Assessing Bacterial, Archaeal, and Fungal Diversity of Spacecraft-Associated Surfaces via Tag-Encoded FLX Amplicon Pyrosequencing

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ABSTRACT

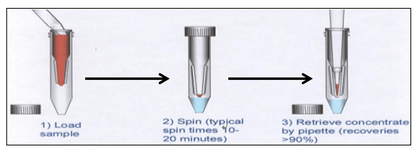
The introduction of microbiota to extraterrestrial settings could have profound repercussions on the scientific integrity of in-situ and sample-return based life detection experiments, and thus a key challenge lies in providing a comprehensive account of the genetic signatures of microbes resident on spacecraft hardware. The advent of high-throughput massively parallel tag-encoded FLX amplicon pyrosequencing (TEFAP) has dramatically increased the resolution of detection of distinct microbial lineages in mixed communities. DNA microarray approaches alone are unlikely to achieve an all-inclusive account of the microbial phylotypes present in a sample, since they cannot fully account for unknown species and are limited to those present in pre-existing databases. In contrast, TEFAP approaches are not predicated on *a priori* information, and thus yield far more comprehensive results. To date, a comprehensive census of the total microbial population associated with spacecraft hardware has yet to be provided.

Surface areas of 250 m^2 were collected from spacecraft hardware and assembly cleanrooms, and total genomic DNA was extracted. Variable regions of the 16S rRNA gene (bacteria and archaea) and ITS region (fungi) were targeted and PCR amplified, and a minimum of 3,000 reads were obtained for each 250 m^2 sample. A MOTHUR-based bioinformatics approach was used to process gene sequences and generate microbial diversity profiles for each of the various types of spacecraft associated samples. Analysis of the TEFAP data enabled cogent inferences to be drawn regarding the complete phylogenetic spectrum of microbes present. Computational population modeling then facilitated predictions about the proportions of innocuous/resistant, transient/recurrent, and ecologically inconsequential/dominant taxa present about the spacecraft surface, thereby empowering forward contamination risk assessments based on microbial recurrence. The successful application of emerging technologies to a spacecraft-relevant microbial census is encouraging, and TEFAP approaches in particular are of immense potential for future analyses of fluctuations in spacecraft-associated microbial populations.

SAMPLE COLLECTION & PROCESSING

Sample Collection. Samples were collected from spacecraft hardware via sampling 1 m^2 surface areas with sterile, water-moistened polyester wipes (Texwipe, Upper Saddle River, NJ). Samples (1m^2) were collected from spacecraft assembly cleanroom floors and ground support equipment via wet-surface sampling with BiSKit devices (QuickSilver Analytics, Abingdon, MD).

Pooling and Concentration of Samples. As spacecraft-associated samples are extremely low in biomass and seldom yield detectable PCR products (Moissl, et al., 2008), the authors pooled multiple samples from each sampling event and/or vicinity. All samples were filter-concentrated using Amicon Ultra-50 Ultracel centrifugal filters (Millipore, Billerica, MA). Each unit has a molecular mass cutoff of 50 kDa, which facilitates the concentration of bacterial cells, spores, and exogenous nucleic acid fragments greater than 100 bp into a final volume of 500 µl.



DNA Purification. Samples were divided into equivalent fractions, one of which was subjected to bead-beating in a FastPrep instrument (MP Biomedicals, Santa Ana, CA). After bead-beating, the fractions were recombined and subjected to total DNA purification via the Maxwell MDx automated nucleic acid extraction system (Promega, Madison, WI).

A typical Class 100,000 spacecraft assembly cleanroom.

ANALYSIS

Tag Encoded FLX Amplicon Pyrosequencing (TEFAP). Bacterial-biased primers 28F and 519R, and archaeal-biased 341F and 958R primers were used to PCR amplify ~550 bp 16S rRNA gene fragments from bacteria and archaea, respectively. Fungal-biased ITS1F and ITS4R primers were employed to amplify ~600 bp fragments of the ITS region. Primer pairs were tailored for TEFAP by adding a fusion linker and a proprietary barcode sequence at the 5' end of the forward primer, and a biotin and fusion linker sequence at the 5' end of the reverse primer (Dowd, et al., 2008). A HotStarTaq Plus master mix kit (QIAGEN) was used to catalyze the PCR under the following thermal cycling conditions: initial denaturing at 95ºC for 5 min, followed by 35 cycles of denaturing at 95ºC for 30 s, annealing at 54ºC for 40 s, and extension at 72ºC for 1 min, finalized by a 10-minute elongation at 72ºC. Resulting PCR products were purified via Rapid Tips (Diffinity Genomics, Inc, West Henrietta, NY), and were pooled accordingly. Small fragments were removed with Ampure Beads (Beckman Coulter, CA).

