	• [option 1] S501, S502, and any other Index 2 adapter (as needed) • [option 2] S503, S504, and any other Index 2 adapter (as needed) • [option 3] S505, S506, and any other Index 2 adapter (as needed)

These strategies represent only some of the acceptable combinations. Alternatively, check the real sequences of each index in the tables to make sure that each base position has a signal in both color channels for the index read:

Good				Bad			
Index 1		Index 2		Index 1		Index 2	
705	GGACTCCT	503	TATCCTCT	705	GGACTCCT	502	CTCTCTAT
706	TAGGCATG	503	TATCCTCT	706	TAGGCATG	502	CTCTCTAT
701	TAAGGCGA	504	AGAGTAGA	701	TAAGGCGA	503	TATCCTCT
702	CGTACTAG	504	AGAGTAGA	702	CGTACTAG	503	TATCCTCT
	√√√√√√√√		√√√√√√√√		√√√√√√√√		√√√√xxxx

√=signal in both color
x=signal missing in one color channel

Prevent PCR Product Contamination

The PCR process is commonly used in the laboratory to amplify specific DNA sequences. Unless proper laboratory hygiene is used, PCR products can contaminate reagents, instrumentation, and genomic DNA samples, causing inaccurate and unreliable results. PCR product contamination can shut down lab processes and significantly delay normal operations.

Make sure that the lab is set up appropriately to reduce the risk of PCR product contamination:

- **Physically Separate Pre-PCR and Post-PCR Areas**
  - Physically separate laboratory space where pre-PCR processes are performed (DNA extraction, quantification, and normalization) from the laboratory space where PCR products are made and processed (post-PCR processes).
  - Never use the same sink to wash pre-PCR and post-PCR troughs.
  - Never share water purification systems for pre-PCR and post-PCR processes.
  - Store all supplies used in the protocols in the pre-PCR area, and transfer to the post-PCR area as needed.
- **Use Dedicated Equipment and Supplies**
  - Dedicate separate full sets of equipment and supplies (pipettes, centrifuges, oven, heat block, etc.) to pre-PCR and post-PCR lab processes, and never share between processes.
  - Dedicate separate storage areas (freezers and refrigerators) to pre-PCR and post-PCR consumables.

Because the pre- and post-amplification reagents are shipped together, it is important to unpack the reagents in the pre-PCR lab area. After unpacking the reagents, move the post-amplification reagents to the proper post-PCR storage area.

Pre-PCR and Post-PCR Lab Procedures

To prevent PCR product contamination, it is important to establish lab procedures and follow best practices. Illumina recommends daily and weekly cleaning of lab areas using

0.5% Sodium Hypochlorite (10% Bleach).

CAUTION
To prevent sample or reagent degradation, make sure that all vapors from the cleaning solution have fully dissipated before beginning any processes.

Daily Cleaning of Pre-PCR Area

A daily cleaning of the pre-PCR area using a 0.5% Sodium Hypochlorite (10% Bleach) solution helps to eliminate PCR product that has entered the pre-PCR area.

Identify pre-PCR areas that pose the highest risk of contamination, and clean these areas with a 0.5% Sodium Hypochlorite (10% Bleach) solution before beginning any pre-PCR processes. High-risk areas might include, but are not limited to, the following items:

- Benchtops
- Door handles
- Refrigerator/freezer door handles
- Computer mouse
- Keyboards

Daily Cleaning of Post-PCR Area

Reducing the amount of PCR product in the post-PCR area helps reduce the risk of contamination in the pre-PCR area. Daily cleaning of the post-PCR area using a 0.5% Sodium Hypochlorite (10% Bleach) solution helps reduce the risk of contamination.

Identify post-PCR areas that pose the highest risk of contamination, and clean these areas with a 0.5% Sodium Hypochlorite (10% Bleach) solution daily. High-risk areas might include, but are not limited to, the following items:

- Thermal cyclers
- Bench space used to process amplified DNA
- Door handles
- Refrigerator/freezer door handles
- Computer mouse
- Keyboards

Weekly Cleaning of All Lab Areas

One time a week, perform a thorough cleaning of the pre-PCR and post-PCR areas using 0.5% Sodium Hypochlorite (10% Bleach).

- Clean all benchtops and laboratory surfaces.
- Clean all instruments that are not cleaned daily.
- Thoroughly mop lab floors.
- Make sure that personnel responsible for weekly cleaning are properly trained on prevention of PCR product contamination.

