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Chapter 1

concept and simulation

1.1 Introduction

In almost all metagenomics projects, diversity analysis plays an important role to supply information about the richness of species, the species abundance distribution in a sample or the similarity and difference between different samples, all of which are crucial to draw insightful and reliable conclusion. Traditionally especially for amplicon metagenomics data set, OTUs(Operational Taxonomic Units) based on 16S rRNA genes are used as the basic units for diversity analysis. OTUs can be good replacement of the concept of "species" in metagenomics. Basically contigs are assembled from reads and are "binned" into OTUs using composition-based or similarity-based approaches. Then the diversity can be estimated by using the abundance information of the OTUs.

Recently there are many more projects generating whole genome shotgun metagenomics data sets. However they are mainly used for assembly and annotation purpose. Less attention was paid to diversity measurement using these whole genome metagenomics data sets. One possible reason is that the whole genome metagenomics data sets are often with low depth given the high diversity of metagenomics samples compared to 16S rRNA ampicon metagenomics data set. Assembly and annotation are always challenging with the low depth and lack of reference sequences. It is also true for diversity measurement. On the other hand, although with low depth, some whole genome metagenomics data sets are with large size because of the high diversity. For instance, there may be 4 petabase pairs of DNA in a gram of soilZarraonaindia:2013aa. Many of those methods for sequence binning or diversity estimation do not scale well and will not work for large metagenomics data sets. For instance, many composition-based binning approach involves k-mer/signature frequency distribution calculation, which is rather computationally expensive. Even basic sequence alignment will be impossible for large metagenomics data set. Many of those statistical software packages to estimate diversity using various estimators are not prepared for the large scale of whole genome metagenomics data.

With the development of next generation sequencing technology, the cost of sequencing is dropping rapidly. Whole genome metagenomics sequencing is more popular and large amount of metagenomics data is being generated with increasing speed, which can not be even met by the increase of computational capacity. Novel methods that can scale well are extremely needed to deal with the increasingly large metagenomics data set.

Here we propose a novel concept - IGS (informative genomic segment) and use IGS as a replacement of OTUs to be the cornerstone for diversity analysis of whole shotgun metagenomics data sets. IGSs represent the unique information in a metagenomics data set and the abundance of IGSs in different samples can be retrieved by the reads coverage through an efficient k-mer counting method. This samples-by-IGS abundance data matrix is a promising replacement of samples-by-OTU data matrix used in 16S rRNA based analysis and all existing statistical methods can be borrowed to work on the samples-by-IGS data matrix to investigate the diversity. We applied the IGS-based method to several simulated data sets and a real data set - Global Ocean Sampling Expedition (GOS) to do beta-diversity analysis and the samples were clustered more accurately than existing alignment-based method. We also tried this novel method to Great Prairie Soil Metagenome Grand Challenge data sets. Furthermore we will show some preliminary results using the IGS-based method for alpha-diversity analysis. Since this method is totally binning-free, assembly-free, annotation-free, reference-free, it is specifically promising to deal with the highly diverse samples, while we are facing large amount of dark matters in it, like soil.

1.2 The concept of IGS(informational genomic segment)

In classic ecology dealing with macroorganisms, diversity measurement is based on the concept of species. For 16S rRNA amplicon metagenomics data set, it is based on the concept of OTUs. While the concept of OTUs can be used to analyze large shotgun metagenomics data set, normally assembly, binning and annotation are required before doing diversity analysis. However these are difficult tasks, lacking of necessary reference genome or being computationally expensive. So we are interested in finding an approach to bypass the difficult tasks like assembly, binning, annotation and use raw reads to make diversity analysis to large metagenomic data possibile. In the beginning we proposed that the concept of k-mers(a DNA segment with the leng of k) can be used to measure diversity. K-mers can be considered as the atom of information in DNA sequences. One of the composition-based approaches to binning is to use the k-mer as the signatures. Suppose the sizes of microbial genomes are similar and the difference between genomic content of microbial genomes is similar, the number of distinct k-mers in the sequence data set correlates to the number of species in a sample. However, because of sequencing error, which is unavoidable due to the limit of sequencing technology, this k-mer based analysis doe not work well. One sequencing error on a read will generate at most k erroneous k-mers. In metagenomics data set, especially with high coverage, most of the distinct observed k-mers are from sequencing errors.

Next we looked at the upper level - reads. A novel method termed as digital normalization was developed to remove abundant reads before assembly. However it also supplies a novel way to distil information from reads by reducing the bad effect of sequencing errors so that we can use those informative reads to measure the microbial diversity. We term those informative reads as IGS(informative genomic segment), which can be considered as a segment of DNA on a microbial genome. Those IGSs should be different enough to represent the abstract information a genome contains. Suppose microbial genomes contain similar number of those IGSs, as they contain similar number of distinct k-mers, the number of IGSs will correlates with the species richness in a sample, and the abundance distribution of IGSs will be related to species evenness in a sample. Furthermore, we can get the abundance of the IGSs across different samples. Many classic diversity estimation methods based on OTUs described in sections above can be applied to estimate the diversity of IGSs and the diversity of actual species subsequently.

The concept of IGS can be the foundation of a novel statistical framwork to evaluate microbial diversity using whole genome shotgun metagenomic data, especially while facing large amount of "dark matters", unknown species. It is almost impossible to do annotation to those "dark matters", since we do not have much information about them. For alpha diversity, we can generate a list of IGSs and the respective abundance in a sample. Then existing estimators like Chao's can be used to estimate the total number of IGSs in the sample. Rarefaction curve based on number of IGSs can also be generated.

For beta diversity, we can generate a samples-by-IGS data matrix from the abundance of IGSs across samples, as a replacement of samples-by-OTU data matrix in OTU-based analysis and samples-by-species data matrix in traditional ecology. From that samples-by-IGS data matrix, we can use existing methods to calculate similarity/disimilarity/distance between samples and do further analysis like clustering and ordination.

1.2.1 IGS(informative genomic segment) can represent the novel information of a genome

Median k-mer abundance can represent sequencing depth of a read(cite diginorm). For a sequencing reads data set with multiple species, the sequencing depth of a read is related to the abundance of species where the read originates.

The Figure 1.1a shows the abundance distribution of reads from 4 simulated sequencing data sets with different sequencing depth - 3 sequencing data sets generated with different sequencing coverage(1x, 10x, 40x) from 3 simulated random genomes respectively and 1 combined data set with all the previously mentioned data sets. No error is introduced in these simulated data sets. Obviously the reads from the three data sets can be separated by estimated sequencing depth. The combined data set can be considered as a sequencing data set with three species with different abundance.

Each point on the curve shows that there are Y reads with a sequencing depth of X. In other word, for each of those Y reads, there are X-1 other reads that cover the same DNA segment in a genome that single read originates. So we can estimate that there are Y/X distinct DNA segments with reads coverage as X. We term these distinct DNA segments in species genome as IGS(informative genomic segment). We can transform the figure in upper position to show the number of IGSs and their respective reads coverage, as shown in figure in lower position. We sum up the numbers of IGSs with different reads coverage for each data set and get the result as shown in below. The sum numbers of IGSs here essentially are the areas below each curve in the figure.

Even though the datasets have different sequencing depth like 10X and 40X, they have similar numbers of IGSs. Dataset with 1X sequencing depth has fewer IGSs because the depth is not enough to cover all the content of the genome(63.2%). The IGSs can be seen as the genomic segment on a genome with the length of reads.(Figure 1.2 Assume the species genome is totally random, which is the case in the simulated data set, the number of IGSs(N) in a species genome is related to the size of genome(G), read length(L) and k size(k), which can be denoted as

$$N = \frac{G}{L}$$

which is the number of reads that can have a 1X coverage of the genome. For the simulated genome with size of 1M bps, read length as 80bps, expected number of IGSs is

1000000/(80 - 22 + 1) = 16949,

which is pretty close to observed value. See Table 1.1 (((((((This needs rewrite, the table will be replaced)))))))))))

Table 1.1: **Total number of IGSs in different simulated reads data sets.**

Data set	total number of IGSs
1X depth	8714
10X depth	16321
40X depth	16794
1X,10X,40X combined	41742

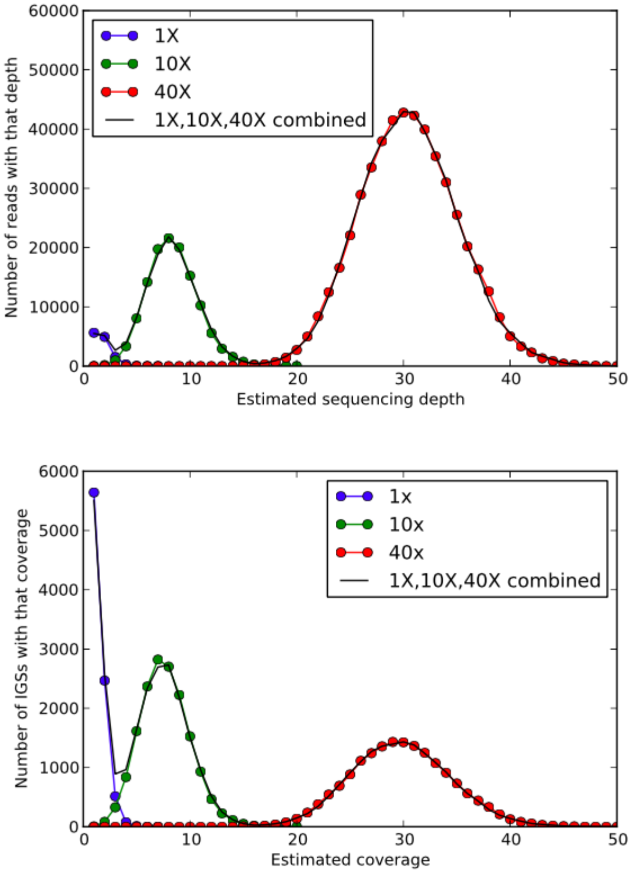

Figure 1.1: **from reads to IGS**

1.2.2 IGS can be used to do alpha diversity analysis

Basically the abundance distribution of IGSs with different coverage in a sample data set is acquired using the method shown above, like:

3 23

4 24

5 25

6 25

...

