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Comparative Study on Morphological, Physiological and Molecular Genetic Diversity Analysis in Rice (*Oryza sativa* L.)

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Abstract: Genetic diversity of 21 rice varieties was analyzed in respect of 13 morphological traits, 14 physiological traits and at molecular level employing 34 Simple Sequence Repeats (SSR) markers. The Spearman's rank correlation value between ranking of morphological genetic distances and ranking of SSR Nei distances were found highly significant (r = 0.321) which reveals strong association between these distances. On the other hand, Spearman's rank correlation value was not significant between ranking of physiological genetic distances and ranking of SSR Nei distances (r = -0.00179*) and between ranking of morphological genetic distances and ranking of physiological genetic distances (r = 0.129). These results reveal that there is not significant association between these genetic distances. From the rank correlation values, it was found that SSR marker based genetic diversity was the most effective in genetic distance determination and grouping of the genotypes, followed by morphological genetic diversity analysis. Physiological genetic diversity was found to be less effective in studying genetic diversity. From the result of this study, it was found that combination of morphological and molecular markers may be useful in studying genetic diversity of rice for conservation, breeding and other crop improvement activities.

Key words: Genetic Diversity % Rank correlation % Rice % SSR marker

INTRODUCTION

Rice (*Oryza sativa* L.) is an important cereal crop belonging to the family Poaceae having chromosome number 2n=24 under the order Cyperales and class Monocotyledon [1] which was domesticated from its wild ancestor about 11,500 years ago [2]. It is consumed exclusively by humans and supplies staple food for nearly 50% of the global population [3]. In many developing countries, rice is the basis of food security and is intimately associated with traditional culture and customs in local regions [4].

For the effective conservation and utilization of rice genetic resources, a clear understanding of genetic diversity and relationships of varieties is essential [5]. Genetic diversity in the available gene pool is the source of variation, which is raw material for the improvement work. Precise information on the nature and degree of genetic divergence of the parents is the prerequisite of an effective breeding program. Inclusion of more diverse parents in hybridization is supposed to increase the chance of obtaining maximum heterosis and gives broad spectrum of variability in segregating generations. Selection of parents based on genetic divergence has been successfully utilized in different crop species also [6-8]. Diverse data sets including morphology [9], physiology [10], isozymes [11] and storage protein profiles [12] have been used to assess genetic diversity. Recently, the utility of DNA markers has been suggested for precise and reliable characterization and discrimination of genotypes [6].

Among different tools of genetic variability assessment, morphological, physiological and DNA markers are widely used. Though several advanced

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techniques are now being used, the use of morphological and physiological traits for plant improvement program is the basis of evaluation for both the conventional and modern breeding approaches. Moreover, it could be said that molecular marker technology can suppliment the conventional breeding efforts [13]. Simple sequence repeats (SSR, also referred as microsatellites) are one kind of DNA marker useful for studying genetic diversity and relationships of organisms, because of the significant level of allelic polymorphism that can be readily revealed [14; 15]. SSR markers have been widely used in rice for studying genetic diversity, cultivar identification, purity analysis, gene mapping, parent selection for crossing and germplasm conservation or utilization [16; 3; 5; 17-19]. The purpose of the study is to compare among morphological, physiological and molecular diversity analysis and to identify the efficacy of these three types of diversity analysis in rice crop.

MATERIALS AND METHODS

Genetic diversity in respect of morphological and physiological traits of 2l modern rice varieties (Table 1) was analyzed from field laboratory study. The experiment was laid out in a RCBD with three replications. The dimension of an individual plot was 4.0 m × 5.0 m having plot to plot and block to block distance of 0.5 m and 1.0 m, respectively. Thirty days old seedlings were transplanted at the rate of three seedlings per hill with the spacing of 20 cm × 20 cm. Morphological data were recorded on 13 traits; plant height (cm), panicle length (cm), maximum number of tillers m^{-2}, number of effective tillers m^{-2}, tiller mortality, number of spikelets $panicle^{-1}$, number of effective spikelets $panicle^{-1}$, number of ineffective spikelets $panicle^{-1}$, spikelet fertility, 1000-grain weight (g), phenotypic acceptability (PACP), straw yield ($t\ ha^{-1}$) and grain yield ($t\ ha^{-1}$). Fourteen physiological data were recorded from the same plots on seedling vigor ($mg\ cm^{-1}$), days to flowering (50%), panicle exsertion rate (%), flag leaf area (cm^2), days to maturity, LAI at panicle initiation and at flowering using length-width method [20]. Crop growth rate (CGR) at panicle initiation and at flowering were measured following Radford [21]. Relative growth rates (RGR) at panicle initiation and at flowering were measured as growth rate per unit plant biomass following Tanaka *et al.*[22]. Net assimilation rates (NAR) at panicle initiation and at flowering were calculated using the formula of Kubota *et al.* [23].

