Integration of Clustering and Multidimensional Scaling to Determine Phylogenetic Trees as Spherical Phylograms Visualized in 3 Dimensions

Yang Ruan1, Geoffrey House2,Saliya Ekanayake1, Ursel Schütte2,3, James Bever2, Haixu Tang1,2, Geoffrey Fox1

1School of Informatics and Computing

2Department of Biology

3School of Public and Environmental Affairs

Indiana University

Bloomington, Indiana, USA

{yangruan, glhouse, sekanaya, xxx, xxx, hatang, gcf}@indiana.edu

*Abstract*— Phylogenetic analysis is commonly used to analyze genetic sequence data from fungal communities, while ordination and clustering techniques commonly are used to analyze sequence data from bacterial communities. However, few studies have attempted to link these two independent approaches. In this paper, we propose a method, called spherical phylogram (SP) to display the phylogenetic tree within the clustering and visualization result. In comparison with traditional tree displaying methods, the correlations between the tree and clustering could be observed directly. In addition, we propose an algorithm called interpolative joining (IJ) to construct and visualize the SP in either 2D or 3D space. In the experiments, we used edge sums to quantify the general fit between the clustering and phylogenetic tree in SP and Mantel tests to determine how well the same grouping of sequences was preserved between the clustering and the SP. Our results show that this clustering and visualization method has similar accuracy as a phylogenetic tree generated using maximum likelihood in classifying the fungal sequences, while having much lower computational cost.

Keywords—Phylogenetic Tree; Multidimensional Scaling; Microbial Communities; Environmental Genomics

# Introduction

The increasing use of high-throughput DNA sequencing techniques to identify microbial communities in the environment has led to a dramatic increase in the size of DNA sequence datasets. The analysis and visualization of these large sequence datasets is a challenge that studies of bacterial diversity and those of fungal diversity have generally approached in different ways. Studies using bacterial DNA sequences typically use clustering approaches such as mothur [[1](#_ENREF_1)]{Schloss, 2009 #1}, ESPRIT [[2](#_ENREF_2)], or UPARSE [[3](#_ENREF_3)] to group DNA sequences from a sample into operational taxonomic units (OTUs) based on a minimum sequence similarity value (similarity-threshold clustering). The differences between OTUs can also be visualized using multidimensional scaling [[4](#_ENREF_4), [5](#_ENREF_5)]. In contrast, studies using fungal DNA sequences have typically used phylogenetic analysis in order to identify groups of similar sequences to visualize the relationships between sequences in the same dataset, and to make inferences about their evolutionary history [[6](#_ENREF_6)].

However there are important limitations to both similarity-threshold clustering techniques and the phylogenetic analysis techniques. Clustering algorithms that use heuristic methods and pairwise sequence alignment (PWA) are computationally faster than creating phylogenetic trees, especially for large numbers of sequences, because they do not require multiple sequence alignment (MSA). Clustering results also allow the clear visualization of extremely large datasets directly. This avoids the frequent need in phylogeny-based analysis to first reduce the number of sequences in the dataset in order to facilitate computation and clear visualization of the resulting trees. However the clustering results cannot infer the evolutionary relationships between sequences that phylogenetic trees can. Also, both methods frequently reduce the size of the dataset being analyzed: for similarity-threshold clustering this reduction is by design through the use of consensus sequences representing each OTU, which are meant to facilitate the visualization and taxonomic identification of the sequences in each cluster; for phylogenetic trees of large datasets, this reduction in the number of sequences is frequently by necessity to allow the computation and clear visualization of the resulting trees.

Here we use a multidimensional scaling (MDS) technique to visualize sequence similarity among all sequences in a dataset as a way to determine clusters of similar sequences directly, without the need to define a sequence similarity-threshold (we will refer to this method as MDS clustering). Because MDS clustering allows the visualization of sequence similarity of extremely large datasets directly, it is a promising technique for determining sequence clusters from high throughput sequencing. However, it is unclear how accurately groups of similar sequences found with MDS clustering correspond with defined taxonomic groups. In order to evaluate the taxonomic accuracy of groups identified with MDS clustering, we combine a computationally efficient clustering pipeline called deterministic annealing clustering and interpolative dimension reduction (DACIDR) [[7](#_ENREF_7)] and a phylogenetic tree created using maximum likelihood based on the same sequence dataset. DACIDR uses MDS clustering for robust and scalable sequence clustering and visualization for over millions of sequences. It is parallelized to process large datasets on multiple compute nodes, using MapReduce [[8](#_ENREF_8)] (MapReduce reference), iterative MapReduce [[9](#_ENREF_9)](Twister reference) and/or MPI frameworks [[10](#_ENREF_10)]. We improved the parallel efficiency of DACIDR by developing a hybrid workflow model to run it under different cluster environments [[11](#_ENREF_11)].

As input for this combined method, we used sequences from the variable D2 domain of the 28S rRNA gene, which is commonly used for taxonomic identification of fungi [[12](#_ENREF_12)]. All sequences were from species of arbuscular mycorrhizal (AM) fungi because they exhibit a large amount of sequence variation both between species as well as within species [[13](#_ENREF_13)], which can make them challenging to analyze [[14](#_ENREF_14)]. The sequence datasets were derived from a combination of: (1) a large-scale AM fungal phylogenetic study [[14](#_ENREF_14)]; (2) additional sequences obtained from GenBank to increase the taxonomic coverage of the dataset; (3) representative 454 sequences from spores of known AM fungal species, where these representative sequences were generated by using DACIDR on over 400k sequences. A more detailed description of this work will be presented in a later paper.

To compare the consistency between the clustering analysis and the phylogenetic tree, we implemented an algorithm we refer to as interpolative joining (IJ) in order to generate a phylogenetic tree called spherical phylogram (SP) using both the clustering result and the traditional phylogenetic tree. To evaluate how well the SP corresponded to the clustering result from the same dataset, we used a combination of edge sums and Mantel tests in our experiments. The different experimental approaches generated similar results that show good agreement between the taxonomic delineations provided by the clustering and those provided by the phylogenetic analysis. This suggests that our proposed clustering technique based on pairwise alignment is a highly suitable alternative to phylogenetic analysis to study microbial communities.