For FLX-Titanium sequencing (Roche, Nutley, New Jersey), resulting PCR amplicon fragment size and concentration were measured with DNA chips using an Experion automated electrophoresis station (Bio-Rad) and a TBS-380 Fluorometer (Turner Biosystems, CA). The total volume of initial PCR product used for subsequent emulsion PCR was 2 uL for strong positives, 5 uL for weak positives, and 20 uL for samples that failed to yield PCR products. This normalization step helped to ensure minimal bias favoring downstream amplification from initially strong PCR products. Approximately 9.6 x 10^6 molecules of ~ 600 bp double-stranded DNA were combined with 9.6 x 10^6 DNA capture beads, and then subjected to emulsion PCR conditions. Following recovery and enrichment, bead-attached DNA molecules were denatured with NaOH and sequencing primers were annealed. A four-region TEFAP run was performed on a GS PicoTiterPlate (PTP) using the Genome Sequencer FLX System in accordance with manufacturer's instructions. Twenty-four to 30 tagged samples were applied to each quarter region of the PTP. All TEFAP procedures were performed at the Research and Testing Laboratory (Lubbock, TX) according to well established protocols (Dowd, et al., 2008).

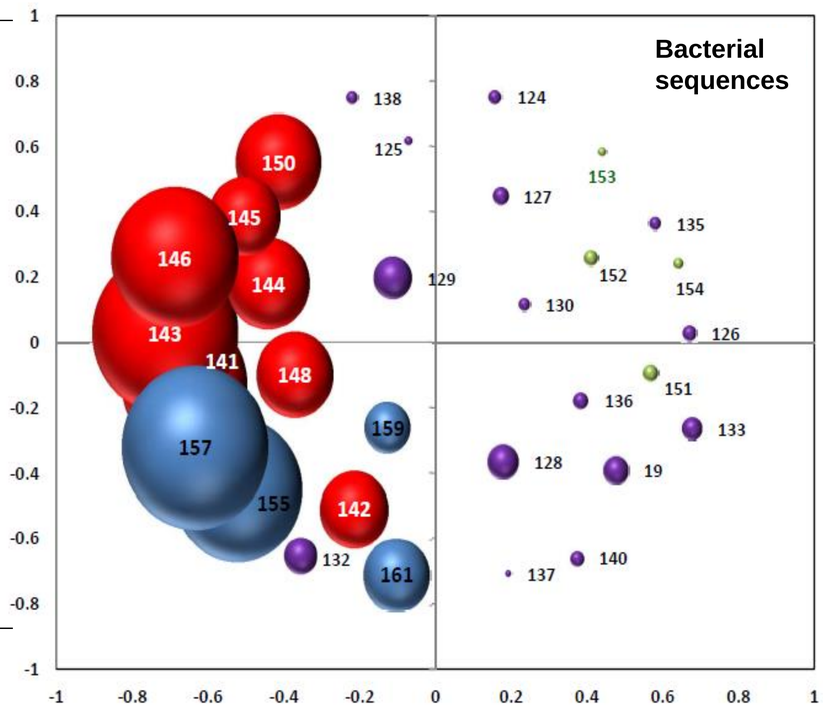
Bioinformatic analyses. Bacterial and archaeal pyrosequences were processed and analyzed using MOTHUR software (Schloss *et al.*, 2009), with the AmpliconNoise algorithm implemented to reduce error. Sequences were removed from consideration if they (a) did not contain the primer sequence, (b) contained an uncorrectable barcode, (c) were < 200 nt in length, or (d) had a quality score < 25. Unique sequences were aligned using the SILVA reference alignment (Schloss, 2009) and trimmed such that only regions of overlapping columns of sequence data were considered. Filtered sequences were assigned to samples according to their barcode. After removing chimeras, sequences were classified in accordance with the new Greengenes training set and taxonomy (McDonald, et al., 2011, Werner, et al., 2012), and clustered into operational taxonomic units at the 0.03 level. The full-length ITS1 sub-region was extracted from the fungal ITS dataset using ITS Extractor v. 1.1 (Nilsson et al., 2010), dismissing entries for which not both of the 3' end of the SSU gene and the 5' end of the 5.8S gene were found. The ITS1 sequences were clustered in CrunchClust v. 43 (Hartmann et al., in press) with pyrosequencing homopolymer correction enabled and at a Levenshtein distance of 7 (approximately 97% sequence similarity, a threshold value known to perform well for a wide range of fungi in terms of ITS distances (Nilsson et al., 2008)). A representative sequence from each OTU as designated by CrunchClust was annotated for taxonomic affiliation using the ITS 454 pipeline of Tedersoo et al. (2010) against the UNITE (Abarenkov et al., 2010) and INSD (Karsch-Mizachi et al., 2012) databases. The results were verified manually for each molecular taxonomic unit (MOTU). An entry was deemed identified to species level if it produced a ≥ 97% match over the full length of the sequence to a fully identified reference sequence whose name was not contradicted by other, equally good matches. The corresponding values for tentative generic and ordinal affiliation were 85% and 70%, respectively; they were deliberately set to be high to avoid false-positive inclusions. In the case of competing names for which neither synonymy nor anamorph-telemorph relationships could be established through MycoBank (Crous et al., 2004), the least inclusive parent nomenclatural level was used (e.g., *Penicillium* sp.).

