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Phylogenetic Relationship of Wild and Cultivated *Oryza* species using SSR Markers

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Research Article

Abstract : Wild plants are important as they are treasure of resistant genes against different types of hurdle stress such as salt tolerance, pest tolerance, flood tolerance, drought tolerance and others. These bio-resources as wild *Oryza* species has been disappearing by different calamities at alarming rate. Therefore an attempt was made to investigate the molecular genetic diversity of wild and cultivated *Oryza* species. 41 microsatellite molecular markers were used to explore the phylogenetic relationships among closely related wild *Oryza* species, elite cultivars and landraces of the genus *Oryza.* The results of SSR analysis, optimization of primers, banding patterns, amplifications, SSR (simple sequence repeat) derived Dendrogram analysis, Jaccard's similarity co-efficient and principal co-ordinate analysis were assessed. 13 species of *Oryza* were taken for the assessment of genetic diversity using SSR markers. 41 SSR primers produced a total 209 bands. 70.03 % was polymorphic in nature and 22.09 % was unique. RM24260 produced maximum number of 11 bands. Resolving power was 0.0672 to 5.733, maximum SSR primer index was 0.500 and maximum PIC (polymorphic information content) value was 0.999. Study highlights the relative genetic diversity of *Oryza* species. It indicates the importance of SSR markers as molecular tools in the assessment of genetic diversity of plant species for conservation. It was validated that the SSR markers used in this investigation were quite efficient to assess the phylogenetic diversity among *Oryza* species.

Keywords: *Oryza* species, SSR Markers, Phylogenetic Relation

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1. Introduction

Phylogenetic diversity represents the heritable variation within and between the population of plant species. Knowledge of the genetic diversity and phylogenic structure within germplasm collection is an important foundation for crop improvement. Progress in plant breeding requires a broad genetic base with a rich and diverse germplasm collection being the backbone of every successful crop improvement program (Sun *et al*.,2001). Growth and development of agricultural resources is mostly depending on genetic diversity among different crop plants and it is estimated that not even 15% of the potential diversity has utilized. This implies that thousands of valuable allelic variations of traits of economic significances remain unutilized. Therefore, landraces of distinct genetic structure are a good promise for the future crop improvement in general and rice in particular (Sajib *et al*., 2012). Rice (*Oryza* spp.) is the staple food of more than 50% of the world's population. Among the rice growing countries in the world. India has the largest area under rice crop and ranks second in production next to china. The cultivated rice (*Oryza sativa* L.) is rich in genetic diversity apart from highly diverse wild progenitors (Behera *et al*., 2012). Identification of genotypes and their inter-relationships is vital. Development of new biotechnological techniques provides increased support to evaluate genetic variations in both phenotypic and genotypic level. Molecular markers are powerful tools in the assessment of genetic variations in the elucidation of genetic relationship within among specie, have demonstrated the potential to detect genetic diversity and to aid in the management of Plant Genetic Resources (Virk *et al*., 2000; Teixeira *et al*., 2005). Simple sequence repeat is an important tool for genetic variation identification of germplasm. SSR marker have some merits such as quickness, simplicity, rich polymorphism and stability, thus being widely applied in genetic diversity analysis, molecular map construction, gene mapping, construction of fingerprints (Xiao *et al*., 2006), genetic purity test (Peng *et al*., 2003), analysis of germplasm diversity (Zhou Xie and Ge,2003), utilization of heterosis, especially in identification of species with closer genetic relationship (Sajib *et al*.,2012). Wild species of the *Oryza* are highly diverse in terms of morphology. Therefore an attempt was made to assess the phylogenetic relationship among wild and cultivated species of *Oryza* using SSR markers for conservation and breeding strategies.

2. Materials and Methods

2.1 Plant materials

Seeds of 30 rice (*Oryza* species) accessions which include 12 wild rice species consisting 14 accessions along with 2 elite rice cultivars and 2 landraces (Table-1) of rice were collected from Rice Gene Bank, Crop Improvement Division, Central Rice Research Institute, Cuttack. The seeds were dehusked and sterilized by washing with 100 % alcohols for 1 min. They were germinated in petriplates by keeping the dehusked seeds in a moist germination paper (Fig-1). Then the petriplates were kept in dark. Four days later the germinated seeds were transferred to individual pots with proper labeling. After three weeks 3 grams of young leaves were collected from each accession for genomic DNA isolation.

Fig.1: Germinated seeds in petriplates

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Table -1: List of Experimental plants (*Oryza* species)