The structure of the paper is organized as follows: Section II discusses existing methods for phylogenetic tree visualization and sequence clustering pipelines; Section III discusses the methods we used for our phylogenetic tree reconstruction, sequence clustering and visualization. Section IV introduces and explains the proposed algorithm for interpolating a phylogenetic tree onto the clustering results; In Section V, we present our experimental result and compare our proposed methods to existing tree generation methods; Followed by our conclusion and future work in Section VI.

# Related work

There are many different existing clustering algorithms, such as greedy heuristic methods, hierarchical clustering, Bayesian clustering, phylogenetically-aware clustering methods, and MDS clustering demonstrated in this paper.

Greedy heuristic methods define seed sequences to represent the clusters it found and compare them with all remaining sequences in order to avoid quadratic time complexity. CD-HIT [[15](#_ENREF_15)] and UCLUST [[16](#_ENREF_16)] are very well known heuristic clustering methods. They can be very fast to cluster large scale of data, but these algorithms overestimate or underestimate the number of clusters since the similarity threshold is very sensitive with large dataset. Hierarchical clustering also uses a greedy algorithm, but it takes a more structured approach to generating clusters by comparing each additional sequence to all of the sequences already in the cluster [[17](#_ENREF_17)]. Mothur and ESPRIT are two popular hierarchical clustering methods, but they suffered from quadratic time and space complexity. Bayesian clustering uses probabilistic approach, which makes it robust to sequencing errors. CROP uses this approach to define clusters based on the sequence variation that is inherent in the dataset [[18](#_ENREF_18)]. It has increased clustering accuracy compared to Mothur and ESPRIT when the sequence divergence between clusters is large [[19](#_ENREF_19)], which we would not expect to be the case with AM fungi [[13](#_ENREF_13)]. Other clustering methods, such as GMYC [[20](#_ENREF_20)] and PTP [[19](#_ENREF_19)], do not require defined sequence similarity thresholds. GMYC uses a maximum likelihood approach to determine the transition point between sequence changes representing speciation events and those representing coalescent events [[21](#_ENREF_21), [22](#_ENREF_22)]. PTP is computationally faster than the GMYC method while also achieving increased clustering accuracy [[19](#_ENREF_19)]. It estimates species clusters using a maximum-likelihood phylogenetic tree produced using the sequences as a guide instead of the coalescent tree, and assumes that each nucleotide substitution has a fixed probability of being the basis for a speciation event [[19](#_ENREF_19)]. The PTP is able to give accurate species determinations regardless of the amount of sequence similarity between the species being compared. However both of these methods require either multiple sequence alignment or a guide phylogenetic tree in order to cluster sequences, and therefore are computationally more costly than a clustering algorithm like DACIDR that uses pairwise sequence alignment.

Many MDS algorithms have been proposed in the past few decades. Newton's method is a simple solution to minimize the STRESS in (1) and SSTRESS in (2)[[23](#_ENREF_23)]. But it uses Hessian to form a basic Newton iteration, and the Hessian construction requires cubic time complexity. Quasi-Newton [[24](#_ENREF_24)] method is proposed to reduce the time complexity of Newton method to sub-cubic by approximating Heissian. Multi-Grid [[25](#_ENREF_25)] MDS (MG-MDS) has been proposed to solve the isometric embedding problems. The performance was increased dramatically compared to other existing methods because of the parallelization. Scaling by Majorizing a Complicated Function (SMACOF) algorithm is one of the MDS algorithms that have been proved to be fast and efficient [[26](#_ENREF_26)]. Another way of solving MDS problem is to treat it as a chi-square problem. And Levenberg–Marquardt (LMA) [[27](#_ENREF_27)] algorithm is a popular curve fitting function to solve that. However, due to the non-linear property of this problem, both of these algorithms could be trapped under local optima. Simulated Annealing and Genetic Algorithm have been used to avoid the local optima in MDS [16] [17]. However, they suffered from long running time due to their Monte Carlo approach. DA-SMACOF [[28](#_ENREF_28)] is then proposed to reduce the time cost find global optima by using deterministic annealing [[29](#_ENREF_29)]. But it assumes all weights equal to one for the input distance matrix. So [[30](#_ENREF_30)] added weighting function to the SMACOF function, called WDA-SMACOF. It uses Conjugate Gradient to avoid the cubic time complexity brought by weighting and matrix inversion, so that it can converge under *O(N2).*

The methods used for phylogenetic tree creation have become more standardized compared to clustering techniques. The most commonly accepted methods are probabilistic approaches including maximum likelihood such as RAxML [[31](#_ENREF_31)] and Bayesian methods such as Mr. Bayes [[32](#_ENREF_32)]. Because both of these methods incorporate uncertainty into phylogenetic tree construction, they are thought to provide phylogenies that are closely aligned with actual patterns of evolutionary history. Neighbor Joining is a classic method [[33](#_ENREF_33)], but not as commonly used as the other two methods nowadays.

# Phylonenetic Tree and clustering

In this section, we discuss the methods we used to generate the phylogenetic tree and the clustering and visualization result. Both of these outputs required sequence alignment beforehand. We did multiple sequence alignment (MSA) for phylogenetic tree and both MSA and pairwise sequence alignment (PWA) for the clustering. We created the phylogenetic tree using 100 iterations in RAxML (version 7.4.2, Stamatakis 2006) with the general time reversible (GTR) nucleotide substitution model and with gamma rate heterogeneity (GTRGAMMA). The clustering result was generated using deterministic annealing (DA) based multidimensional scaling (MDS) with on the all pair distance matrix. Note that we only describe part of the DACIDR pipeline used in this particular paper.

## Sequence Alignment

As mentioned previously, we were using both MSA and PWA. MSA is used for three or more sequences and it is usually more computational complex than PWA. And it is commonly used in phylogenetic analysis so we chose this method to generate input for RAxML. PWA aims to find an overlapping region of the given two sequences that has the highest similarity as computed by a score measure. The overlap may either be defined over the entire length or over a portion of the two sequences. The former is known as global alignment and latter as local alignment. Needleman-Wunsch (NW) [[4](#_ENREF_4)] and Smith-Waterman Gotoh (SWG) [[34](#_ENREF_34)] are two popular algorithms performing these alignments respectively.