RESULTS

Table 1. Sample characteristics of various spacecraft and associated surfaces

Sample (total area sampled)	Sample ID	Sampling devices	Sample type	Area (m2)	Cleanroom type	Description
Cleanroom types (33 m^2)						
GI-37	**150**	BisKit	Floor-70A	10	Non-NASA Cleanroom	Non-NASA cleanroom (LBNL; #70A)
GI-35-6	**143**	BisKit	Entrance floor	1	Ordinary room	Ordinary room adjacent to JPL-SAF
GI-35-4	**141**	BisKit	Shoe Cleaner	1	Ordinary room	Ordinary room adjacent to JPL-SAF
GI-35-7	**144**	BisKit	Floor 1	1	Ordinary room	Ordinary room adjacent to JPL-SAF
GI-35-8	**145**	BisKit	Floor 2	1	Ordinary room	Ordinary room adjacent to JPL-SAF
GI-35-5	**142**	BisKit	Air-lock	1	Ordinary room	Ordinary room adjacent to JPL-SAF
GI-36-3	**146**	BisKit	JPL-SAF GSE	9	Class 100K	During spacecraft assembly (JPL-SAF)
GI-36-4	**148**	BisKit	JPL-SAF Floor	9	Class 100K	During spacecraft assembly (JPL-SAF)
Cleaning vs prior to cleaning (38 m^2)						
GI-42-1	**155**	BisKit	Floor	9	Class 100K	JPL-144 cleanroom prior to cleaning
GI-42-2	**157**	BisKit	GSE	9	Class 100K	JPL-144 cleanroom prior to cleaning
GI-43-1	**159**	Polyester wipe	Floor	10	Class 100K	JPL-144 cleanroom after cleaning
GI-43-2	**161**	Polyester wipe	GSE	10	Class 100K	JPL-144 cleanroom after cleaning
Spacecraft surfaces [early ATLO; spore count-based (110 m^2)]						
GI-16	**124**	Polyester wipe	spacecraft	6	Class 100K	No spore count
GI-17	**125**	Polyester wipe	spacecraft	10	Class 100K	No spore count
GI-25	**133**	Polyester wipe	spacecraft	8	Class 100K	No spore count
GI-26	**134**	Polyester wipe	spacecraft	7	Class 100K	No spore count
GI-27	**135**	Polyester wipe	spacecraft	4	Class 100K	No spore count
GI-28	**136**	Polyester wipe	spacecraft	6	Class 100K	No spore count
GI-29	**137**	Polyester wipe	spacecraft	18	Class 100K	No spore count
GI-18	**126**	Polyester wipe	spacecraft	10	Class 100K	1 to 5 spore per m2
GI-19	**127**	Polyester wipe	spacecraft	14	Class 100K	1 to 5 spore per m2
GI-20	**128**	Polyester wipe	spacecraft	5	Class 100K	1 to 5 spore per m2
GI-21	**129**	Polyester wipe	spacecraft	4	Class 100K	1 to 5 spore per m2
GI-22	**130**	Polyester wipe	spacecraft	1	Class 100K	1 to 5 spore per m2
GI-30	**138**	Polyester wipe	spacecraft	13	Class 100K	1 to 5 spore per m2
GI-32	**140**	Polyester wipe	spacecraft	3	Class 100K	5 to 10 spore per m2
GI-24	**132**	Polyester wipe	spacecraft	1	Class 100K	300 spores per m2
Spacecraft surfaces [late ATLO; component-based (52 m^2)]						
GI-38	**151**	Polyester wipe	spacecraft	26	Class 100K	Component CS (0.2 spores per m2)
GI-39	**152**	Polyester wipe	spacecraft	9	Class 100K	Component DS (0.4 spores per m2)
GI-40	**153**	Polyester wipe	spacecraft	16	Class 100K	Component R (0.3 spores per m2)
GI-41	**154**	Polyester wipe	spacecraft	1	Class 100K	Non-flight samples (14 spores per m2)