Table 1: List of rice varieties (*Oryza sativa* L.) used in the study

July 28, 2011SL. No.	Name of variety	Year of release
01	BR3	1973
02	BR4	1975
03	BR5	1976
04	BR10	1980
05	BR11	1980
06	BR22	1988
07	BR23	1988
08	BR25	1992
09	BRRI dhan30	1994
10	BRRI dhan31	1994
11	BRRI dhan32	1994
12	BRRI dhan33	1997
13	BRRI dhan34	1997
14	BRRI dhan37	1998
15	BRRI dhan38	1998
16	BRRI dhan39	1999
17	BRRI dhan40	2003
18	BRRI dhan41	2003
19	BRRI dhan44	2005
20	BRRI dhan46	2007
21	Rajasail	Local variety

BR = Bangladesh Rice, BRRI = Bangladesh Rice Research Institute

Table 2: SSR markers used for the determination of molecular diversity

Sl. No.	Primer	Sequence of the primer		Chromosome Number	Position (MB)
		Forward	Reverse		
01.	RM05	CACACTCCCATGCTAACAACTGG	CATCAAGAAGAGCAGTCCTGTGC	01	24.3
02.	RM490	ATCTGCACACTGCAAACACC	AGCAAGCAGTGCTTTCAGAG	01	-
03.	RM1287	CCATTTGCAGTATGAACCATGC	ATCATGCAATAGCCGGTAGAGG	01	10.8
04.	RM3412	TGATGGATCTCTGAGGTGTAAAGAGC	TGCACTAATCTTTCTGCCACAGC	01	11.6
05.	RM8094	AAGTTTGTACACATCGTATACA	CGCGACCAGTACTACTACTA	01	11.2
06.	RM7075	GCGTTGCAGCGGAATTTGTAGG	CCCTGCTTCTCTCGTGCAGTCG	01	15.1
07.	RM10696B	TCCAGATCAACCAGCACATC	CCTGAAGGG.AGGGAGTATTTG	01	-
08.	RM10696	CCTTCGACTCCATGAAACAAACG	TCTCTTTGCCCTAACCCTATGTCC	01	11.0
09.	RM10713	ATGAACCCGGCGAACTGAAAGG	CTGGCTCCCTCAAGGTGATTGC	01	11.2
10.	RM10720	GCAAACGTCTACGTGAGAAACAAGC	GCATGTGGTGCCTTAACATTTGG	01	11.4
11.	RM10927	TGGATCCCACTAATCCAAATGC	GAAAGACTCCTTCCAATGTTAGGC	01	15.7
12.	RM279	GCGGGAGAGGGATCTCCT	GGCTAGGAGTTAACCTCGCG	02	2.9
13.	RM424	GATTCCACGTCAGGATCTTCTGG	GCTCACCAGTTGAGATTGAAAGG	02	11.38
14.	RM489	GAACAGGGACACAATGATGAGG	GACGATCGGACACCTAATTACAGC	03	4.3
15.	RM6266	CACCTTCTTGAGAAGCTCCTTCG	GACATCGAGAGCGAGGACAGC	03	23.6
16.	RM401	GCATGAGCTGCTCTCATTATTGTCC	GAAACGAACCAAACGTTCATCG	04	13.2
17.	RM1155	GACAGGGAGTGTGGCAACTATGC	GATCACAGACAATCATGGGTTGG	04	20.5
18.	RM1024	AACTGCCATCTCTGAAACTCTGC	CATCTCACTTCAGAAGGATCATAGCC	05	1.2
19.	RM289	TTCCATGGCACACAAGCC	CTGTGCACGAACTTCCAAAG	05	7.78
20.	RM469	TTACGTGATCACACAGGCTCTCC	AAGCTGAACAAGCCCTGAAAGG	06	0.6
21.	RM20224	AGTATGAAAGTCGGTGACGATGG	GAGATGTCACGTCTTCACTTAGGG	06	20.6
22.	RM5371	GCAGAGGATGCCCACTTAATTCC	GGGCTAGCTTTAGCTGCGTTGC	06	25.4
23.	RM436	ATTCCTGCAGTAAAGCACGG	CTTCGTGTACCTCCCCAAAC	07	22.09
24.	RM455	CCACAAATTAATCCGGATCACACC	AGCATTGTGCAATCACGAGAAGG	07	22.3
25.	RM38	ACGAGCTCTCGATCAGCCTAGC	CACTCCATGGAAGAGGCAAGC	08	2.1
26.	RM256	GACAGGGAGTGATTGAAGGC	GTTGATTTCGCCAAGGGC	08	24.14
27.	RM566	AATATGGTGGCGCGTACATCC	TGATCGAGCCAACAACAACTGG	09	14.7
28.	RM242	AAACACATGCTGCTGACACTTGC	TTACTAGATTTACCACGGCCAACG	09	18.6
29.	RM258	CTCCCTGGCCTTTAAAGCTGTCG	GACGAACAGCAGCAGAAGAGAAGC	10	17.6
30.	RM590	GAGATCGAGGAGGAGGTGAGG	AGTACTGCCGATCATATGGAAGC	10	22.6
31.	RM3428	GCCATTGACACCAAATGATCACC	GGCATATAAGGTCCATGGTGAATTGG	11	13.4
32.	RM286	CTGGCCTCTAGCTACAACCTTGC	AAACTCTCGCTGGATTCGATAGG	11	0.38
33.	RM17	GGAGAAAGAGAGGTGATCCTTTCC	CATGTCTTGGTGAGTGATGTTGC	12	26.95
34.	RM463	GAGGATTAATTAGCGTGTGACC	GTCGTGACATCTACTCAAATGG	12	22.09