Figure 1 Illustration of Sequence alignment

Figure 1 shows a general sequence alignment with possible end gaps (note a local alignment will not result end gaps). We consider the region excluding end gaps as the aligned region. Pairs of boxes with the same color indicate a match and others indicate mismatches. Pairs with one box and one dash indicate a character being aligned with a gap. Two parameters governing NW and SWG are scoring matrix and gap penalties, namely gap open (GO) and gap extension (GE). Alignment algorithm maximizes a score measure calculated as in Figure 2.

The choice of alignment algorithm may depend on the particular dataset and in certain cases it is possible to result in alignments, which are optimal from the algorithm’s point of view, but has little practical value [[35](#_ENREF_35)].

|  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | A | T | C | G |
| A | 5 | -4 | -4 | -4 |
| T | -4 | 5 | -4 | -4 |
| C | -4 | -4 | 5 | -4 |
| G | -4 | -4 | -4 | 5 |

 | GO = -16 GE = -4 |
| T C A A C C A -T T - - - C T G 5 -4 -16 -4 -4 5 -4 -16  |
| $$S=5+\left(-4\right)+\left(-16\right)+\left(-4\right)+\left(-4\right)+5+\left(-4\right)+\left(-16\right)$$$$= -38$$ |

**Figure 2 Score of an alignment**

## All Pair Distance Calculation

We align each pair of sequences and compute a distance for each such alignment resulting an all-pairs distance matrix. This serves as the input for remaining algorithms in DACIDR pipeline. It is possible to define different distance measures [[35](#_ENREF_35)] for an alignment and we have chosen percent identity (PID) as the distance in this analysis.

Given the alignment between two sequences, let the number of matching pairs in aligned region be $N'$ and the total number pairs in the aligned region be $N$. The PID distance, $δ\_{PID}$, is then computed as given below.

|  |  |
| --- | --- |
| $δ\_{PID}=1.0-\frac{N'}{N}$  | (1) |

## Multidimensional Scaling with Deterministic Annealing

MDS is a set of techniques used in dimension reduction. It is used to mapping original high dimensional data into target dimension space while preserving the proximity observed in the original dimension space as much as possible. Given a target dimension $L$, the mapping of points in *L-dimension* can be given by an $N×L$ matrix $X$, where each point in the target dimension space is represented as the $i$th row in $X$. It is a non-linear optimization problem and the object function that MDS is trying optimize is given as the following:

|  |  |  |
| --- | --- | --- |
|  | $ σ\left(X\right)=\sum\_{i<j\leq N}^{}w\_{ij}(d\_{ij}(X)-δ\_{ij})^{2}$  | (2) |
|  | $σ\left(X\right)=\sum\_{i<j\leq N}^{}w\_{ij}(d\_{ij}^{2}(X)-δ\_{ij}^{2})^{2 }$  | (3) |

where *w* denotes a possible weight, $d\_{ij}\left(X\right)$ is the Euclidean distance from point *i* to *j* in the mapping and $δ\_{ij}$ is the original distance from point *i* to *j.* This object function is also referred as STRESS or SSTRESS [[23](#_ENREF_23)]. Note that the original pairwise distance matrix, denoted as $Δ$ must follows the rules: (1) Symmetric: $δ\_{ij}= δ\_{ji}$. (2) Positivity: $δ\_{ij}>0$. (3) Zero Diagnosal: $δ\_{ii}=0$. We use WDA-SMACOF [[30](#_ENREF_30)] for our MDS clustering since it can avoid local optima by using DA. DA [[29](#_ENREF_29)] is an annealing process that finds the global optima of an optimization process instead of local optima by adding a computational temperature to the target object function. By lowering the temperature during the annealing process, the problem space gradually reveals to the original object function. It uses an effective energy function, which is derived through expectation and is deterministically optimized at successively reduced temperatures.

|  |  |  |
| --- | --- | --- |
|  (a) The cladogram of a tree with 5 nodes | (b) The leaf nodes of the tree in 2D space after dimension reduction | **(c) The tree in 2D space after interpolation of the internal nodes** |
| Figure 3 The illustration of a phylogenetic tree in a 2D space |

##  Parallezation of Pipeline

We have improved the efficiency of the parallelization of the pipeline by using a hybrid MapReduce workflow management system [[11](#_ENREF_11)]. Because all-pair distance calculation is a task independent application, so we used Hadoop [[36](#_ENREF_36)] for its parallelization inside the workflow. However, it is well-known that Hadoop has a large overhead while running iterative parallel applications, such as MDS applications. Therefore, to avoid that extra computational cost, we use Twister [[9](#_ENREF_9)], which is an iterative MapReduce framework for parallelization of WDA-SMACOF. The detailed parallelization can be found in [[7](#_ENREF_7)] and [[30](#_ENREF_30)]. Finally, since this entire workflow is written in JAVA, so it is easy to migrate it to either an HPC cluster or to a Cloud environment.

# Phylogenetic Tree Display with Clustering

As mentioned previously, by using DACIDR, each sequence is represented as a point in the target dimension space, i.e. the 3D space. Also, by using RAxML, all the sequences are represented as leaf nodes in phylogenetic tree. Therefore each leaf node in the phylogenetic tree corresponds to a point in the 3D dimension reduction result. However, traditional tree display software, such as MEGA5 [[37](#_ENREF_37)] and FigTree [[38](#_ENREF_38)] only display trees separately from the clustering result, so it is difficult to observe the relationships between the phylogenetic tree and the clustering result.

 In this section, we proposed a method, called Interpolative Joining (IJ) to display an existing phylogenetic tree by using the clustered sequences from the same, dataset as leaf nodes of the tree. This allows for direct visual comparison between the phylogenetic tree and the sequence clusters. The generated tree can be in either 2D or 3D depends on the target dimension. And it is referred either circular phylogram in 2D or spherical phylogram (SP) in 3D. In our study, as our target dimension is 3D, the generated tree will be referred as SP in this paper.

## Distance Calculation

The internal nodes cannot be directly observed because they represent hypothetical ancestor sequences, and therefore the distances from internal nodes to leaf nodes of the generated phylogenetic tree are unknown. By using RAxML, it is possible to calculate distance from an internal node to another node by using the summation over all the edges between them. For example, in figure 3(a), the distance between point C and E can be calculated by summing over edge(C, B), edge(B, A) and edge(A, E). This distance calculation can generate a pairwise distance matrix for all the nodes in the based on all the edge lengths. However, this edge sum method does not work to find the distance between pairs of leaf nodes since the pairwise distances between leaf nodes are already known from the MDS clustering results. For example, the distance between leaf node C and D shown in figure 3(b) is clearly not equal to edge(B, C) + edge(B, D). Therefore if the summation over the edges is used for defining distances during interpolation, the result will have a high bias because different distances were used for leaf nodes. Therefore, we chose the distance calculation method used in neighbor joining (NJ) algorithm to calculate the distances between internal nodes based on the existing distances between leaf nodes so that all distances used for visualization are consistent.