Here 23 IGSs with coverage as 3, this number is calculated from dividing the total number of reads with coverage as 3, which is 69, by the coverage 3:

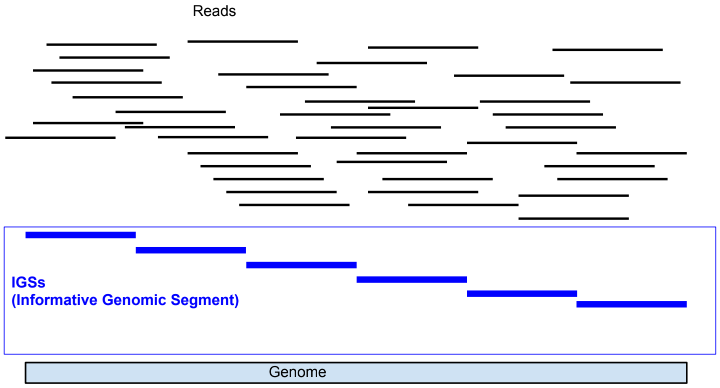

Figure 1.2: **the concept of IGS**

69/3. Similarly there are 96/4 = 24 IGSs with coverage as 4.

If we draw an analogy between IGSs and OTUs, this is like there are 23 different OTUs with 3 reads mapped to, and 24 different OTUs with 4 reads mapped to.

Then list all the different IGSs and the corresponding count,and we can get a long list with each IGS and the corresponding coverage.The coverage of an IGS can be considered as the abundance of such IGS in a sample. The list looks like:

IGS_ID abundance
1 3
2 3
3 3
...
23 3
24 4
25 4
...
47 4
48 5
...
...

This list is the counterpart of an OTU table in OTU based diversity analysis.

With such table at hand, numerous existing statistical methods and software packages can be used to investigate the alpha diversity.

1.2.3 IGS can be used to do beta diversity analysis

As in alpha diversity analysis, OTU table is also a cornerstone for beta diversity analysis. As long as we get a reliable OTU table, there are existing pipelines to do the beta diversity analysis.

A typical OTU table across different samples is like this, which is also called samples-by-OTU data matrix.

OTU_ID	Sample1_ID	Sample2_ID	Sample3_ID
OTU1	3	4	2
OTU2	2	5	0
OTU3	3	1	4

Like a OTU table, we hope to have the IGS table for the IGSs:

IGS_ID	SampleA	SampleB	SampleC	SampleD
IGS1	5	1	2	1
IGS2	5	1	2	1

So now the problem is how we can generate a sample-by-IGS data matrix as the counterpart of samples-by-OTU data matrix so many of the existing tools/methods used for OTU-based diversity can be borrowed for this kind of IGS-based analysis, just as what is shown above for alpha diversity analysis.

Firstly, as how we get the coverage of a read from a sample dataset in this sample dataset, we can get the coverage of a read from a sample A dataset in another sample B dataset. We can still use the median k-mer count to represent the coverage. The basic idea is the same.

Because a read must derive from a segment in the genome of some species in a sample, if a read R from sample A with a coverage C_A in sample A has a coverage as C_B in sample B, that means that segment of genome in sample A from which read R derive also exists in sample B. That genomic segment has a coverage as C_A in sample A and has a coverage as C_B in sample B. Roughly there should be about C_A reads (read R should be one of them) in sampleA covering that genomic segment and C_B reads in sampleB covering that genomic segment. Meanwhile, the C_A reads in sampleA should all have a coverage as C_B in sampleB, just like read R as one of them. Similarly, the C_B reads in sampleB should all have a coverage as C_A in sampleA.

Ok, now let's make an example.

Suppose there are 6 reads in sample A, all have a coverage as 3 in sampleA, and have a coverage as 2 in sampleB.

According to the discussion about IGS in previous section, the 6 reads cover 2 IGSs with a coverage as 3 for each IGSs. There should be 4 reads in sampleB covering the exact same 2 IGSs, with a coverage as 2 in sampleB.

So now we have 2 distinct IGSs with redundancy as 3 and 2 in the two samples respectively.

Note: small number is used in the analysis above as example, but it should be emphasized that the analysis is based on large number statistically.

Let's expand this example from 2 samples to 4 samples(A,B,C,D), as shown in figure above.

Let's say we find 10 reads in sampleA, with coverage as 5-1-2-1 in samples A-B-C-D respectively. (We call ”5-1-2-1” ”coverage spectrum” across samples.) So there should be **about** 2 reads in sampleB, 4 reads in sampleC, 2 reads in sampleD, all of which have a ”coverage spectrum” as ”5-1-2-1”. Basically these 18 reads altogether cover 2 distinct IGSs, which apparently exist in all the 4 samples. The 2 distinct IGSs has a redundancy as 5,1,2,1 in the 4 samples respectively.

If we draw an analogy between IGSs and OTUs, this is like there are 2 OTUs, both with 5,1,2,1 reads mapped to in sample A,B,C,D respectively.

Like a OTU table, here we can have the IGS table for the two IGSs:

IGS_ID	SampleA	SampleB	SampleC	SampleD
IGS1	5	1	2	1
IGS2	5	1	2	1

1.3 Evaluating IGS method using simulated data sets

1.3.1 An experiment using a simple simulated data sets

For this experiment, firstly we create 6 synthetic samples (Sample 1-6) based on 9 synthetic 10K genomes (genome A-I), with different composition of species and diversity.

The species composition for each synthetic sample is as below:

sample1: AAAB
sample2: AABC
sample3: ABCD
sample4: ABCE
sample5: AFGH
sample6: IFGH

For sample1, there are two species - A and B, with abundance distribution as 3:1.

The sequencing depth of all the synthetic data sets is 10X. So the species abundance in each sample is as below:

sample1: genomeA - 30, genomeB - 10
sample2: genomeA - 20, genomeB - 10, genomeC - 10
sample3: genomeA - 10, genomeB - 10, genomeC - 10, genomeD - 10
sample4: genomeA - 10, genomeB - 10, genomeC - 10, genomeE - 10
sample5: genomeA - 10, genomeF - 10, genomeG - 10, genomeH - 10
sample6: genomeI - 10, genomeF - 10, genomeG - 10, genomeH - 10

An a simple experiment, there is no sequencing errors introduced in the synthetic reads data sets.

Figure 1.3 and Figure 1.4 show that IGS method can yield the information about the difference of samples correctly. Sample 5 and sample 6 and very close to each other on the figure, which is true if we check the species composition of the two samples shown above.

Figure 1.5 shows the method can yield the richness information correctly. From the figure, samples with 4 different species have the richness almost twice as large as the sample with 2 different species.

From these results, we show the IGS method can work well to a simplest scenario, with high sequencing depth (10X) and no sequencing error. Next we will check the influence to the analysis accuracy of variable sequencing depth and sequencing error and introduce new ways to preprocess the data to decrease the influence of sequencing error.

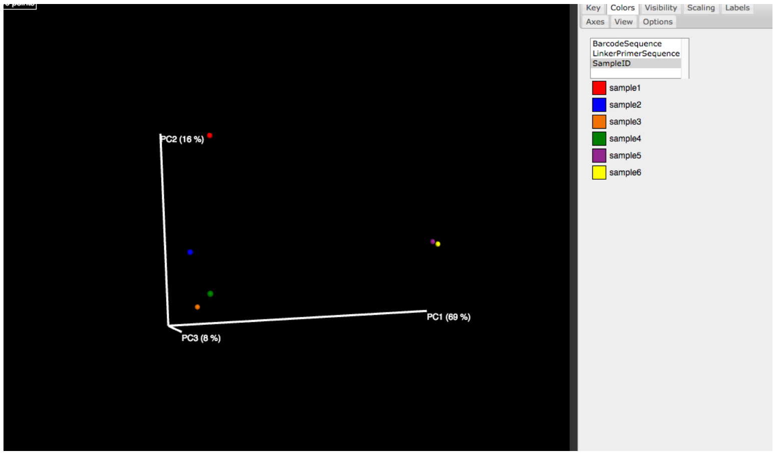

Figure 1.3: **Ordination of the 6 synthetic samples using IGS method**

1.3.2 Improving the accuracy of this method in real world analysis

Previously we have shown the IGS method generally works to a simple simulated data sets, with high sequencing depth and no sequencing error. In real world, in many situations we have to deal with the metagenomic data sets with relatively low sequencing depth, like soil or sea water samples. Also it is a fact that all sequencing technology will generate some errors. As discussed in the background section, one of the reasons we develop the IGS method is that based on the abundance counting of reads rather than k-mers, it is expected that the IGS method is less prone to sequencing error. However the effect of those factors jeopardizing the accuracy is still observable.

In this section, we will analyze the effect of these factors to the accuracy of the IGS method and investigate the ways to reduce the effect to increase the accuracy of analysis.

As in last section, six synthetic samples were generated with the same species composition . For each sample, sequencing reads data sets with different sequencing depth(0.1X, 1X, and 10X) and different sequencing error rate (0.5%, 1.0%, 1.5%, and 0% - no error at all) are simulated.

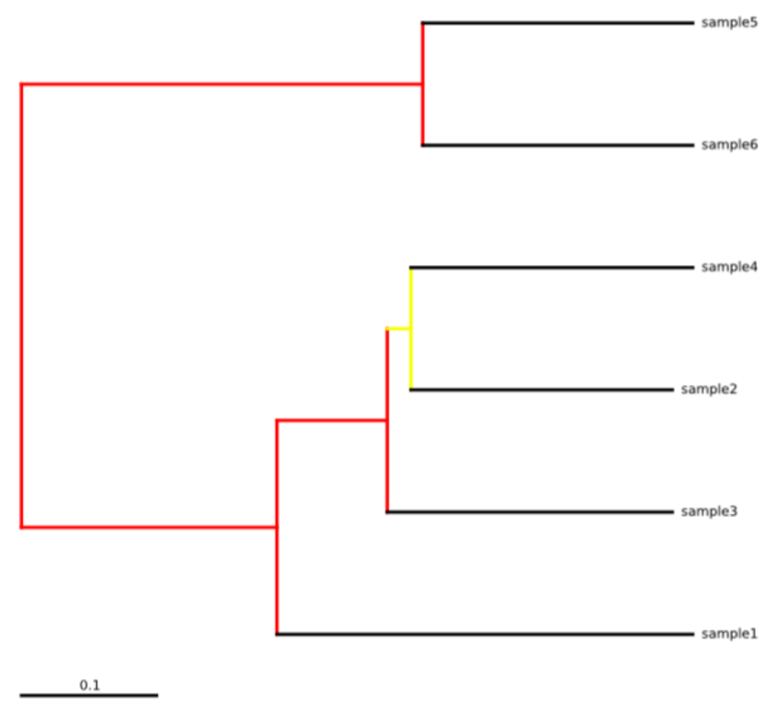

Figure 1.4: **Clustering of the 6 synthetic samples using IGS method**

To show the effect of sequencing error to accuracy of the analysis, we compared the richness estimation using reads with different sequencing error rate, as shown in Figure 1.6. For data set without error (error rate = 0), the esimated size of metagenome matches real size perfectly. With increasing error rate, the size of metagenome is over estimated more and more seriously. This is due to several factors,which will be discussed below.