(Source: http://www.gramene.org)

Morphological and physiological data were analyzed using GENSTAT 5.5 program where clustering was done using non-hierarchical classification using covariance matrix and genetic distance between two genotypes was calculated using the formula proposed by Mahalanobis [24]:

$$pD^2 = W^{ij}(xG^1_i - xG^2_i)(xG^1_j - xG^2_j)$$

Where,

pD^2 = Genetic divergence between two genotypes.

W^{ij} = The inverse of estimated variance and co-variance matrix.

x_i and x_j = The multiple measurements available on each individual.

Molecular diversity was conducted using 34 Simple Sequence Repeat (SSR) markers (Table 2). Seeds were germinated in germination chamber and after 3 days, germinated seedlings were sown in pots. Then the pots were kept in the net house. DNA was collected from the leaf of 28 days old seedlings following modified miniscale protocol [25]. Thirty four SSR markers distributed in 12 chromosomes were used for diversity analysis of the varieties (Table 1). Molecular weight for each amplified allele was measured in base pair using Alpha-Ease FC 5.0

software. The allele frequency data from Power Marker version 3.25 [26] was used to export the data in binary format (allele presence ="1" and allele absence = "0") for analysis with NTSYS-pc version 2.2 [27]. A similarity matrix was calculated with the Simqual subprogram using the Dice coefficient, followed by cluster analysis with the SAHN subprogram using the UPGMA clustering method as implemented in NTSYS-pc was used to construct a dendogram showing relationship among the genotypes. Genetic distance was calculated using the "Nei distance" [28].

Distances of 21 varieties were estimated for morphological, physiological and SSR analysis. Two hundred ten [{n × (n-l)}/2] genetic distances between the varieties were ranked separately for these three methods. Among these rankings, rank coefficients (r_s) were calculated by Spearman's rank correlation test. In order to measure and compare the association between two criteria of rankings, Spearman has devised the following formula:

$$r_S = 1 - \frac{6\sum_i di^2}{(n-1)n(n+1)}$$

Where,

di = Differences between two sets of rankings
n = Number of observation
r_s = Spearman's rank correlation coefficient

In this study, the number of pairs was large; the estimated r_s were tested using the criterion [29].