The NJ algorithm starts with a completely unresolved tree, whose topology corresponds to that of a star network, and ends once the tree is completely resolved and all branch lengths are known. The core idea of this algorithm is to find a way of constructing a tree that follows the balanced minimum evolution (BME) criterion, which generates the optimal tree topology and minimizes the edge lengths of the tree. And our algorithm IJ used the same strategy to interpolate the phylogenetic tree based on MDS clustering result to generate SP, so that the SP will has a minimum total branch lengths. Nevertheless, if the SP matches the original phylogenetic tree better, the sum of all the edges will be shorter.

The distance calculation used in IJ is similar to the one used NJ, and it can be formulated according to the following: suppose we have *n* existing points, denoted as $P=\{p\_{1},p\_{2},p\_{3},…,p\_{n}\}$. And a point $p\_{i}$can be represented as a vector $[x\_{i1},x\_{i2}, … ,x\_{iL}]$ in *L-dimension*. The distance between two points $p\_{i}$ and $p\_{j}$is denoted as $d(p\_{i}, p\_{j})$ and can be calculated as Euclidean distance using the following equation:

|  |  |  |
| --- | --- | --- |
|  | $$d\left(p\_{i}, p\_{j}\right)=\sqrt{\sum\_{l=1}^{L}(x\_{il}-x\_{jl})^{2}}$$ | (4) |

Given any two points $p\_{i},p\_{j}\in P$, there are two corresponding leaf nodes in the phylogenetic tree. Their parent is denoted as a new point $\hat{p}$ that can be interpolated into the target dimension space. The distance from $\hat{p}$ to $p\_{i}$ and $p\_{j}$ can be given in following equations:

|  |  |
| --- | --- |
| $$ d\left(\hat{p}, p\_{i}\right)=\frac{1}{2}d\left(p\_{i}, p\_{j}\right)$$$$+\frac{1}{n-2}\sum\_{k=1}^{n}(d\left(p\_{i},p\_{k}\right)-d\left(p\_{j},p\_{k}\right))$$ | (5) |

Because all of the distances follows three basic rules for $Δ$ mentioned in Section III(C). Thus all distances are symmetric, i.e. $d\left(p\_{i}, p\_{j}\right)=d\left(p\_{j}, p\_{i}\right)$, and $d\left(\hat{p}, p\_{i}\right)$ can be calculated as

|  |  |  |
| --- | --- | --- |
|  | $$d\left(\hat{p}, p\_{j}\right)=d\left(p\_{i}, p\_{j}\right)-d\left(\hat{p}, p\_{i}\right)$$ | (6) |

The distances from $p$ to all other points, except pi and pj, can be obtained using the following equation where $1 \leq k \leq n$ where $k \ne i$ and $k \ne j$:

|  |  |
| --- | --- |
| $$d\left(\hat{p}, p\_{k}\right)=\frac{1}{2}(d\left(p\_{i},p\_{k}\right)+d\left(p\_{j},p\_{k}\right)-d\left(p\_{i}, p\_{j}\right))$$ | (7) |

Note that equation (4) is Euclidean distance calculation and equation (5) to equation (7) were calculation of the minimum evolution path for any given two points in *P*, so that for any internal nodes in the phylogenetic tree, its distances to all other points can be obtained using the equations above.

## Interpolation

When the distances from the internal nodes to all other points are obtained, we can then interpolate the internal node as a point into the target dimension space. The interpolation [[39](#_ENREF_39)]was first introduced into the fields of data visualization and clustering to solve the large-scale data problem (ref), also referred as the *in-sample* and *out-of-sample* problem. First, the original input dataset is split into two parts, one is called the *in-sample* dataset, and the other one is referred to as the *out-of-sample* dataset. Then a clustering or dimension reduction algorithm with a high accuracy can be applied on the *in-sample* dataset to generate the *in-sample* result. Based on the *in-sample* result, an interpolation algorithm with lower time and space cost can be used to generate the result from the *out-of-sample* dataset. The tradeoff of this method is that the interpolation algorithm usually has a lower accuracy then the algorithm applied on the *in-sample* dataset.

In our case, the phylogenetic tree’s leaf nodes corresponding points in the 3D space are the *in-sample* data, denoted as *P*, and the points represent internal nodes are the *out-of-sample* data, denoted as $\overbar{P}$. By using equations (4) to (7), the distance of an *out-of-sample* point $\hat{p}$ to all other *in-sample* points is calculated as the original distance for interpolation, which is denoted as $\hat{Δ}$. After $\hat{p}$ is interpolated to *L-dimension*, it can be represented as a vector $\hat{x}$ with length *L*. Nevertheless, the *in-sample* points and *out-of-sample* points in the *L-dimension* can be defined as $X=\{X\_{1}, X\_{2}\}$, where $X\_{1}=\left\{x\_{1},x\_{2},x\_{3},…,x\_{N}\right\}$ and $X\_{2}=\left\{x\_{N+1}\right\}$.

The distance from $\hat{p}$ to all other points can be obtained using equation (1), which is the Euclidean distance in 3D space, denoted as *d(X)*. So for each *out-of-sample* point $\hat{p}$, there is a difference between the Euclidean distance in the *L-dimension* and the original distance, and the object function is given by the following:

|  |  |  |
| --- | --- | --- |
|  | $$σ\left(X\right)=\sum\_{i\leq N}^{}w\_{i\hat{x}}\left(d\_{i\hat{x}}\left(X\right)-δ\_{i\hat{x}}\right)^{2}$$ | (8) |

The goal of interpolation is to minimize the STRESS value for each of the given *out-of-sample* point. So that each *out-of-sample* point can be interpolated to a place where the original distances differs least compared to *L-dimension* distances. WDA-MI-MDS is a robust iterative algorithm that can interpolate *out-of-sample* points into the target dimension space one by one (ref). For every *out-of-sample* point, the algorithm finds a majorizing function for equation (8), and by using the estimated value of $\hat{x}$ in previous iteration, it can guarantee a non-increasing STRESS value for $\hat{p}$ as the number of iterations increases. Additionally, it can avoid possible local optima for STRESS function by using DA. The detailed equations for this algorithm can be found in [[30](#_ENREF_30)].