the effect of sequencing error to the accuracy of analysis

The first factor to take into account is sequencing error. One sequencing error will generate k erroneous k-mers. This is the reason why it is difficult to use k-mer counting only to do diversity analysis, as a large proportion of k-mers in a reads data set are erroneous, especially for low coverage reads data. As discussed in the section about digital normalization, using median k-mer count to retrieve the coverage of a read is less prone to sequencing

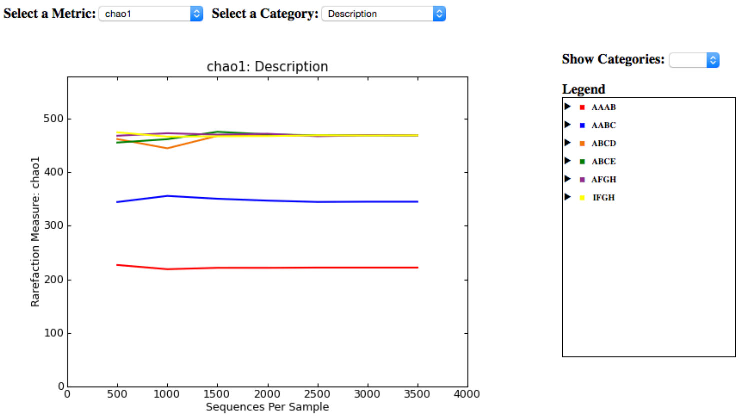

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Figure 1.5: **Richness estimation of the 6 synthetic samples using IGS method**

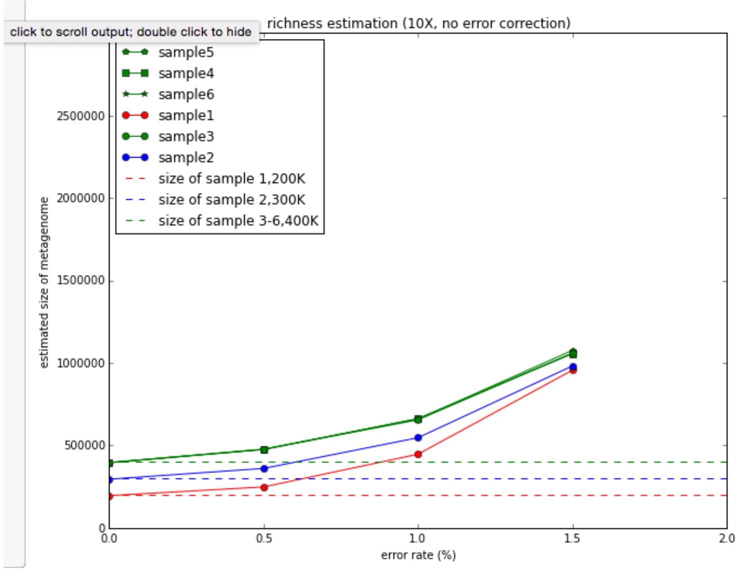


Figure 1.6: **Richness estimation using IGS method without adjustment**

error, because sequencing error will decrease the count of some reads to 1 incorrectly, but this does not always affect the median k-mer count.

Take the experiment we did previously as an example, for read length as

100bp and k as 19, one sequencing error will affect the count of 19 k-mers at the most, two sequencing errors will affect the count of at most 38 k-mers. The count of these k-mers will be retrieved as 1 incorrectly, suppose it is highly impossible that an erroneous k-mer is the same as another real k-mer in the data set accidently, as long as the k is large enough compared to the size of data set. So out of the 82 k-mers in the 100bp read, at most 38 k-mers will have count as 1 incorrectly, but this will not affect the median k-mer count, which is the count of the 41th k-mer if ranked by count. However, if there are three or more errors in the read, the situation is more complicated. For 3 errors in a read, 3 to 57 k-mers will be affected by the errors to have an incorrect count as 1. The distribution of the probability about the number of affected k-mers can be acquired by a model similar to Lander-Waterman model used in genome sequencing theory. Here we got the distribution using simulation, as shown in Figure 1.7. From this probability distribution, we can get the probability that 3 errors will affect more than 40 k-mers is 0.43. In this case, 3 errors will affect the median k-mer count of a read. We can also get such probability for 4 errors or more. Combining to the probability that a certain number of errors occur in a read with a specific sequencing error rate, which is easy to derive from binomial distribution, we can get the probability that the coverage of a read is incorrectly assessed as 1. Still for the example here, this probability is the probability that 3 erorrs occur in a read multiplied by the probability that 3 errors will affect median k-mer count, plus the probability that 4 erorrs occur in a read multiplied by the probability that 4 errors will affect median k-mer count,and so on.

Generally, let $P\_error(n, e, L)$ is the probability that n errors occur in a read with length as L, with error rate as e and $P\_effect(n, k, L)$ is the probability that n errors in a read with length of L affect median k-mer count. The probability that the coverage of a read is incorrectly assessed as 1 is

$$\sum_{n=3}^{\infty} P\_error(n, e, L) \times P\_effect(n, k, L)$$

, and by binomial distribution,

$$P\_error(n, e, L) = f(n; L, e) = Pr(X = n) = \binom{L}{n} e^n (1 - e)^{L-n}$$

Practically, when $n > 5$ and $e < 0.015$, $P\_error(n, e, L)$ is very small, we only consider number of errors in a read as 3, 4 and 5.

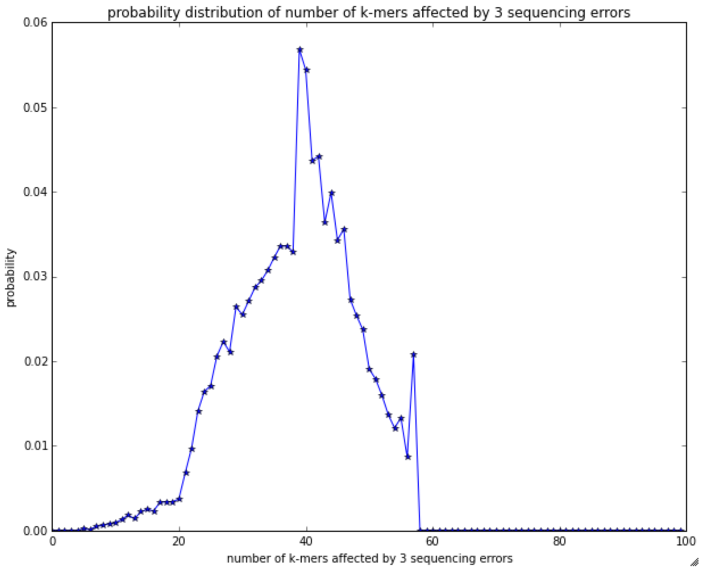


Figure 1.7: **Richness estimation using IGS method without adjustment**

From the discussion above, the sequencing errors reduce the coverage of some reads incorrectly to 1 and the probability this occurs to a read can be estimated. So to reduce the effect of sequencing error on this aspect, we can calculate the expected number of reads that are affected and remove those reads from the set of reads with coverage as 1 before generating list of IGS from the reads abundance distribution.

Also, we want to make sure 2 errors in a read will not affect median k-mer count, since it is more common to have 2 errors in a read practically. In this case,

$$2 \times k < \lfloor \frac{L - k + 1}{2} \rfloor$$

, we can get $k < L/5$,basically. For L as 100, k will be 19, which is what we choose in the testing. However, the k should not be too small, or the k-mers can not handle the information of a large data set.

Taking the sequencing error into account, we used the methods introduced above to adjust the estimation of metagenome size of the 6 synthetic samples. The estimation after adjustment is closer to real number, as shown in Figure 1.8..

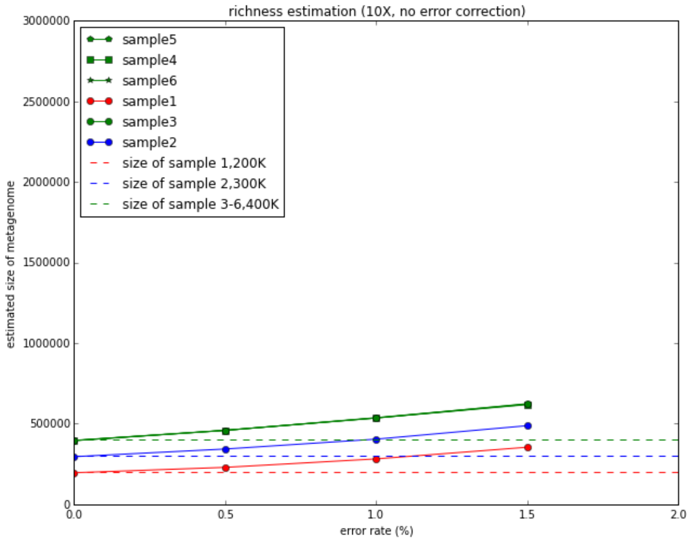


Figure 1.8: **Richness estimation using IGS method adjusted by sequencing error rate**

the effect of collision in bloom filter to analysis accuracy

As discussed in the chapter about k-mer counting, the collision in bloom filter which we use for efficient k-mer counting will result in counting error. If the false positive rate for a specific bloom filter we use for k-mer counting is 0.1, 10% of the k-mers will have incorrect counts. When we use median k-mer count to get read coverage, such incorrect count has the effect on two aspects. On one hand, some k-mers in a read will have incorrect higher count. But if the false positive rate is low, this will not affect median k-mer count. This shows the method of using median k-mer count to get read coverage is not only less prone to sequencing error, but also less prone to the inaccuracy characteristics of underlying data structure. One the other hand, this inaccurate count also affects the counts of those erroneous k-mers generated by sequencing error. For example, 3 errors in a read affect the count of 43 k-mers, the counts for these 43 k-mers are supposed to be 1. But because of the collision in bloom filter and the resulting incorrect k-mer counting, if the false positive rate is 0.1, about 4 out of the 43 k-mers will have inflated count, mostly as 2. So the combined effect of sequencing error and collision in bloom filter is that some reads will have incorrect coverage as

1 and some reads will have incorrect as 2. We can get the percentage of total reads that will have such incorrect coverage, using statistical model similar to that discussed in last section. Using same example, 3 errors occur in a read, if the 3 errors affect 41-45 k-mers(with a chance of 0.20), the median k-mer count will be 2, due to the collison in bloom filter, while if he 3 errors affect more than 45 k-mers(with a chance of 0.24), the median k-mer count will be 1, purely due to sequencing errors.