$$t = r_2\sqrt{\frac{n-2}{1-r_s^2}} \text{ with n-2 df}$$

RESULTS

Two hundred ten {n x (n-l)/2} genetic distances were measured among the genotypes for each diversity analysis (morphological diversity, physiological diversity and molecular diversity). The morphological, physiological and SSR distances were ranked separately and was compared. The rankings appeared to be quite different among these three methods. Of course, some pairs of varieties were consistently close over the three methods, but most variety pairs, however, behaved rather irregularly from one system of measurement to another. Then Spearman's coefficient of rank correlation analysis is carried out by following three ways:

- Correlation between morphological genetic distances and SSR Nei distances.
- Correlation between physiological genetic distances and SSR Nei distances and
- Correlation between morphological and physiological genetic distances.

Correlation Between Morphological Genetic Distances and SSR Nei Distances: Relative ranking was done separately on the basis of morphological genetic distances and molecular distances and correlation was estimated through Spearman's coefficient of rank correlation formula. The rank correlation value (r_s) was found 0.318 and 't' value was found 4.89 with n-2 degree of freedom. This value was highly s ignificant which indicated strong association between these rankings (Table 3). This highly significant correlation coefficient revealed that both techniques were effective in estimating the genetic distances and grouping of genotypes. Several previous results also showed significant correlation between morphological and molecular diversity analysis. The significant correlations indicate that these independent sets of data likely reflect the same pattern of genetic diversity and validate the use of these data to calculate diversity statistics.

Correlation Between Physiological Genetic Distances and SSR Nei Distances: Ranks of physiological genetic distances showed nonsignificant correlation with the ranks of molecular distances. The rank correlation value (r_s) was -0.002 and "t" value was found -0.026. This result indicated that the ranking of divergence between the genotypes based on physiological diversity analysis differs from the molecular diversity analysis and there is absence of association between these genetic distances (Table 3). This result also revealed that one of the rankings more efficiently ranked the genetic distances then another one. It also suggests that the two systems give different estimates of genetic relations among the varieties.

Correlation Between Morphological and Physiological Genetic Distances: Ranking were done on the basis of morphological genetic distances and physiological genetic distances separately. Their rank correlation was determined using Spearman's coefficient of rank correlation formula. Here, the rank correlation values r_s = 0.130 and t= 1.88 which was very close to the significant correlation coefficient value. However, this result was not significant which indicates absence of association between these rankings.

Table 3: Spearman's coefficient of rank correlation among the ranking of different genetic diversity analysis

Comparison between	Spearman's correlation value	"t" value	Level of significance
Morphological genetic distances and SSR distances	0.321	4.885	Highly significant
Physiological genetic distances and SSR distances	-0.00179	-0.026	Not significant
Morphological and Physiological genetic distances	0.129	1.879	Not significant

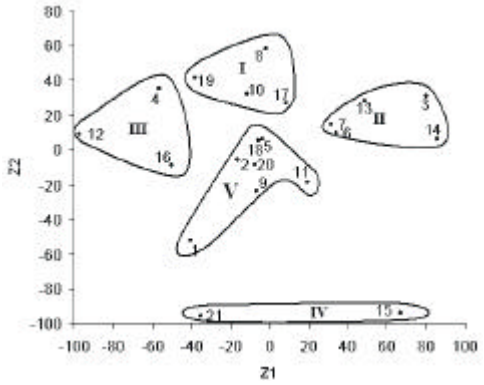

Fig. 1: Five clusters of 21 T. Aman rice varieties based on their morphological genetic distances

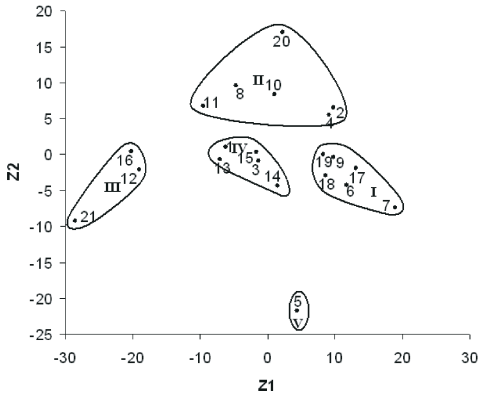

Fig. 2: Five clusters of 21 T. Aman rice varieties based on their physiological genetic distances