## Tree Generation

Equation (4) and equation (7) gives the distance calculation formulas for the internal nodes, which is also referred to as the *out-of-sample* point in previous section. And equation (8) gives the STRESS value of using interpolation for the internal nodes. For each internal node, WDA-MI-MDS can be applied to find its location in the target dimension space. However, not all internal nodes from the phylogenetic tree were selected only based on the leaf nodes. Since in traditional *out-of-sample* problems, the *in-sample* dataset remains the same during interpolation, it is not applicable to use those kinds of algorithms for internal nodes interpolation. Figure 3(c) gives an example of how the internal nodes are interpolated during neighbor joining. Node A is interpolated based on node E and node B, which is also an internal node for the entire phylogenetic tree shown in Figure 3(a).

**Algorithm 1 Interpolative Joining algorithm**

Input: $P$, $\overbar{P}$, $T$, $\overbar{T}$

For each pair of siblings ($t\_{i}$,$t\_{j}$) in *T*

 Find their parent $\hat{t}$ in $\overbar{T}$

 Find point$p\_{i}$ and $p\_{j}$ in *P*

 For other point $p\_{k}$ in *P*

 Compute $d\left(p\_{k}, p\_{i}\right)$, $d\left(p\_{k}, p\_{j}\right)$ using (4)

 End for

 Compute $d\left(\hat{p}, p\_{i}\right)$and $d\left(\hat{p}, p\_{j}\right)$using (5) and (65)

 For other point $p\_{k}$ in *P*

 Compute $d\left(\hat{p}, p\_{k}\right)$ using (7)

 End for

 Use (8) as object function and WDA-MI-MDS to compute $\hat{p}$

 Remove $t\_{i}$ and$t\_{j}$ from *T*

 Add $\hat{t}$ into *T* and remove $\hat{t}$ from $\overbar{T}$

 Add $\hat{p}$into *P* and remove $\hat{p}$ from $\overbar{P}$,

End for

Return P

To solve that problem, we proposed an algorithm called Interpolative Joining (IJ). In IJ, the *in-sample* dataset needs to be modified during the interpolation process. Because the *out-of-sample* points are interpolated one by one, each *out-of-sample* point that is already interpolated is added into the *in-sample* dataset and will be considered as an *in-sample* point for subsequent *out-of-sample* points. To do this, the IJ algorithm searches the tree from the bottom up. Every time two leaf nodes are found that share the same parent, those two leaf nodes are used to calculate the coordinates for the internal node. The two leaf nodes will then be removed from the tree, and the newly interpolated internal node will be considered a new leaf node. This is demonstrated in figure 3(c), point C and D are discard from the leaf node set once node B is interpolated. However, these two *in-sample* points, which correspond to the two leaf nodes, will remain in the *in-sample* dataset. Therefore, the total number of nodes for the input phylogenetic tree will be decreasing and the size of the *in-sample* dataset will be increasing during the interpolation process.

In formal definition, $P$ and $\overbar{P}$ are used in terms of *in-sample* and *out-of-sample* points in *L-dimension*; $T$ is the set of leaf nodes and $\overbar{T}$ is the set of internal nodes from the phylogenetic tree. Therefore $p\_{i}$ is the representation of $t\_{i}$ in the target dimension space. For each pair of leaf nodes $t\_{i}$ and $t\_{j}$ that have the same parent $\hat{t}$, there is a pair of *in-sample* points which are denoted as point$p\_{i}$ and $p\_{j}$ in *P* that represents them. Immediately after $\hat{t}$ is found, the $\hat{p}$ that represents it is initialized as a random point and added into $\overbar{P}$. After $\hat{t}$ is interpolated into the *L-dimension* space, $\hat{p}$ is removed from $\overbar{P}$ and added into *P*. The $t\_{i}$ and $t\_{j}$ will be removed from *T*, and $\hat{t}$ is added into *T* and removed from $\overbar{T}$. Nevertheless, $\overbar{P}$ will always contain only one *out-of-sample* point during each iteration, where the iteration number equals the number of internal nodes in $\overbar{T}$ at the beginning. The detailed process of IJ is illustrated in Algorithm 1. As calculation of Euclidean distance and WDA-MI-MDS is very fast, to generate the SP with a predefined phylogenetic tree and MDS clustering result only takes a few seconds on a single core.

# Experiments

The experiments were carried out on BigRed II, which is a hybrid cluster with a total number of 344 CPU nodes and 676 CPU/GPU nodes at Indiana University. Each CPU node contains two AMD Opteron 16-core Abu Dhabi x86\_64 CPUs and 64 GB of RAM. It’s a Cray Linux Environment and we used the cluster compatibility mode to process the data with the help of Twister and Hadoop. The clustering and visualization of the sequence datasets was completed using DACIDR. We created a maximum likelihood unrooted phylogenetic tree from the multiple sequence alignment (MSA) with RAxML (Stamatakis 2006) using 100 iterations with the general time reversible (GTR) nucleotide substitution model and with gamma rate heterogeneity (GTRGAMMA). We then used the tree to guide the generation a pairwise distance matrix between all sequences in each of the two MSA datasets using RAxML. These pairwise distance matrices were then used as the reference when testing for the effect of alignment technique and sequence length on consistency between clustering and phylogeny. Finally, the IJ was run on local machine to generate spherical phylogram (SP). And the SPs were displayed using a data visualization software called PlotViz3 [[40](#_ENREF_40)].

|  |
| --- |
| Figure 4 The comparison using Mantel between distances generated by three sequence alignment methods and RAxML |