We did the same experiment but also adjusted the estimation according to the false positive rate of bloom filter and got better estimation, as shown in Figure 1.9.

With adjustment to estimation taking sequencing error and collision in bloom filter into account, as shown in Figure 1.9, the estimated genome size is closer to real number. With error rate as 1%, false positiver rate as 0.1, with 10X coverage data, the estimated genome size is about 20-25% more than real number. However the estimation is still increasing with higher error rate. This means there are still other factors influencing this accuracy of the estimation but we failed to take into account.

The other problem is that the result of low coverage data(0.1x) is worse than before adjustment. This may be due to the smaller number of reads with abundance of 2 after adjustment. It may also be due to small size of simulated data (with only hundreds of reads).

comparison of dissimilarity matrix from beta diversity analysis

To evaluate the effectiveness of beta diversity analysis using IGS based method, we compare the dissimilarity matrix generated by IGS based method with that generated from another metagenomics comparison tool - Commet(Compareads), and the true matrix,since we know exactly the species composition of the simulated data sets.

The clustering and ordination are all from the dissimilarity matrix. We think comparing matrix directly makes more sense than comparing the clustering and ordination plot. So we will not show the clustering and ordination figure in this evaluation. If the matrix can reflect the real relationship between samples reliably, the clustering and ordination will only be routine job.

The true dissimilarity matrix of the 6 simulated samples using bray-cutis metric from species composition directly is shown in Figure 1.10. For a simulated data set with 10x coverage and no error introduced (which will

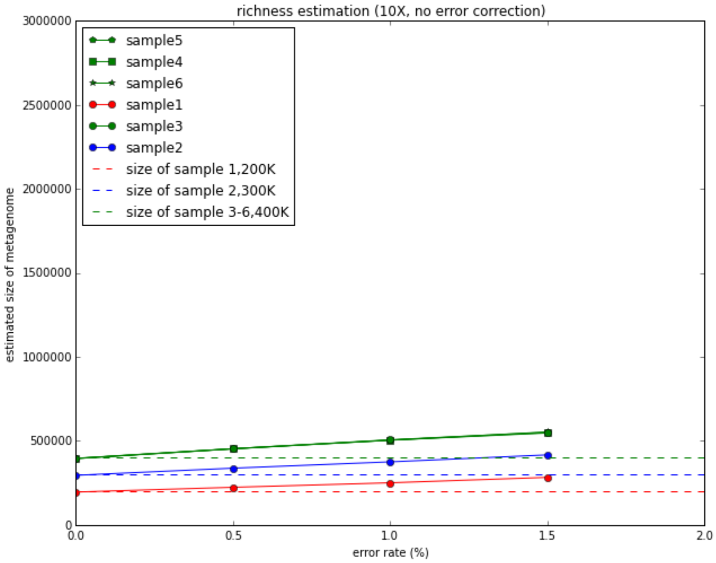


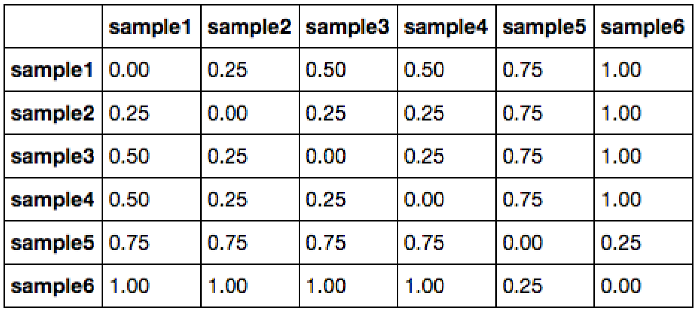
Figure 1.9: **Richness estimation using IGS method adjusted by sequencing error rate and false positive rate of bloom filter**

tell us the optimal performance of IGS method), the dissimilarity matrix can be calculated by using IGS method, as shown in Figure 1.11. We can see the absolute values in the matrix are not very close to that in the real matrix.But the relative values correspond to that in the real matrix well to show the relative distance between each pair of samples. To get a objective metric, we use Mantel (citing)test to calculate the correlation value between the two matrixes. The correlation is 0.9714, which means a very positive correlation between the two matrices. We are very confident that the matrix from IGS method can reflect the true relationship between samples pretty well.

Next we test how well the matrix calculated by various methods can reflect the real relationship between samples. The simulated data sets with sequencing depth as 1 and 10, with sequencing error as 3% and without sequencing error are used in this experiment. For the data sets with sequencing error, we use a HMM based error correction tool to preprocess the reads to check the effectiveness of error correction. We also compare the performance of IGS based method and another metagenome comparing tool - Comet.

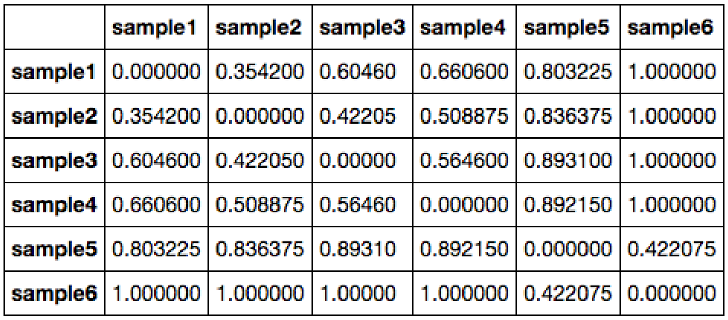
As shown in Figure 1.12, firstly, for all data sets, the matrix from IGS method has a higher correlation to golden standard than that from Comet. As expected, the matrix from data sets with sequencing error has a lower correlation than that from error-free data sets. Also Comet is more sensitive to sequencing error rate, compared to IGS method. However, error correction can increase the correlation significantly. Also higher coverage will yield more accurate matrix, which is not surprising.

Figure 1.13 shows how well the matrix calculated from data set with variable coverage can reflect the real relationship between samples. It is as expected that higher coverage data will yield better/more accurate distance matrix. Note even with a coverage as low as 0.1, the correlation is 0.89. This can give us the hint how reliable the result will be if we only use a small proportion of data from a large metagenomic data set.



	sample1	**sample2**	**sample3**	**sample4**	**sample5**	**sample6**
sample1	0.00	0.25	0.50	0.50	0.75	1.00
sample2	0.25	0.00	0.25	0.25	0.75	1.00
sample3	0.50	0.25	0.00	0.25	0.75	1.00
sample4	0.50	0.25	0.25	0.00	0.75	1.00
sample5	0.75	0.75	0.75	0.75	0.00	0.25
sample6	1.00	1.00	1.00	1.00	0.25	0.00

Figure 1.10: **Dissimilarity matrix between synthetic samples using Bray-cutis from species composition directly**



	sample1	**sample2**	**sample3**	**sample4**	**sample5**	**sample6**
sample1	0.000000	0.354200	0.60460	0.660600	0.803225	1.000000
sample2	0.354200	0.000000	0.42205	0.508875	0.836375	1.000000
sample3	0.604600	0.422050	0.00000	0.564600	0.893100	1.000000
sample4	0.660600	0.508875	0.56460	0.000000	0.892150	1.000000
sample5	0.803225	0.836375	0.89310	0.892150	0.000000	0.422075
sample6	1.000000	1.000000	1.00000	1.000000	0.422075	0.000000

Figure 1.11: **Dissimilarity matrix between synthetic samples using Bray-cutis from sequencing reads using IGS method**

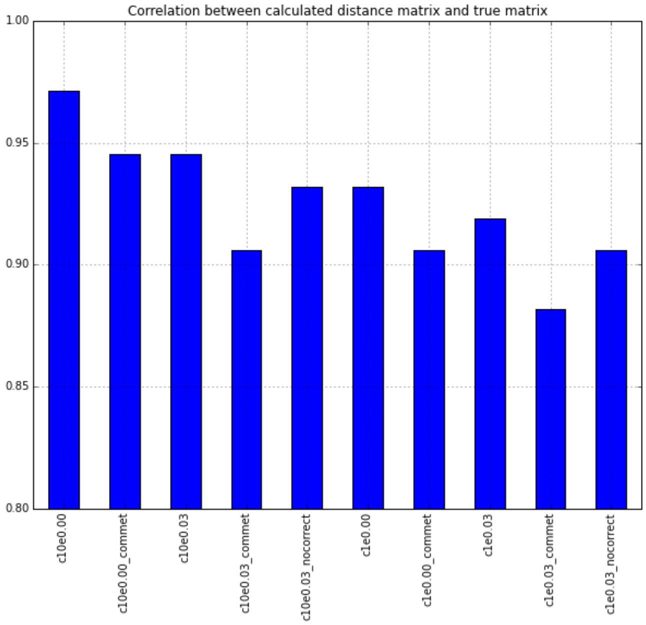


Figure 1.12: **Correlation between calculated distance matrix and true matrix from different data sets and using different methods**

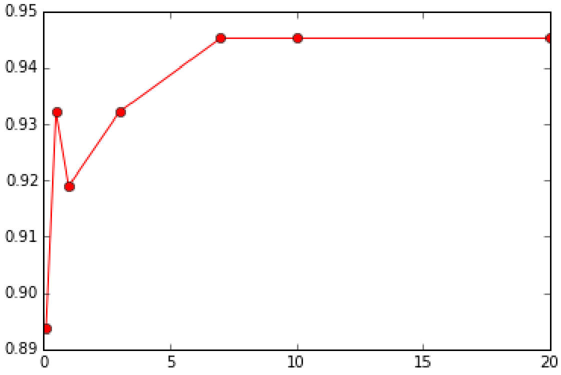


Figure 1.13: **Correlation between calculated distance matrix and true matrix from different dat sets with different sequencing depth**

Evaluate alpha diversity analysis by estimating size of metagenome

We can use statistic metric to estimate the total number of IGSs in a sample, which can be used to calculate the estimated genome size of a sample using the formula below:
size of genome = number of IGS x (reads_length - k-size +1)

Here we check how accurate the estimated size of genome and coverage is using different data sets with variable coverage/sequencing depth.