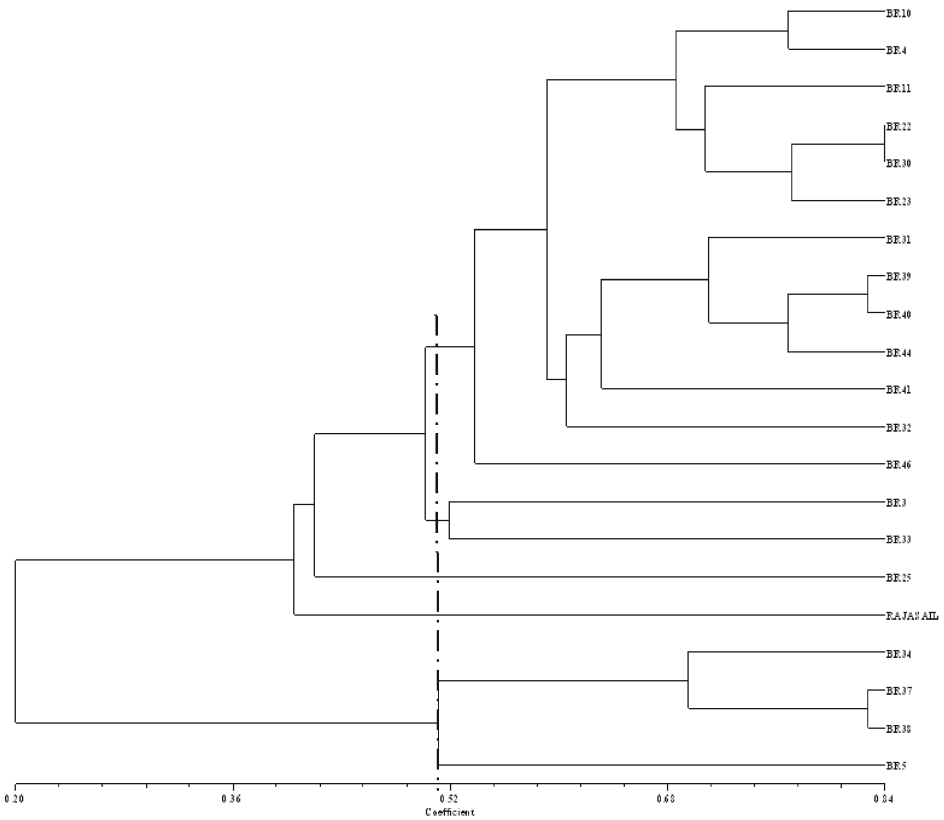

Fig. 3: A UPGMA cluster dendrogram showing the genetic relationships among 21 rice cultivars based on the alleles detected by 34 SSR markers.

Clustering Patterns: Cluster analysis based on morphological traits provides five clusters (Figure 1). Clustering pattern showed that cluster V is composed of the highest number of genotypes (7) followed by cluster II consisting of 5 genotypes, cluster I consisting of 4 genotypes, cluster III consisting of 3 genotypes and cluster IV consisting of 2 genotypes. Fourteen physiological characters based clustering also provides five clusters (Figure 2). Cluster I and cluster II is composed of the highest genotypes (6) followed by cluster IV (5 genotypes), cluster III (3 genotypes) and cluster V (1 genotypes), respectively. Same clustering pattern was previously reported in several reports [30-33; 10]. Molecular clustering provides five clusters at 50 % coefficient of similarity level (Figure 3).