##  Obtaining sequences

 We first downloaded the sequence alignment of AMF sequences from a recent large-scale phylogeny of AMF (Krüger et al. 2012) and only retained sequences that contained at least a portion of the 28S rRNA gene. We then collected two sets of additional AM fungal sequences: (1) sequences from GenBank that had confident species attribution in order to supplement the species coverage within the sequence dataset; (2) representative sequences for known AM fungal species obtained from spores using 454 sequencing (Roche, Indianapolis, IN) of the variable and phylogenetically informative D2 domain of the 28S rRNA gene. We applied DACDIR on this dataset to find 126 clusters and then picked a representative sequence for each cluster as part of the dataset. The additional sequences from GenBank were added to the original sequence alignment from [[14](#_ENREF_14)] using MAFFT [[41](#_ENREF_41)]. In order to evaluate how different sequence lengths affected the correspondence between phylogenetic trees and clustering, we then created two datasets with sequences that shared the same starting location on the 28S rRNA gene: one dataset contained longer sequences, and the other contained shorter sequences. We first trimmed the MSA and only retained the unique sequences that spanned an extended region beyond the D2 domain (dataset 1, roughly 675 bases long without gaps); then from that subset we retained only the unique sequences that spanned the 454 sequencing start site and the average end position of the 454 sequences (roughly 425 bases long without gaps). Finally, we added the representative 454 sequences to this trimmed alignment using MAFFT as described above to create dataset 2. This gave a MSA for dataset 1 (999nts) with: 801 sequences from [[14](#_ENREF_14)] and 505 sequences from GenBank for a total of 1306 sequences, and for dataset 2 (599nts with 454 optimized) with: 514 sequences from [[14](#_ENREF_14)], 380 sequences from GenBank, and 126 representative 454 sequences for a total of 1020 sequences. We note that DACIDR is capable of efficiently clustering millions of sequences [[7](#_ENREF_7)], but for this phylogenetic comparison test we selected a smaller set of sequences that still represents the expected range of genetic variability within AM fungi.

|  |  |
| --- | --- |
| Figure 5 Maximum likelihood phylogenetic tree from dataset 2 that is collapsed into clades at the genus level as denoted by colored triangles at the end of the branches. Branch lengths denote levels of sequence divergence between genera and nodes are labeled with bootstrap confidence values. 454 sequences from spores that are not part of another clade are denoted with the label ‘454 sequence from spore’. Two sequences in the *Claroideoglomus* clade are instead attributed to *Rhizophagus*, and one sequence in the *Funneliformis* clade is instead attributed to *Septoglomus* (denoted by arrows at the blunt end of the colored triangles). This figure is generated by FigTree. |  |
| **(a) Multiple sequence alignment (MSA) result** |
|  |
| **(b) Smith-Waterman pairwise alignment (SWG) result** |
|  |
| **(c) Needleman-Wunsch pairwise alignment (NW) result** |
| E:\Sali\InCloud\Dropbox\PhylogeneticTreewithClustering\invisible_square.pngFigure 6 The screenshots of spherical phylogram for using the phylogenetic tree shown in Figure 5 with three different sequence alignments. The colors of the branches in these figures are as same as the colors of the branches shown in Figure 5. |

## Sequence Alignment Comparison

We used the Mantel test in order to evaluate whether pairs of experimental treatments retained the same structure of sequence differences between them.

Mantel tests determine whether a correlation between the entries contained in two different pairwise distance matrices is statistically significant by permuting the distance matrices to obtain an empirical *p-*value for the correlation. The treatments consisted of different alignment techniques applied to each of the two different length datasets; comparisons were then made to the RAxML distance matrix from the same dataset. The Mantel tests were performed using the *vegan* package in R (version 3.0.2, R Core Team 2013), and none of the tests had *p*-values greater than 0.001, suggesting all of the measured correlations were likely significant despite the increased type I error (false-positive) rate that can occur with Mantel tests [[42](#_ENREF_42)].

|  |  |
| --- | --- |
| **(a) Edge sum of the SP generated in 3D space on** **599nts dataset optimized with 454 sequences** | **(b) Edge sum of the SP generated in 3D space on** **999nts dataset** |
| **(c) Correlation between the distances generated in 3D space and RAxML on 599nts dataset optimized with 454 sequences from Mantel test** | **(d) Correlation between the distances generated in 3D space and RAxML on 999nts dataset from Mantel test** |
| **Figure 7 The edge sum and Mantel comparison of three different MDS methods using distance input** **generated from three different types of sequence alignments on two dataset** |

Figure 4 illustrates the result of the Mantel test applied on MSA and the Pairwise Sequence Alignment (PWA) which includes both the Smith Waterman Gotoh (SWG) and Needle-Wunsch (NW). Using longer sequences (dataset 1) consistently resulted in higher correlations between the reference distance matrix and either of the pairwise alignment techniques. However, both the SWG and the NW pairwise alignment methods gave comparable correlation values for dataset 1 and for shorter sequences (dataset 2). The very high correlations between the RAxML reference matrix and the MSA distance matrix used for MDS clustering regardless of sequence length are expected because the input alignment is identical for both matrices and only the distance calculation method is different. Using pairwise alignments for the same datasets resulted in lower correlations with the RAxML reference matrix, although they still provided a reasonably good fit.

The relationships between genera of AM fungi from the phylogenetic tree created with dataset 2 (Figure 5), was consistent with the current understanding of AM fungal phylogenetic relationships, with the exception of *Racocetra*, *Scutellospora*, and *Gigaspora* all being assigned to the same evolutionary group. By comparing the phylogenetic tree (Figure 5) and the SPs (Figure 6), it is possible to visualize the how the branches of the tree correlate with the sequences after MDS. If long branches are required in the interpolated tree in order to connect points that are the same color in the MDS visualization, then the tree does not match well with the MDS result. This is because the sequences on the same branch of the phylogenetic tree are more similar to each other than to other sequences in the dataset, and therefore they should be located close to each other in the MDS visualization as well. The SPs (Figure 6) show that the points with the same color (as color-coded from the genera in the phylogenetic tree, Figure 5), generally group together. There are a few points in the SPs using pairwise alignments (Figure 5(b) and Figure 5(c)) that have longer branches than the points from the SP using the MSA. This is consistent with the fact that the SP generated from the MSA has a better correlation with the phylogenetic tree than the SPs generated from the pairwise alignments (Figure 7(c) and Figure 7(d)). However, the SPs also verify that using pairwise alignments for the MDS generally gives a good fit with the interpolated phylogenetic tree.