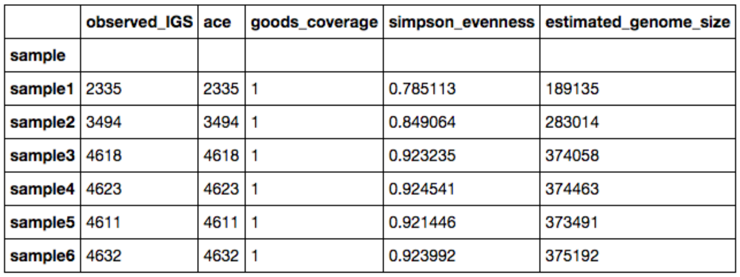
The genome size of the 6 samples should be:
sample1: AAAB 2x100K = 200K bp
sample2: AABC 3x100K = 300K bp
sample3: ABCD 4x100K = 400K bp
sample4: ABCE 4x100K = 400K bp
sample5: AFGH 4x100K = 400K bp
sample6: IFGH 4x100K = 400K bp

In this experiment, we use ACE metric since we find it is more accurate than Chao1, since it uses more abundance information.

Table 1.14 shows the estimated genome size of samples using error-free simulated sequencing data sets is close to true size shown above. If the sequencing error is introduced, the estimated genome size is inflated dramatically as shown in Table 1.15, which is not surprising. However after applying error correction, we can still get good estimation of genome size, as shown in Table 1.16. This proves again error correction can improve the effectiveness of IGS method.

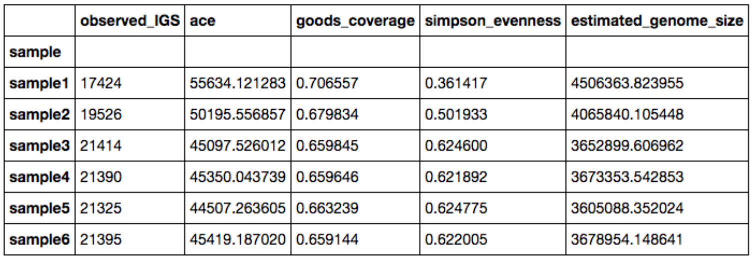
Figure 1.16 shows the estimated genome size from data sets with variable coverage. The estimated genome size keeps increasing as we use more reads, with higher coverage,which is higher than actual genome size. It's interesting that the estimated genome size is very high with low coverage, (probably due to more unique k-mers, which makes error correction more difficult. Then the estimated genome size drops a little bit as coverage from 0.1X to 1-3X, then starts to climb again as coverage increases, probably because there are always more erroneous k-mers that cannot be corrected. The good thing is that the climbing rate is not that high, this is believed to be due to the effectiveness of our error correction algorithms.

It is important to point that even though the absolute value of estimated genome size may be overestimated. The relative relationship between samples are reliable, as shown in the figure. Sample3,4,5,6 all have 4 species, while sample 2 has 3 species, and sample 1 has 2 species. They can be separately pretty well.



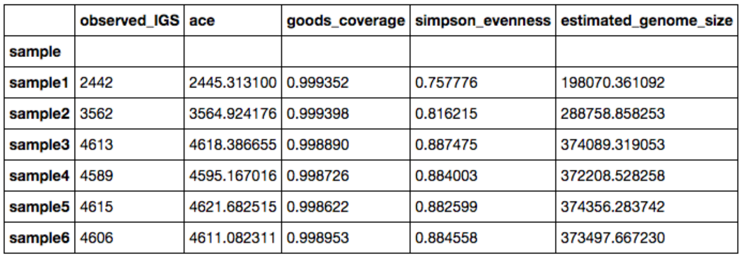
	observed_IGS	ace	goods_coverage	simpson_evenness	estimated_genome_size
sample					
sample1	2335	2335	1	0.785113	189135
sample2	3494	3494	1	0.849064	283014
sample3	4618	4618	1	0.923235	374058
sample4	4623	4623	1	0.924541	374463
sample5	4611	4611	1	0.921446	373491
sample6	4632	4632	1	0.923992	375192

Figure 1.14: **Coverage = 10x, No error**



	observed_IGS	ace	goods_coverage	simpson_evenness	estimated_genome_size
sample					
sample1	17424	55634.121283	0.706557	0.361417	4506363.823955
sample2	19526	50195.556857	0.679834	0.501933	4065840.105448
sample3	21414	45097.526012	0.659845	0.624600	3652899.606962
sample4	21390	45350.043739	0.659646	0.621892	3673353.542853
sample5	21325	44507.263605	0.663239	0.624775	3605088.352024
sample6	21395	45419.187020	0.659144	0.622005	3678954.148641

Figure 1.15: **Coverage = 10x, error = 3%, no error correction**



	observed_IGS	ace	goods_coverage	simpson_evenness	estimated_genome_size
sample					
sample1	2442	2445.313100	0.999352	0.757776	198070.361092
sample2	3562	3564.924176	0.999398	0.816215	288758.858253
sample3	4613	4618.386655	0.998890	0.887475	374089.319053
sample4	4589	4595.167016	0.998726	0.884003	372208.528258
sample5	4615	4621.682515	0.998622	0.882599	374356.283742
sample6	4606	4611.082311	0.998953	0.884558	373497.667230

Figure 1.16: **Coverage = 10x, error = 3%, with error correction**

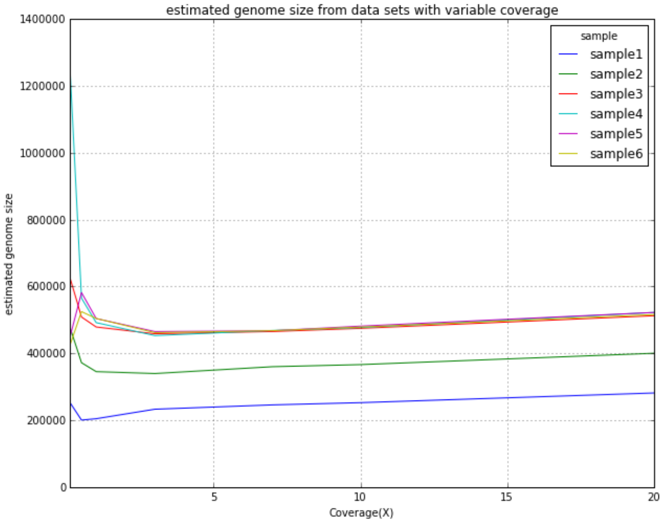


Figure 1.17: **estimated genome size from data sets with variable coverage**

1.3.3 The IGS method can provide a whole framework to do alpha or beta diversity, with good versatility.

From the testing using simulated data sets shown here, we are confident that our IGS method works well and can give reliable results from data sets with error and low sequencing depth.

The IGS method can provide a whole framework to do alpha or beta diversity. Here we tested beta diversity using only bray-curtis metric and alpha diversity on richness only. Actually any metric can be applied to the IGS-by-samples table, abundance-based or incidence-baserd, richness or evenness.

Compareads(Commet) based on reads overlap between samples can get a matrix reflecting the real relationship between samples pretty good but it is stuck with one metric, which is based on the percentage of overlap reads between samples. This metric is like bray curtis , but not exactly the same.

Chapter 2

real data sets

2.1 Applying IGS method to real metagenome data sets

Having shown that the IGS method gives good results about microbial diversity to simulated synthetic data sets, we will now evaluate the novel method on several published metagenomic datasets, with samples from ocean, human microbiome and soil. For the ocean sample and human microbiome data sets, we will compare the result from IGS method with that from original publication. For soil sample, since there is no other diversity analysis that has been done to these data sets, we will show the result we got from IGS and try to interpret the ecological meaning of the result.

2.1.1 GOS data sets: Sorcerer II Global Ocean Sampling Expedition

We tested the IGS method on a famous public dataset from the Sorcerer II Global Ocean Sampling expedition. During the expedition, 44 water samples were collected from different locations across Atlantic Ocean and Pacific Ocean and were sequenced using Sanger technology. The whole dataset is composed of XXXX reads, out of 44 samples. A whole metagenomic comparison of the samples has been done using a sequence alignment method in original research.

The IGS method took XX hours on a XXX hardware to generate the dissimilarity matrix of the samples. After clustering, Figure 2.1 shows that, consistently with the original study, the samples are clustered according to their geographical origin. The group with yellow color contains samples from Tropical- Galapogas. The group with light purple color contains samples from Tropical -Open Ocean. The group with dark purple color contains samples from Sargasso. The group with green color contains samples from Temperate.

If we compare the cluster we got from IGS method with the cluster in original study, we can see the IGS method yield a cluster with better resolution and accuracy than the method used in original study. For example, in original study, sample 14,21 and 22 from Tropical - Galapogas are separated from other Tropical- Galapagos samples, while in Figure 2.1 they are grouped together. Also, samples 00a,00b,00c,00d, obviously from the same location, are grouped together in our result,while in original research, sample 00a is separated from the other three samples.

Compared with the cluster by Compareads, our method is comparable, with some distinct differences. For example, sample 16 is clustered together with 15,17,18,19 in our result, but in the result by Compareads, sample 16 is clustered with 23,26 inaccurately, considering the geographical origin.

Next we use IGS method to analyze the alpha diversity. Figure 2.2 shows the rarefaction curve of IGSs of the samples. As expected, we can not see the saturation,which means the sequencing data set is still far from enough overage. Because the data sets for different samples have dramatically different size, we estimate the total number of IGSs using Chao1 estimator with limited number of reads in each sample(50000) to make sure the smallest data set has enough reads for comparison, as shown in Figure 2.3 .

It is obvious that the richness of samples is related to the geographical origin. The sample from tropical area has a higher richness than the samples from northern area. The relationship between samples is consistent to the cluster in beta diversity analysis shown above.

As discussed in the section above about alpha diversity analysis to synthetic data, such number of total IGSs may over-estimated but the relative relationship between samples on richness should be reliable.