JPL-SAF: Jet Propulsion Laboratory-Spacecraft Assembly Facility. LBNL: Lawrence Berkeley National Laboratory



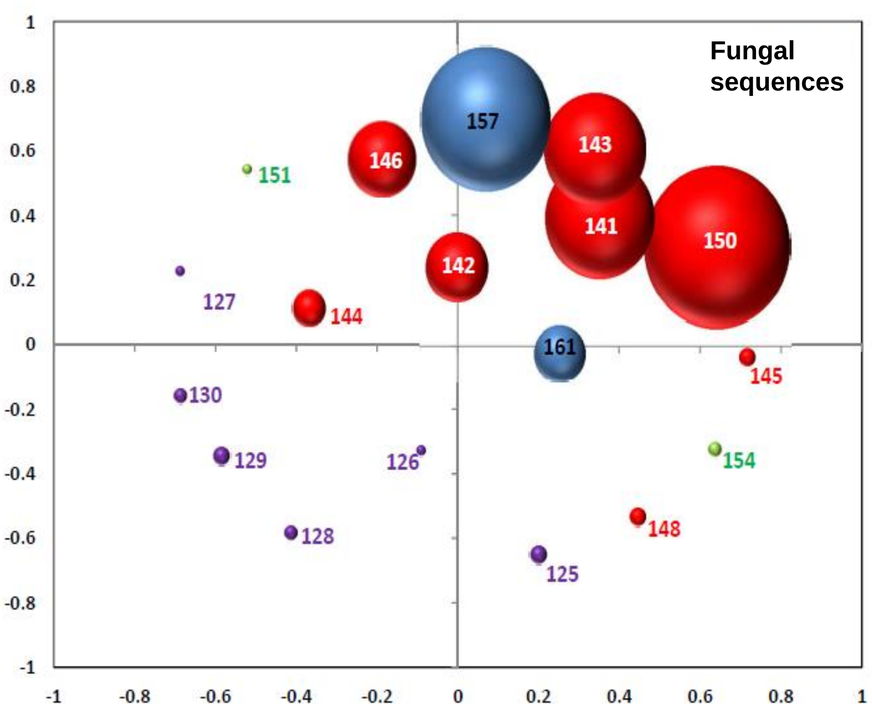


Fig.1. Nonmetric multidimensional scaling analysis depicting variations in diversity of bacterial and fungal sequences across samples. Spheres are labeled according to numerical sample identification, and sized according to relative MOTU abundance. Red: Cleanroom types, Blue: Prior to vs. post-cleaning, Magenta: general spacecraft hardware surfaces, and Green: mission componentry samples.

Table 4. Archaeal pyrosequences and MOTU retrieved from various spacecraft and associated surfaces

Archaeal taxa	Number of archaeal pyrosequences/MOTU from: Category I					Cat II		Category III					
	150	143	141	142	148	157	161	124	126	127	128	130	132
	Floor	Entrance floor	Shoe Cleaner	Air-lock	JPL-GSE	GSE	GSE	Spacecraft	Spacecraft	Spacecraft	Spacecraft	Spacecraft	Spacecraft
Nitrosphaeraceae - SCA114-1	17		35	47		5	2						
Nitrosphaeraceae - SCA114-2			1										
Nitrosphaeraceae - SCA117		6											
Methanobacteriaceae - 1								1	2	7	3	16	1
Methanobacteriaceae - 3												1	
Number of archaeal MOTUs	1	1	2	1		1	1	1	1	1	1	2	1

Table 2. Bacterial pyrosquences generated from various spacecraft and associated surfaces