## MDS method comparison

The different methods of MDS affected how well the phylogenetic tree projected using IJ matched the sequences in 3D. WDA-SMACOF is a robust MDS method that can always find the global optima, whereas EM-SMACOF can be easily trapped under local optima. The LMA usually had a result that was very similar to EM-SMACOF (Figure 7). The normalized STRESS value for each different input using the different methods was from 0.021 to 0.023, which suggests the distances after dimension reduction have a high similarity to the original distances, and therefore sequence differences were preserved well during MDS.

We use edge sums over all the branches of the phylogenetic tree in the SP and correlations from the Mantel test to evaluate the differences between these three dimension reduction methods. As mentioned before, the points of the dimensional reduction that connect to the same branches of the SP should be shorter if they match the tree better, which will result in a lower edge sum. WDA-SMACOF had a much lower edge sum compared to both LMA and EM-SMACOF (Figure 7 (a) and (b)). This is because the clusters naturally appeared when the STRESS value becomes lower, and LMA and EM-SMACOF are trapped under the local optimum, so there are some points from very small branches of the tree could still be far away from each other in the 3D space and not clustered. In contrast, WDA-SMACOF can always find the global optimum so that these points from very small branches are always converged into clusters. This is why there were not any excessively long branches for the SP plots generated by using WDA-SMACOF (Figure 6). From the Mantel test correlations (Figure 7 (c) and (d)), although WDA-SMACOF performs better than the other two methods, it shows very little difference between the three.

From Figure 7, the edge sum is a more sensitive measurement then Mantel test while evaluating the SPs. However, it also has a higher variance than Mantel test because it was calculated after IJ. On the other hand, Mantel test is more robust and shows very little differences while comparing the dimension reduction methods. Therefore, we combine the usage of edge sums and pairwise correlations from the Mantel test to demonstrate that the interpolated phylogenetic trees closely fit the MDS using WDA-SMACOF, even with pairwise alignments.

# ConclusionS and Future work

In this paper, we proposed a method called Interpolative Joining (IJ) that can be used to project existing phylogenetic trees onto a MDS clustering result in order to generate spherical phylogram (SP), which is much more efficient that traditional displaying method.

Unlike traditional clustering methods that require a similarity-threshold, the WDA-SMACOF used by DACIDR for MDS clustering uses the full range of genetic variability contained in a dataset when determining taxonomic groups because it considers each sequence separately, yet it is still computationally fast. This allows a more natural clustering approach given the inherent variability in the sequence dataset than is possible when using similarity-threshold clustering. In addition, because the taxonomic groups delimited by the MDS clustering matched those from the phylogenetic tree so closely for AM fungi, computationally slower clustering methods such as GMYC or PTP that use phylogenetic relationships to guide cluster generation may not be required for studies of genetically diverse fungi. Other than that, WDA-SMACOF can always find global optima and be scaled for large dataset. Together these characteristics make MDS clustering using DACIDR a promising option for determining taxonomic groups from the increasingly large environmental sequence datasets that are generated by high throughput sequencing.

Overall, even with the genetically diverse AM fungal DNA dataset, we found that the MDS clustering generated by DACIDR using pairwise sequence alignments accurately defined different taxonomic groups in a way that is in close agreement with a phylogenetic tree generated independently from a multiple sequence alignment of the same dataset. Therefore in our future work, the MDS clustering using DACIDR appears able to replace the traditional phylogenetic method for the taxonomic analysis of large fungal sequence datasets in studies where either: 1) evolutionary relationships are not of primary interest, or 2) the sequences represent taxonomic groups that are poorly defined in existing sequence databases, which is common when obtaining sequences from environmental samples.

Other than just displaying the representative or consensus sequences from each cluster found from the original input dataset, it is possible to display the tree with entire dataset in the 3D space with the help of IJ. And the interpolation algorithm used in DACIDR could also be improved to help identify the sequences which are poorly defined.

##### Acknowledgment

##### References

[1] P. D. Schloss, S. L. Westcott, T. Ryabin *et al.*, “Introducing mothur: Open-Source, Platform-Independent, Community-Supported Software for Describing and Comparing Microbial Communities,” *Applied and Environmental Microbiology,* vol. 75, no. 23, pp. 7537-7541, December 1, 2009, 2009.

[2] Y. Sun, Y. Cai, L. Liu *et al.*, “ESPRIT: estimating species richness using large collections of 16S rRNA pyrosequences,” *Nucleic Acids Research,* vol. 37, no. 10, pp. e76, June 1, 2009, 2009.

[3] R. C. Edgar, “UPARSE: highly accurate OTU sequences from microbial amplicon reads,” *Nat Meth,* vol. 10, no. 10, pp. 996-998, 10//print, 2013.

[4] A. Hughes, Y. Ruan, S. Ekanayake *et al.*, “Interpolative multidimensional scaling techniques for the identification of clusters in very large sequence sets,” *BMC bioinformatics,* vol. 13, no. Suppl 2, pp. S9, 2012.

[5] L. Stanberry, R. Higdon, W. Haynes *et al.*, “Visualizing the protein sequence universe,” *Concurrency and Computation: Practice and Experience*, 2013.

[6] U. Koljalg, R. H. Nilsson, K. Abarenkov *et al.*, “Towards a unified paradigm for sequence-based identification of fungi,” *Mol Ecol,* vol. 22, no. 21, pp. 5271-7, Nov, 2013.

[7] Y. Ruan, S. Ekanayake, M. Rho *et al.*, “DACIDR: deterministic annealed clustering with interpolative dimension reduction using a large collection of 16S rRNA sequences,” in Proceedings of the ACM Conference on Bioinformatics, Computational Biology and Biomedicine, Orlando, Florida, 2012, pp. 329-336.

[8] J. Dean, and S. Ghemawat, “MapReduce: simplified data processing on large clusters,” *Communications of the ACM,* vol. 51, no. 1, pp. 107-113, 2008.

[9] J. Ekanayake, H. Li, B. Zhang *et al.*, "Twister: a runtime for iterative mapreduce." pp. 810-818.

[10] J. Willcock, A. Lumsdaine, and A. Robison, “Using mpi with c# and the common language infrastructure,” *Concurrency and Computation: Practice and Experience,* vol. 17, no. 7‐8, pp. 895-917, 2005.

[11] Y. Ruan, Z. Guo, Y. Zhou *et al.*, "Hymr: a hybrid mapreduce workflow system." pp. 39-48.

[12] C. L. Schoch, K. A. Seifert, S. Huhndorf *et al.*, “Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi,” *Proceedings of the National Academy of Sciences,* vol. 109, no. 16, pp. 6241-6246, April 17, 2012, 2012.