(This is not discussed in original research of GOS)

2.1.2 HMP metagenomics data set

Here we test IGS method on 12 HMP(Human Microbiome Project) samples from different body parts like skin, oral or vaginal. Principal component

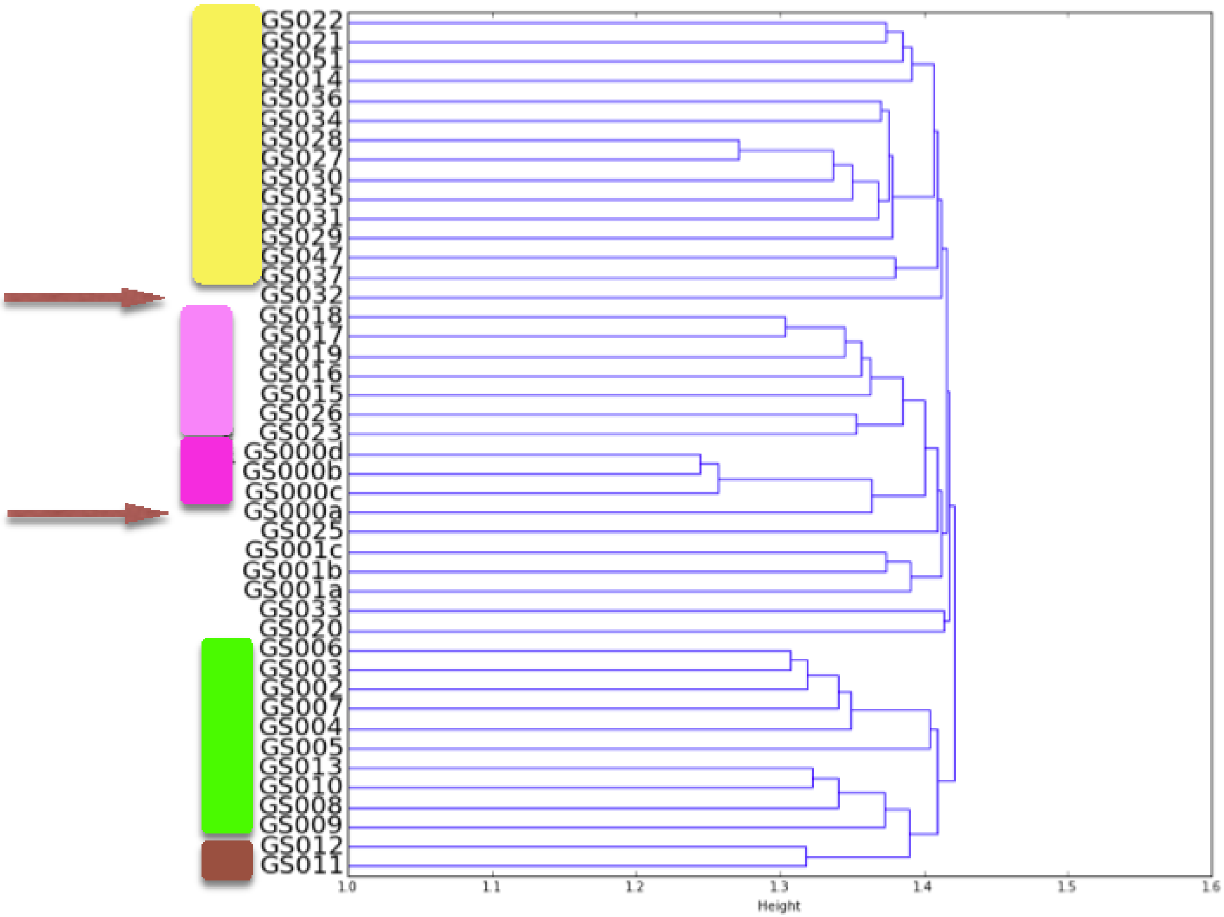


Figure 2.1: **cluster of GOS samples using IGS method**

analysis(Figure 2.4) shows the samples are separated well by the body parts where they are collected. (P value: XX)

Rarefaction curve and estimated number of IGSs shows the richness of samples is related to the body part where they are collected. The oral samples have higher richness than skin or vaginal samples, which is consistent to other research.

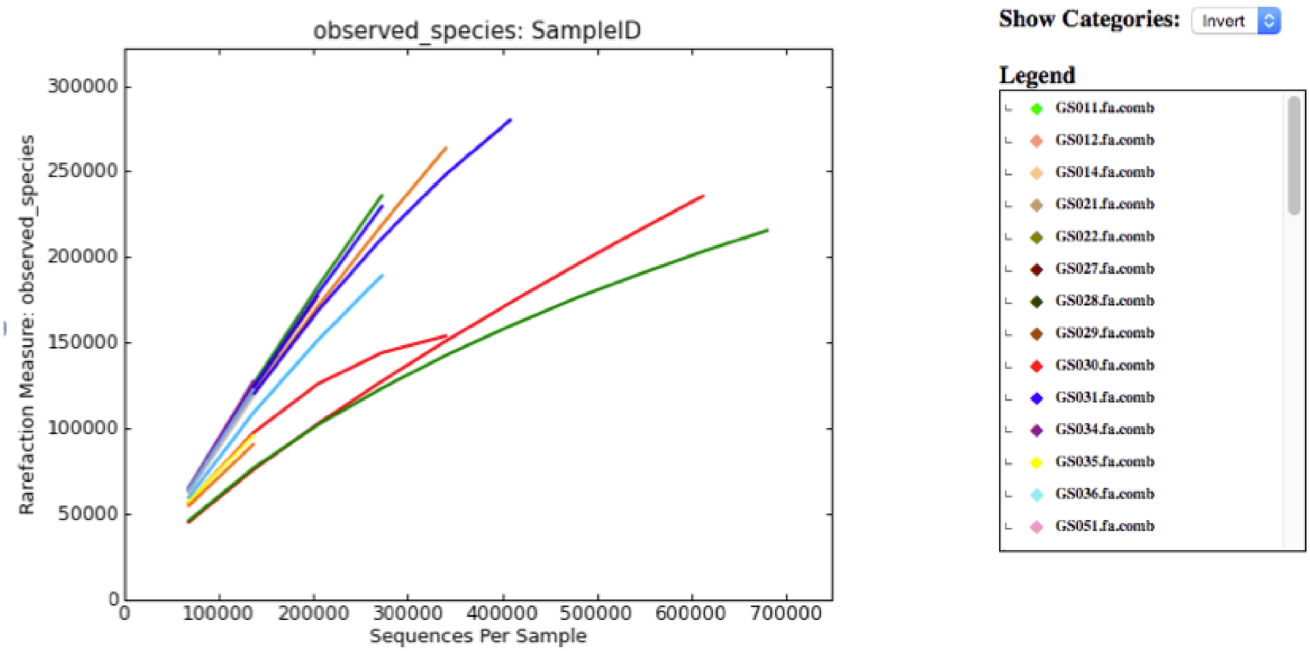


Figure 2.2: **Rarefaction curve of IGSs of the GOS samples**

2.1.3 GPGC - Great Prairie Soil Metagenome Grand Challenge

Having tested the IGS method on two relatively smaller metagenomic data sets, we will now use it to analyze a larger data set from soil samples collected from different treatment and different location across the great prairie region in the US. (Table 2.2).

Using 1m and 2m randomly selected subsets can yield pretty good results.

As discussed above with simulated data sets, using reads data sets with lower sequencing coverage will reduce the accuracy of the analysis. But as shown in Figure 1.13, with sequencing depth as 0.1x, the calculated distance matrix using IGS method still has a reasonably high correlation with golden standard distance matrix. So we can use subset of a large data set to acquire the diversity information, with the trade-off of lower accuracy.