Bacterial Taxa	Number of pyrosequences from: Category I								Category II				Category III																Category IV			
	150	143	141	144	145	142	146	148	155	157	159	161	124	125	133	134	135	136	137	126	127	128	129	130	138	140	132	151	152	153	154
	Floor-70A	Entrance floor	Shoe Cleaner	Floor 1	Floor 2	Air-lock	JPL-SAF GSE	JPL-SAF Floor	Floor	GSE	Floor	GSE	spacecraft	spacecraft	spacecraft	spacecraft	spacecraft	spacecraft	spacecraft	spacecraft	spacecraft	spacecraft	spacecraft	spacecraft	spacecraft	spacecraft	spacecraft	spacecraft	spacecraft	spacecraft	spacecraft
Actinobacteria	81	798	315	279	106	222	1764	55	88	618	8	120			1	76		3	1	49	60	105			79	7		5			
Armatimonadetes	1	21	6	7					6	1					27																
Bacteroidetes	7	252	101	64	104	62	214	108	2477	436	59	97						4			18						47				
Chlorobi										3																					
Verrucomicrobia		1							8		5																				
Chloroflexi	1	10	15	7	17	5	26	4		27												25									
Deinococcus-Thermus	3	6	1	7	2		26		7	41		11										1									
Acidobacteria	4	23	10	11	16	1	113	350	1	37		11			3																
Firmicutes	13	322	186	174	15	17	383	157	17	157		8				33				2		19		3	60						
Fusobacteria		67	10	17	6		1	1		2												25									
Gemmatimonadetes	1	13	4				17		3	2		2																			
Nitrospirae									1	1																					
Planctomycetes		11	2			3	1		2	4		3									2	1									
Proteobacteria																															
Alpha	5776	2465	693	1065	785	196	1120	606	7495	2517	692	73		1	23	22						26	129		15		127		54		
Beta	1478	431	215	154	215	104	523	100	1031	565	291	271	9	2	42	101	33	139		32	77	139	329	26	3		103	37	2	5	
Delta		3	3	2			5	36	6	7					12																
Gamma	1211	3374	962	1165	796	100	251	357	4833	1127	456	250			3	43	30	25		27	91	96	36	7			175	15	36	74	15
Unidentified		13	6	24	12	7			104	13			4																		
Spirochaetes								1																							
Tenericutes	2		1																												
Unidentified division																															
SC4							24			6												3									
TM7		6	3				3															1						12			
WPS-2		14							2																						
Unclassified bacteria	20	277	89	61	10	78	224	8	159	384		737			1							29									
Total # of sequences	8598	8107	2622	3037	2084	795	4695	1783	16240	5948	1511	1583	13	3	112	275	63	171	1	59	219	339	698	33	21	139	459	69	92	79	15

Table 3. Bacterial MOTU retrieved from various spacecraft and associated surfaces

Bacterial Taxa	Number of MOTU retrieved from: Category I								Category II				Category III																Category IV			
	150	143	141	144	145	142	146	148	155	157	159	161	124	125	133	134	135	136	137	126	127	128	129	130	138	140	132	151	152	153	154
	Floor-70A	Entrance floor	Shoe Cleaner	Floor 1	Floor 2	Air-lock	JPL-SAF GSE	JPL-SAF Floor	Floor	GSE	Floor	GSE	spacecraft	spacecraft	spacecraft	spacecraft	spacecraft	spacecraft	spacecraft	spacecraft	spacecraft	spacecraft	spacecraft	spacecraft	spacecraft	spacecraft	spacecraft	spacecraft	spacecraft	spacecraft	spacecraft
Actinobacteria	31	169	122	50	31	54	185	25	51	186	3	30			1	6		1	1		3	10	15			5	1		1		
Armatimonadetes	1	6	4	1					3	1					1																
Bacteroidetes	6	65	45	9	21	10	43	8	39	75	4	22						1				1				4					
Chlorobi										1																					
Verrucomicrobia		1							4		2																				
Chloroflexi	1	4	6	2	2	1	5	1		8												3									
Deinococcus-Thermus	2	3	1	3	2		8		5	15		3										1									
Acidobacteria	2	6	7	2	1	1	4	1	1	5		1			2																
Firmicutes	7	49	50	15	4	3	27	11	10	33		2				1					1		4		2	1					
Fusobacteria		2	2	3	1		1	1		2													1								
Gemmatimonadetes	1	3	3				3		3	2		1																			
Nitrospirae									1	1																					
Planctomycetes		4	1			2	1		2	2		1																			
Proteobacteria																															
Alpha	113	156	113	49	50	33	124	41	233	188	38	22		1	3	3						7	9		1		14		2		
Beta	38	61	47	23	17	13	35	9	48	61	7	23	4	1	2	5	2	3		4	1	3	5	2	1		9	2	1	1	
Delta		1	3	1			1	1	3	3					1																
Gamma	11	67	39	38	23	15	29	19	76	40	12	16			2	4	2	2		2	3	8	5	2			6	3	3	1	3
Unidentified		3	4	4	3	1			4	3			1																		
Spirochaetes								1																							
Tenericutes	2		1																												
Unidentified division																															
SC4							3			3																					
TM7		2	3				1																								
WPS-2		2							2																						
Unclassified bacteria	12	50	35	12	4	12	27	4	28	33		15			1							2									
Total # of sequences	227	654	486	212	159	145	497	122	513	662	66	136	5	2	13	19	4	7	1	6	8	29	45	4	4	6	34	7	6	2	3