[13] R. H. Nilsson, E. Kristiansson, M. Ryberg *et al.*, “Intraspecific ITS variability in the kingdom Fungi as expressed in the international sequence databases and its implications for molecular species identification,” *Evolutionary bioinformatics online,* vol. 4, pp. 193, 2008.

[14] M. Krüger, C. Krüger, C. Walker *et al.*, “Phylogenetic reference data for systematics and phylotaxonomy of arbuscular mycorrhizal fungi from phylum to species level,” *New Phytologist,* vol. 193, no. 4, pp. 970-984, 2012.

[15] W. Li, and A. Godzik, “Cd-hit: a fast program for clustering and comparing large sets of protein or nucleotide sequences,” *Bioinformatics,* vol. 22, no. 13, pp. 1658-1659, July 1, 2006, 2006.

[16] R. C. Edgar, “Search and clustering orders of magnitude faster than BLAST,” *Bioinformatics,* vol. 26, no. 19, pp. 2460-2461, October 1, 2010, 2010.

[17] S. M. Huse, D. M. Welch, H. G. Morrison *et al.*, “Ironing out the wrinkles in the rare biosphere through improved OTU clustering,” *Environmental Microbiology,* vol. 12, no. 7, pp. 1889-1898, 2010.

[18] X. Hao, R. Jiang, and T. Chen, “Clustering 16S rRNA for OTU prediction: a method of unsupervised Bayesian clustering,” *Bioinformatics,* vol. 27, no. 5, pp. 611-618, March 1, 2011, 2011.

[19] J. Zhang, P. Kapli, P. Pavlidis *et al.*, “A general species delimitation method with applications to phylogenetic placements,” *Bioinformatics,* vol. 29, no. 22, pp. 2869-2876, November 15, 2013, 2013.

[20] J. Pons, T. G. Barraclough, J. Gomez-Zurita *et al.*, “Sequence-Based Species Delimitation for the DNA Taxonomy of Undescribed Insects,” *Systematic Biology,* vol. 55, no. 4, pp. 595-609, August 1, 2006, 2006.

[21] J. R. Powell, “Accounting for uncertainty in species delineation during the analysis of environmental DNA sequence data,” *Methods in Ecology and Evolution,* vol. 3, no. 1, pp. 1-11, 2012.

[22] T. Fujisawa, and T. G. Barraclough, “Delimiting Species Using Single-Locus Data and the Generalized Mixed Yule Coalescent Approach: A Revised Method and Evaluation on Simulated Data Sets,” *Systematic Biology,* vol. 62, no. 5, pp. 707-724, September 1, 2013, 2013.

[23] A. J. Kearsley, R. A. Tapia, and M. W. Trosset, *The solution of the metric STRESS and SSTRESS problems in multidimensional scaling using Newton's method*, DTIC Document, 1995.

[24] C. T. Kelley, *Iterative methods for optimization*: Siam, 1999.

[25] M. M. Bronstein, A. M. Bronstein, R. Kimmel *et al.*, “Multigrid multidimensional scaling,” *Numerical linear algebra with applications,* vol. 13, no. 2‐3, pp. 149-171, 2006.

[26] I. Borg, and P. J. Groenen, *Modern multidimensional scaling: Theory and applications*: Springer, 2005.

[27] J. J. Moré, "The Levenberg-Marquardt algorithm: implementation and theory," *Numerical analysis*, pp. 105-116: Springer, 1978.

[28] S.-H. Bae, J. Qiu, and G. C. Fox, "Multidimensional Scaling by Deterministic Annealing with Iterative Majorization Algorithm." pp. 222-229.

[29] K. Rose, E. Gurewitz, and G. Fox, “A deterministic annealing approach to clustering,” *Pattern Recognition Letters,* vol. 11, no. 9, pp. 589-594, 1990.

[30] Y. Ruan, and G. Fox, "A Robust and Scalable Solution for Interpolative Multidimensional Scaling with Weighting." pp. 61-69.

[31] A. Stamatakis, “RAxML Version 8: A tool for Phylogenetic Analysis and Post-Analysis of Large Phylogenies,” *Bioinformatics*, January 21, 2014, 2014.

[32] F. Ronquist, M. Teslenko, P. van der Mark *et al.*, “MrBayes 3.2: Efficient Bayesian Phylogenetic Inference and Model Choice Across a Large Model Space,” *Systematic Biology,* vol. 61, no. 3, pp. 539-542, May 1, 2012, 2012.

[33] N. Saitou, and M. Nei, “The neighbor-joining method: a new method for reconstructing phylogenetic trees,” *Molecular biology and evolution,* vol. 4, no. 4, pp. 406-425, 1987.

[34] T. F. Smith, and M. S. Waterman, “Identification of common molecular subsequences,” *J Mol Biol,* vol. 147, no. 1, pp. 195-7, Mar 25, 1981.

[35] S. Ekanayake, *Study of Biological Sequence Clustering*, Pervasive Technology Institute, Indiana University, Bloomington, 2013.

[36] T. White, *Hadoop: The Definitive Guide: The Definitive Guide*: O'Reilly Media, 2009.

[37] K. Tamura, D. Peterson, N. Peterson *et al.*, “MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods,” *Molecular biology and evolution,* vol. 28, no. 10, pp. 2731-2739, 2011.

[38] A. Rambaut, “FigTree, a graphical viewer of phylogenetic trees,” *See* [*http://tree*](http://tree)*. bio. ed. ac. uk/software/figtree*, 2007.

[39] S.-H. Bae, J. Y. Choi, J. Qiu *et al.*, "Dimension reduction and visualization of large high-dimensional data via interpolation." pp. 203-214.

[40] J. Y. Choi, Y. Ruan, S.-H. Bae *et al.*, "PlotViz3: A cross-platform tool for visualizing large and high-dimensional data," 2010.

[41] K. Katoh, and M. C. Frith, “Adding unaligned sequences into an existing alignment using MAFFT and LAST,” *Bioinformatics,* vol. 28, no. 23, pp. 3144-3146, December 1, 2012, 2012.

[42] G. Guillot, and F. Rousset, “Dismantling the Mantel tests,” *Methods in Ecology and Evolution,* vol. 4, no. 4, pp. 336-344, 2013.