For the GPGC datasets, we make a subset with 2 million reads from each

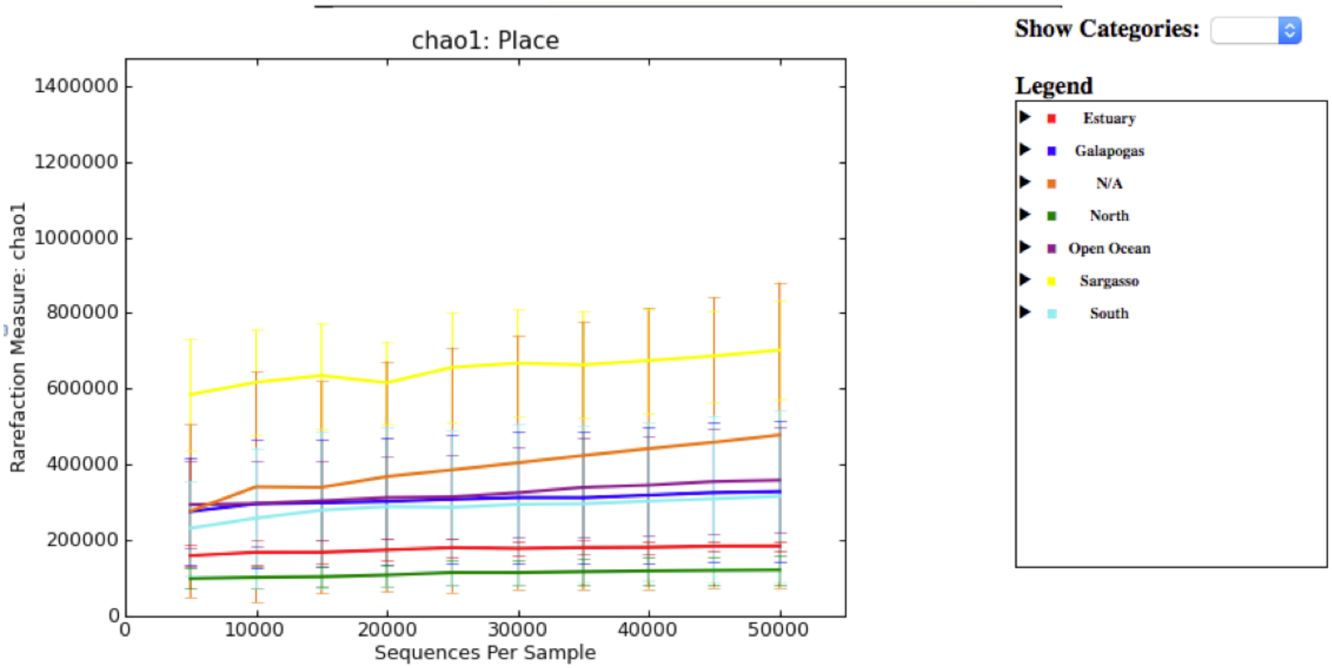


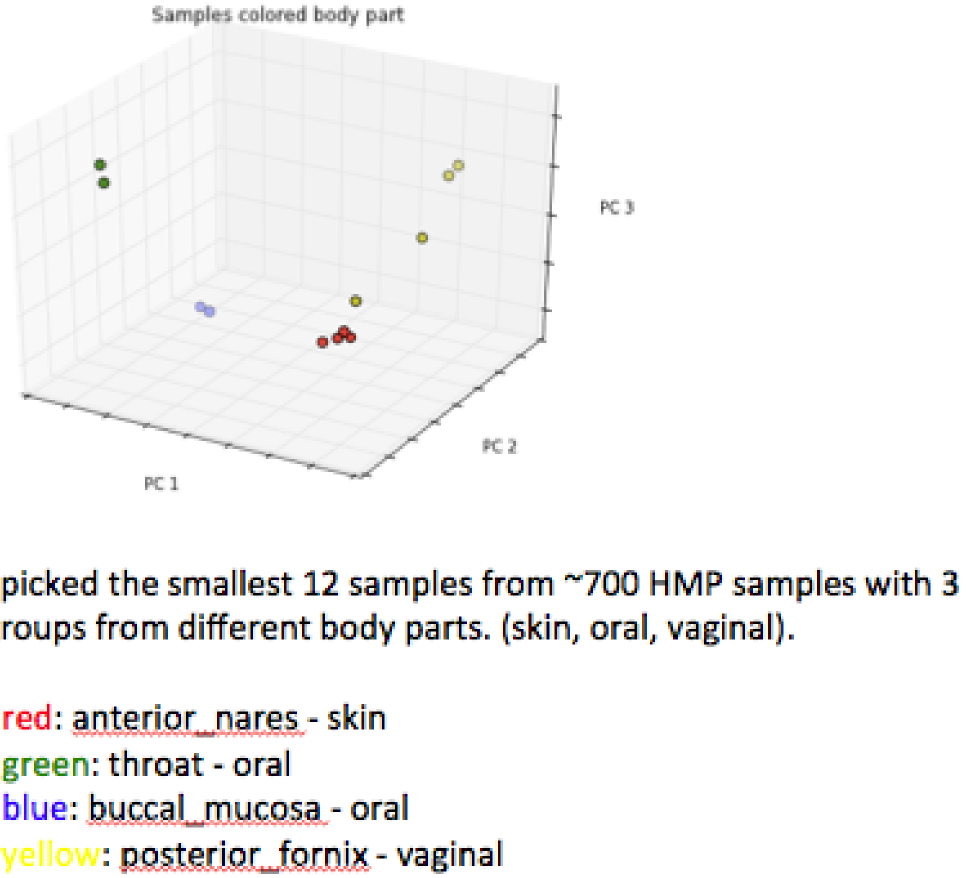
Figure 2.3: **Estimated number of IGSs of the GOS samples**

sample and do the diversity analysis using IGS method.

Principal component analysis(Figure 2.6) shows the samples are separated well by location where they are collected. (P value: XX) This proves that the geographical origin plays a more important part in determining the similarity of genomic composition of samples, compared to different treatment. Note this is from a relatively small subset. (1-2 million reads).

Figure 2.7 shows the rarefaction curve and estimated number of IGSs of the samples. Basically the "corn" and "switchgrass' samples have higher richness than "restored" and "prairie" samples. This observation that cultivation increases the richness of soil is consistent with the ?intermediate disturbance hypothesis?. The disturbance from treatment like cultivation opens more niches and the stable community like prairie eliminates some populations by the principle of competitive exclusion. .

quotes TJ's comments - Its harder to explain the rank by state. The Kansas site experiences more drought stress and higher temps. Maybe that selects for some more divergent physiologies? The Iowa and Wisconsin sites experience more cold, esp freezing conditions arresting their biology for 3-



I picked the smallest 12 samples from ~700 HMP samples with 3 groups from different body parts. (skin, oral, vaginal).

- red: anterior_nares - skin
- green: throat - oral
- blue: buccal_mucosa - oral
- yellow: posterior_fornix - vaginal

Figure 2.4: **PCoA of HMP**

4 months, but the freeze-thaw cycles also kill off some each cycle (sound like intermediate disturbance!!), and with new growth each spring, this new growth would the fast growers, i.e. less diverse. Why Iowa is the least diverse, I don?t know - they planned it to help Adina and Titus with assembly.

Also from the alpha diversity, we can have a rough estimation of the total size of metagenome in iowa soil, which is about 540G basepairs. This proves the high complexity of soil sample and we still need more sequencing effort to achieve a reasonable high coverage.

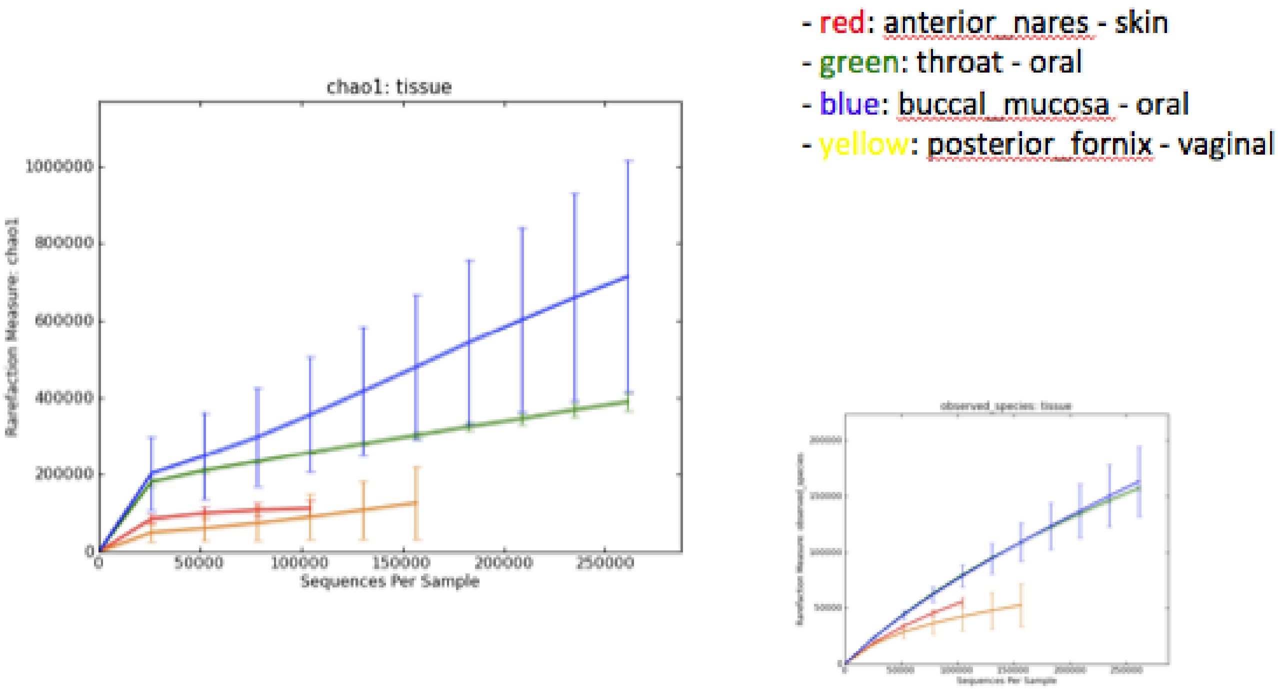


Figure 2.5: **alpha diversity of HMP**

2.1.4 more soil metagenomic samples

Additionally we test the IGS method on two other unpublished data sets. One is a series of soil samples collected from KBS with different treatment. Figure 2.8 shows the IGS method can separate the samples by treatment well.

The other data set is a series of soil samples from Amazon rainforest. The samples are separated well by the treatment. (Figure 2.9) It is also obvious that samples from forest have lower richness than prairie. (Figure 2.10)

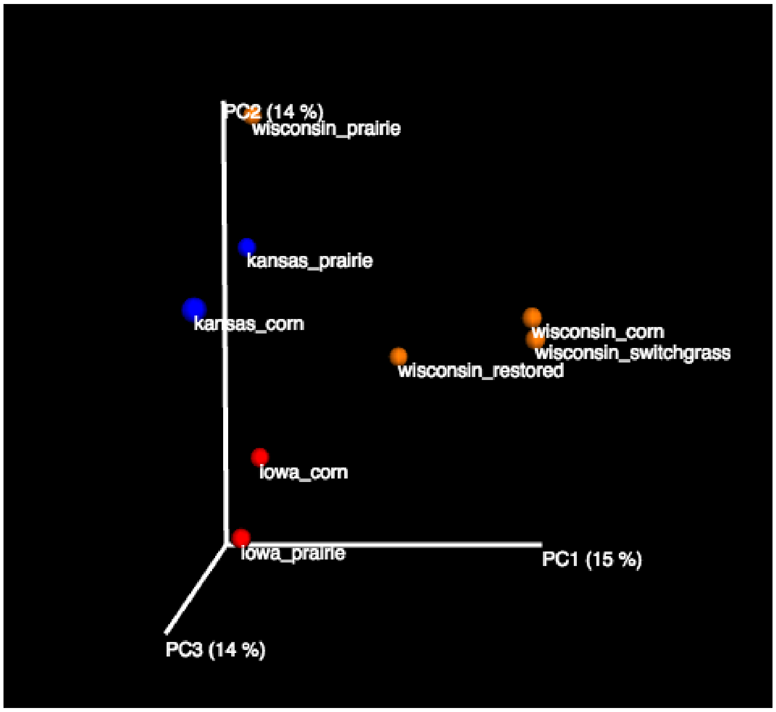


Figure 2.6: **PCoA of GPGC samples**

2.2 Future Direction

- Extract reads that are unique/common between samples and do annotation to those reads.

- sequencing depth evaluation

- genome size estimation

- better choosing diginorm parameters(size of hashtables, etc.) with genome size estimation as upper limit.