Table 5. Abundance and diversity of various fungal sequences associated with spacecraft surfaces and assembly environments

Fungal taxa* that are:	Number of pyrosequences retrieved from: Category I								Cat II		Category III							Cat IV			Number of MOTU present in: Category I								Cat II		Category III							Cat IV	
	150	141	142	143	144	145	146	148	157	161	125	126	127	128	129	130		151	154		150	141	142	143	144	145	146	148	157	161	125	126	127	128	129	130		151	154
Phylum / *Sub-Phylum* / *Class*	Floor-70A	Entrance floor	Shoe Cleaner	Floor 1	Floor 2	Air-lock	JPL-SAF GSE	JPL-SAF Floor	GSE	GSE	Spacecraft	Spacecraft	Spacecraft	Spacecraft	Spacecraft	Spacecraft		Spacecraft	Spacecraft		Floor-70A	Entrance floor	Shoe Cleaner	Floor 1	Floor 2	Air-lock	JPL-SAF GSE	JPL-SAF Floor	GSE	GSE	Spacecraft	Spacecraft	Spacecraft	Spacecraft	Spacecraft	Spacecraft		Spacecraft	Spacecraft
Ascomycota	844	76	357		4	467	44		535	28											12	7	3		1	4	4		3	1									
Pezizomycotina																																							
Unclassified Pezizomycotina	564	1994	1093	31		587	741		4426	71											23	17	14	2		6	7		28	2									
Dothideomycetes	2545	6749	3979	152	3	1866	1702		14204	2129	59	2	492	8							59	36	35	3	1	13	17		56	16	2	1	1	1					
Eurotiomycetes	803	898	498		3	163	87	54	857	95					1	18		69			27	11	5		1	3	2	2	12	1					1	1		1	
Lecanoromycetes	689	504	264			114	442		1598	15											9	1	1			1	1		1	1									
Leotiomycetes	168	522	192			137	141		1256												16	6	5			2	4		9										
Pezizomycetes	10								71												1								2										
Sordariomycetes	186	898	406	270		249	64		1633	101					110	3					21	10	10	4		3	3		19	2					1	1			
Saccharomycotina																																							
Saccharomycetes	325	406	442			137	17		150												1	6	4			1	1		2										
Taphrinomycotina																																							
Unclassified Taphrinomycotina	2								44												1								1										
Basidiomycota	17																				1																		
Agaricomycotina																																							
Unclassified Agaricomycotina									13																				1										
Agaricomycetes	82	251	110			341	5		234					39	1						11	4	3			2	1		3					1	1				
Tremellomycetes	62584	3053	1366			864	248	7	2805	526											28	17	16			5	9	1	23	3									
Unclassified Tremellomycetes	62519	2909	1353			864	248	7	2738	526											21	16	14			5	9	1	22	3									
Pucciniomycotina			6																			1																	
Agaricostilbomycetes		122	9																			1	1																
Cystobasidiomycetes	76	1109	355	18		175			301	45											4	5	4	1		1			3	1									
Microbotryomycetes	12		50																		1	2																	
Pucciniomycetes	324	3465	909			432	150		1324	292	3								2		16	8	12			3	1		14	2	1								2
Ustilaginomycotina																																							
Exobasidiomycetes	255	108	93	71			133		166												4	1	1	2			1		3										
Ustilaginomycetes	4	2							15												1		1						4										
Chytridiomycota																																							
Chytridiomycetes		36							52	30													1						3	1									
Former Zygomycota																																							
Mortierellomycotina	3																				1																		
	69493	20193	10129	542	10	5532	3774	61	29684	3332	62	2	492	47	112	21		69	2		236	133	116	12	3	44	51	3	186	30	3	1	1	2	3	2		1	2

*NCBI Taxonomy

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