DISCUSSION

Among morphological, physiological and molecular methods of diversity analysis, the molecular diversity is based on the naturally occurring polymorphism which escapes the limitations of environmental influences and gene expression. On the other hand, both the morphological and physiological traits are largely influenced by the environmental conditions and cultural practices [31]. Morphology is the visual expression of the plant where the influence of environment, cultural practices and gene-environment interaction plays an important role. In most of the cases, several genes and several complex biochemical processes are involved for a morphological or a physiological trait in rice plant. Beyene *et al.* [30] reported significant correlation between SSR and morphological ($r = 0.43$; $p = 0.001$) diversity analysis and also showed correlation between AFLP data and morphological data ($r = 0.39$, $p = 0.001$) in describing genetic relationships in traditional Ethiopian highland maize. Pfrender *et al.* [34] reported strong correlation between morphological (quantitative traits) and molecular based divergences ($r = 0.88$, $P<0.01$). Ghalmi *et al.* [32] reported significant correlation between morphological genetic matrices and molecular genetic matrices ($r = 0.22$ and $P<0.05$). Present study revealed significant correlation between morphological diversity and molecular diversity which may be due to the expression of gene to respective phenotype of morphology. Gilliland, Coll *et al.* [35] and Roldán-Ruiz *et al.* [36] reported that when varieties with shared genepools were examined using molecular markers, extremely high similarity measures were produced and were also linked to morphological similarities. The significant correlations indicate that these two independent sets of data likely reflect the same pattern of genetic diversity and validate the use of these methods for diversity estimation and also in grouping of genotypes. This result also indicate that the combination of morphological and molecular markers may be useful in studying genetic diversity as reported by Cortese *et al.* [37].

Molecular distances and physiological distances showed nonsignificant correlation coefficient between the ranks of genetic distances. The same result was reported by Tar'an *et al.* [38] where he found nonsignificant correlation between the distances based on molecular markers and distance based on morphological and physiological characters. Such observation should not be regarded as indicating a weakness or limitation of these systems as reported by Roldán-Ruiz *et al.*[39]. These results may have arisen because the diversity at the molecular level, which is a priori neutral, may not reflect the diversity at the physiological level, as described by Karhu *et al.* [40]. Another possibility of this nonsignificant correlation may be due to the number of physiological traits collected for diversity analysis which might be few to reflect the actual diversity of the varieties. If the number of traits would be high enough, then the correlation might be changed to be significant. Furthermore, most of the physiological traits also vary sharply in harmony with the change of their micro-environment and cultural practices, for which the estimates may provide diverse result in field condition which may affect the distance estimation among the studied genotypes. The correlation coefficient of diversity estimates was not significantly correlated with those based on the morphological and physiological characters, suggesting that these systems give different estimates of genetic distance among the varieties.

Compared to morphological and biochemical characteristics, the DNA genome provides more powerful source of genetic polymorphism [41]. It allows direct comparison of genetic diversity to be made at the DNA level, have the potential to identify a large number of polymorphic loci with an excellent coverage of an entire genome, are phenotypically neutral, allow scoring of plants at any developmental stage and are not modified by environment and management practices [42]. A number of studies reported that DNA markers are the most promising technique used to diversity analysis and to differentiate among genotypes at species and subspecies level [43-45]. Since molecular studies represent the actual genotypic constituents and are independent of

environment, so we can consider it as the most powerful method of diversity analysis. Furthermore, the others whose ranking show significant correlation with DNA marker based distances can be considered to be effective also. Considering this view, we can suggest morphological genetic diversity as second choice of diversity analysis. Beyene *et al.* [30] also suggested morphological traits as relatively less reliable and efficient for precise discrimination and analysis of their genetic relationships then molecular diversity. Despite this, morphological traits are important for its fast, simple and as a general approach for assessing genetic diversity. It was found that ranking using physiological genetic distances showed insignificant rank correlation with both the ranking of SSR marker based distances and the ranking of morphological genetic distances. So, it could be said that physiological diversity might be less efficient compared to molecular and morphological diversity. Finally it could be concluded that, for genetic diversity analysis and grouping the genotypes, molecular distances is the most effective followed by morphological genetic distances and physiological genetic distances was less effective.

In breeding program, generally parents are selected based on the genetic divergence for obtaining transgressive segregants and superior genotypes. Parent selection for hybridization can be done by inclusion of distant parents [46-49]. Breeding program perform better if parents are selected based on specific objectives considering positive common criterion as additional benefit. Moreover, selection of parents from each cluster and crossing them in a series of diallel cross were proved to be highly fruitful [50]. Different clustering pattern have also been reported by different methods of diversity analysis in some previous studies [51; 7; 52]. So the method which provide accurate assessment of genetic diversity and efficiently group the genotypes will be selected for parent selection in future breeding program. Since molecular diversity based on SSR marker provide the most accurate genetic diversity, so molecular diversity is to be given preference over morphogenetic and physiogenetic diversity analysis. Morphogenetic diversity analysis will be given second choice which also provides diversity estimates comparable with the molecular diversity.

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