- reads binning/classification (after clustering)(if number of samples is small, may not be effective)

- co assembly (by extracting the reads with total coverage across samples greater than 10, for example)

- iterative diversity analysis - loading more reads to get higher accuracy, but stops as pattern/clustering is significant enough (only for diversity analysis)

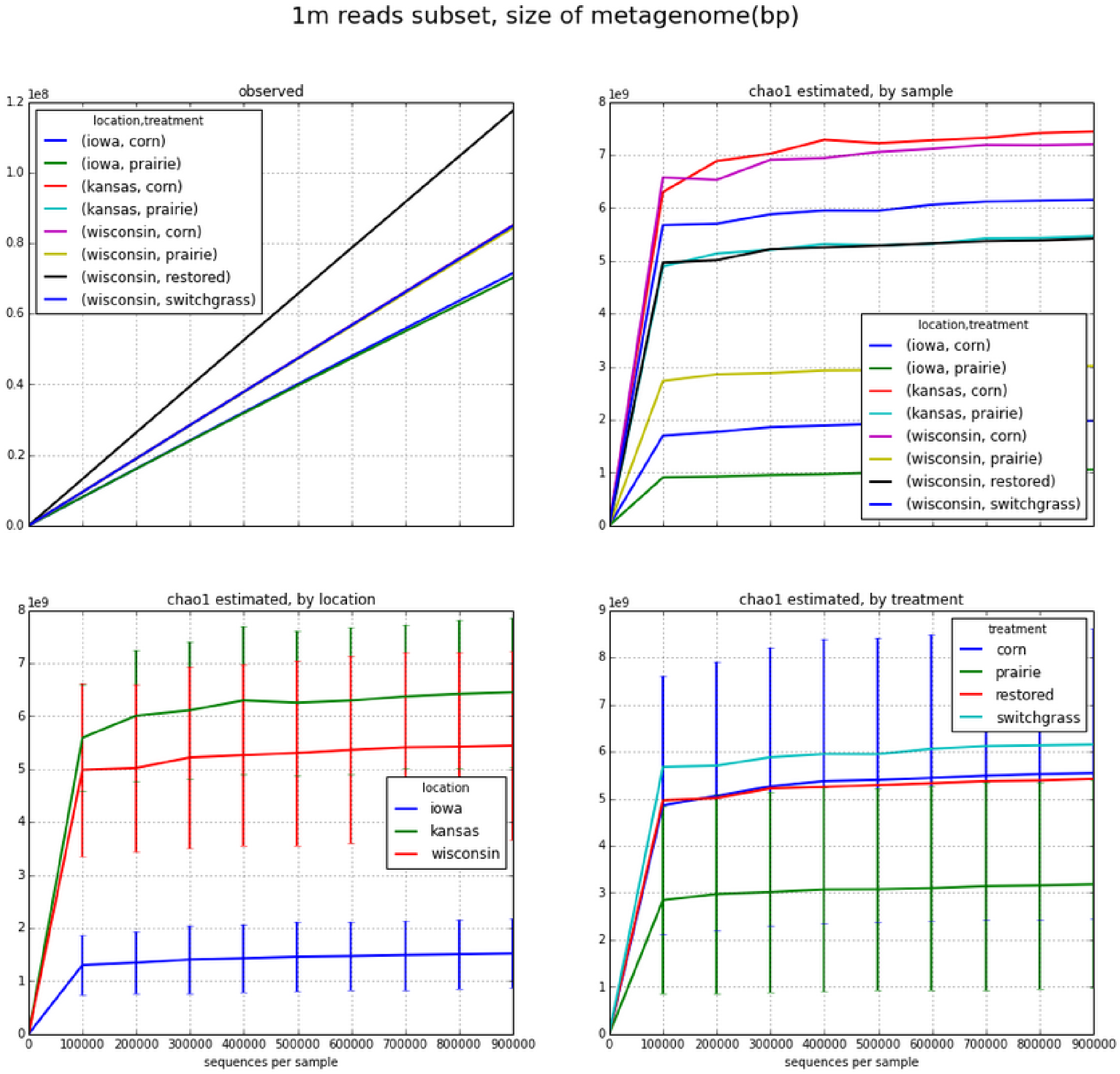


Figure 2.7: **alpha diversity of GPGC samples**

2.3 Conclusion

2.4 Data

2.4.1 Four simulated reads data sets with different species abundance distribution

2.4.2 Simulated sequencing reads of e.coli

Here we simulated 4 sequencing reads data sets with read length as 100bp of e.coli with different sequencing depth(50x and 150x) and different sequencing

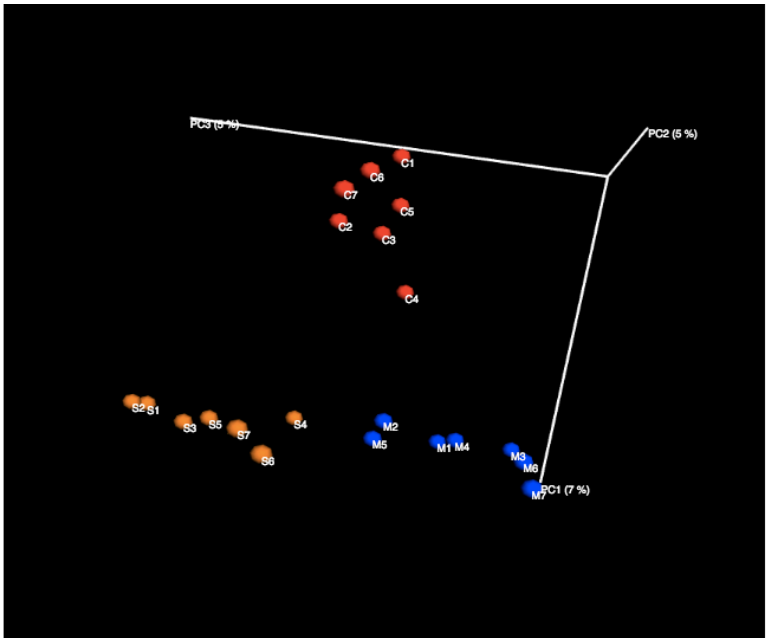


Figure 2.8: **PCoA of soil samples collected from KBS**

error rate(1%,2% and 0%). Table 2.1

Table 2.1: **Simulated sequencing reads data sets of e.coli**

sample	coverage	error rate
A	150	0.01
B	50	0.01
C	50	0.01
D	50	0.02

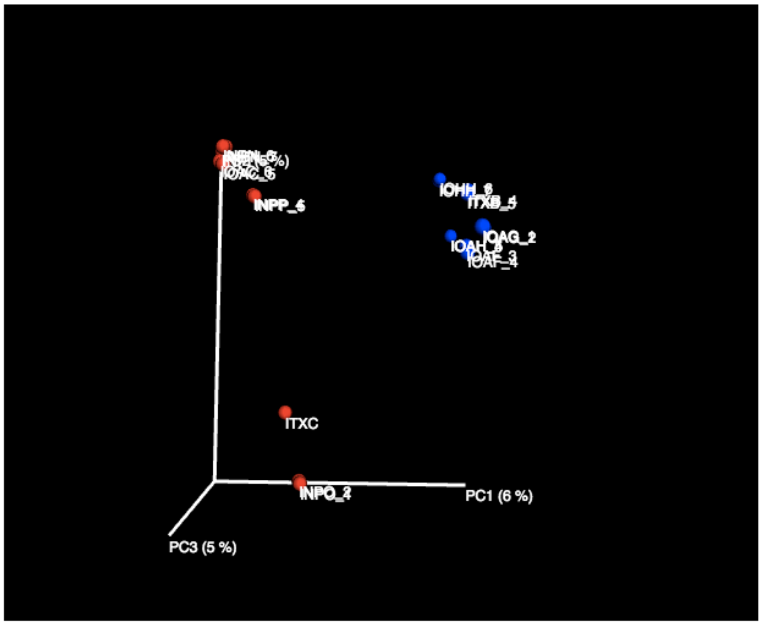


Figure 2.9: **PCoA of soil samples collected from Amazon rainforest**

Table 2.2: GPGC Data sets

sample	# of reads	size of .gz file	# of bps	ave. length
iowa corn	1514290825	46G	144202427079	95.2
iowa prairie	2597093273	74G	226815059143	87.3
kansas_corn	2029883371	66G	206933829048	101.9
kansas_prairie	0	145G	0	0
wisconsin_corn	1616440116	51G	162257698471	100.4
wisconsin_prairie	1653557590	53G	166467901724	100.7
wisconsin_restored	226830595	11G	34241520930	151.0
wisconsin_switchgrass	310966735	13G	40259619921	129.5

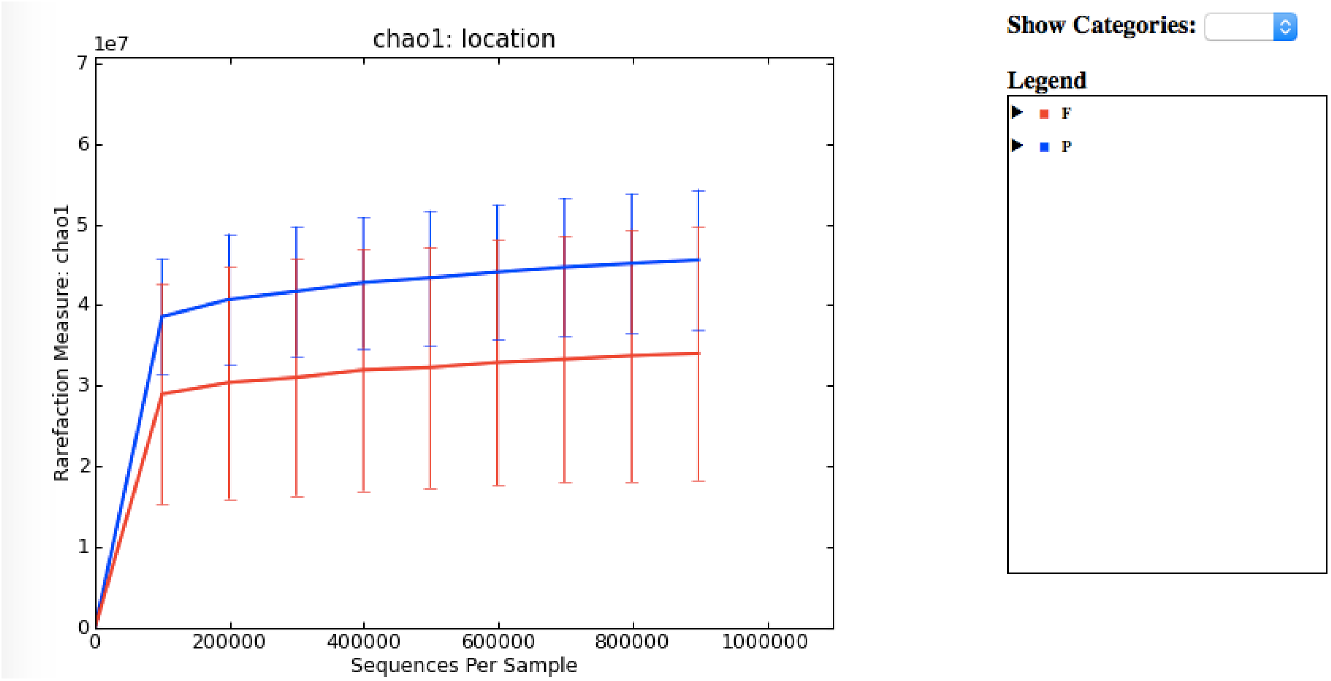


Figure 2.10: **PCoA of soil samples collected from Amazon rainforest**

Chapter 3

Conclusion

3.1 Summary

3.2 Future Work