Items Fallen to the Floor

The floor is contaminated with PCR product transferred on the shoes of individuals coming from the post-PCR area; therefore, anything falling to the floor must be treated as contaminated.

- Disposable items that have fallen to the floor, such as empty tubes, pipette tips, gloves, lab coat hangers, must be discarded.

- Non-disposable items that have fallen to the floor, such as a pipette or an important sample container, must be immediately and thoroughly cleaned. Use a 0.5% Sodium Hypochlorite (10% Bleach) solution to remove PCR product contamination.
- Clean any lab surface that has come in contact with the contaminated item. Individuals handling anything that has fallen to the floor, disposable or non-disposable, must discard their lab gloves and put on a new pair.

Best Practices

When preparing libraries for sequencing, always adhere to good molecular biology practices. Read through the entire protocol before starting to make sure that all of the required materials are available and your equipment is programmed and ready to use.

Handling Liquids

Good liquid handling measures are essential, particularly when quantifying libraries or diluting concentrated libraries for making clusters.

- Small differences in volumes (±0.5 µl) can sometimes cause large differences in cluster numbers (~100,000).
- Small volume pipetting can be a source of potential error in protocols requiring the generation of standard curves, such as qPCR, or small but precise volumes, such as the Agilent Bioanalyzer.
- If small volumes are unavoidable, use due diligence to make sure that pipettes are correctly calibrated.
- Make sure that pipettes are not used at the volume extremes of their performance specifications.
- Prepare the reagents for multiple samples simultaneously, to minimize pipetting errors, especially with small volume enzyme additions. As a result, pipette one time from the reagent tubes with a larger volume, rather than many times with small volumes. Aliquot to individual samples in a single pipetting movement to allow for standardization across multiple samples.

Handling Magnetic Beads

NOTE
Cleanup procedures have only been validated using the 96-well plates and the magnetic stand specified in the *Consumables and Equipment* list. Comparable performance is not guaranteed when using a microcentrifuge tube or other formats, or other magnets.

- Before use, allow the beads to come to room temperature.
- Do not reuse beads. Always add fresh beads when performing these procedures.
- Immediately before use, vortex the beads until they are well dispersed and the color of the liquid is homogeneous.
- When pipetting the beads, pipette slowly and dispense slowly due to the viscosity of the solution.
- Take care to minimize bead loss, which can affect final yields.
- Change the tips for each sample, unless specified otherwise.
- Let the mixed samples incubate at room temperature for the time indicated in the protocol for maximum recovery.

- When removing and discarding supernatant from the wells, use a single channel or multichannel pipette and take care not to disturb the beads.
- When aspirating the cleared solution from the reaction plate and wash step, it is important to keep the plate on the magnetic stand and not disturb the separated magnetic beads. Aspirate slowly to prevent the beads from sliding down the sides of the wells and into the pipette tips.
- To prevent the carryover of beads after elution, approximately 2.5 µl of supernatant is left when the eluates are removed from the bead pellet.
- Be sure to remove all of the ethanol from the bottom of the wells, as it can contain residual contaminants.
- Keep the reaction plate on the magnetic stand and let it air-dry at room temperature to prevent potential bead loss due to electrostatic forces. Allow for the complete evaporation of residual ethanol, because the presence of ethanol affects the performance of the subsequent reactions. Illumina recommends at least minutes drying time, but a longer drying time can be required. Remaining ethanol can be removed with a 10 µl pipette.
- Avoid over drying the beads, which can impact final yields.
- Do not scrape the beads from the edge of the well using the pipette tip.
- To maximize sample recovery during elution, incubate the sample/bead mix for 2 minutes at room temperature before placing the samples onto the magnet.

Avoiding Cross-Contamination

Practice the following to avoid cross-contamination:

- Open only one adapter tube at a time.
- Change the tips for each sample, unless specified otherwise.
- Pipette carefully to avoid spillage.
- Clean pipettes and change gloves between handling different adapter stocks.
- Clean work surfaces thoroughly before and after the procedure.

Potential DNA Contaminants

When handling and processing samples using this protocol, use best practices to avoid PCR contamination, as you would when preparing PCR amplicons.

Temperature Considerations

Temperature is an important consideration for making libraries:

- Keep libraries at temperatures ≤37°C, except where specifically noted.
- Place reagents on ice after thawing at room temperature.

Equipment

- Review the programming instructions for your thermal cycler user guide to make sure that it is programmed appropriately using the heated lid function.
- It is acceptable to use the thermal cycler tracked heating lid function.