SL. No.	Accession No.	Species	Site of collection
1.	Unknown	O.sativa 'Savitri'	CRRI, Cuttack
2.	100118	O.nivara	Bhubaneshwar
3.	100517	O.nivara	Belari, Puri
4.	100518	O.nivara	Hatia, Puri
5.	100522	O.nivara	Nandankanan, Bhubaneshwar
6.	100001	O.rufipogon	Bhubaneshwar
7.	100528	O.sativa 'Khasa'	Dulki,West Tripura
8.	100530	O.sativa 'Maibarak'	Dugli,West Tripura
9.	100531	O.rufipogon	South Tripura
10.	100531	O.rufipogon	South Tripura
11.	100531	O.rufipogon	South Tripura
12.	100534	O.rufipogon	Matarbari,South Tripura
13.	100517	O.rufipogon	Melaghar,West Tripura
14.	100518	O.rufipogon	West Tripura
15.	100518	O.rufipogon	West Tripura
16.	100518	O.rufipogon	West Tripura
17.	100504	O.longistaminata	IRRI, Phillipines
18.	100506	O.minuta	IRRI, Phillipines
19.	100507	O.officinalis	IRRI, Phillipines
20.	100500	O.eichengeri	IRRI, Phillipines
21.	100502	O.grandiglumis	IRRI, Phillipines
22.	100496	O.alta	IRRI, Phillipines
23.	100497	O.australiensis	IRRI, Phillipines
24.	100499	O.brachyantha	IRRI, Phillipines
25.	100512	O.ridleyi	IRRI, Phillipines
26.	100532	O.coarctata	Atharbari, Paradip
27.	100533	O.coarctata	Ghanagalia, Paradip
28.	100534	O.coarctata	Nehrubangla, Paradip
29.	100535	O.coarctata	Bhutamundai, Paradip
30.	Unknown	O.sativa 'Swarna Sub-1	CRRI, Cuttack

Table -2 : A List of SSR primer pairs used for the present study

SL. No.	Chr. No.	Primer	Forward Sequence	Reverse Sequence
1	1	RM5443	CTCATTGGCACATCTACATACAGG	TCCAACTAAGCAGAAGAACTAGGG
2	1	RM11229	TGACAGAAACAAAGCGGAAGG	TCCAAACCGCTATTCTTGTAGC
3	1	RM3148	GCTTTGGTATTTGCAGGTTCACG	CTATTGCTCGAACACTTTGCTTCTCC
4	1	RM11278	ACTTCTTGTAGCACTGCACCTTCG	CCTCGGCAACTGCTTCAAGG
5	1	RM11258	GCTCCACCATTCATCCATACAGC	ATTCGATTGGTTCCTTGGCTTCC
6	2	RM12349	CCGATTAGCGATTGATATGGAGTAGG	AGTGCACAGCCATGGAATTATGC
7	2	RM13097	GGGCTTAAGGACTTCTGCGAACC	AGCGATCCACATCATCAAATCG
8	2	RM13260	AGTCCAAGAAAGGCACGAGAGG	CTGCATCGAAGAAGAAGAAGAAGC
9	2	RM13357	CATAAAGGGACCCACTTGTCAGC	TGATGTCTGGTCCACAGTCTGC
10	2	RM13679	AGATGACAAGGTGAGAGCACTGG	TGGAGCCCAGAATTTCTAGATCG
11	3	RM14860	GGAAGGTGATTTCATCCGGTAGC	TGGCATGTTTAATGCTGGTTCG
12	3	RM14981	GGCGAGCAGAAGTATAATCCAGAAGG	CGCTTGTGGCTTACTGGCTTGG
13	3	RM15809	AAAGCTGCGACGAACACGAACG	CGCCGCAGCAGAGAAGAGAAGG
14	3	RM15981	GGTTAAAGGGAGGACACCACTCG	TCTAGCCAGGCATGACAAGAACC
15	4	RM16770	AACCGGCCATGCCAGAGAGG	CCAAATCCTATCCGCCACACACC
16	4	RM17201	GATCGTTGCTGCTTTCAATGAGG	AGTGTTTCACCTTGGACCCATGC
17	4	RM17480	GAGTTCGTCCCTGACAAACAGAAACG	GTGAGCGAGCGAGTGAGTGAGC
18	4	RM17600	CCTCGAAATGAATTGCAGTCGAACG	GTCTTGTGCCTTGTGCCGATGG
19	5	RM18065	CGATGGTGAGTGGTGATTCATGC	ATCATCCGCGCATTAGCATTTCC
20	5	RM18691	GCAGTTCGTTGTGGAGGAACACC	ATCGGCCACCCAAATCTTAATGG
21	5	RM19029	AAATATCTATCGGCCTCTCCAAGC	GGAGGAATCGAACCAGAGAAGC
22	6	RM19255	TTAAGCTAGGGAATCAGCGGTTAGC	GGAGTTGCAGTGTGGTGTGTGG
23	6	RM19844	CGTTCGAGCAGAACCATCTACC	CCTCTTCCGCTCCACTCTCC
24	6	RM20615	TTACACCAGGCTACCCAAACTCG	TTGCTGAGTTCCCTCGTCTATCC
25	7	RM21024	ATTAAGCTACTGTCTGCCTCCTTCG	GCTTCGTTTCAGGTGGTCAGG
26	7	RM21258	TATCATTCCGGTCCAAAGTGTCG	TCCGGTCCAAAGTCTCATTTGC
27	7	RM21559	GTATAGCCAACCAAGCAAGATAGC	GATGCCTAGACACACATGTAAGC
28	8	RM22252	AAGGAGAAGTTCTTCGCCCAGTGC	GCCCATTAGTGACTGCTCCTAGTCG
29	8	RM22571	TCCTCCTCCACCTCAATCACACC	CCGAGCTCGCCATTAGCTTGC
30	8	RM22914	GGAGCAGCTAAGGCAGATAAGAGG	GCCTTCATGCTTCAGAAGACAGC
31	9	RM24260	GATCTCTCACCCACATGCCTAGC	TCCCATCTTGATCGATCTCTTCG
32	9	RM24683	CAGTGGCGTGGAGAGAAATTTGG	CTCACCTGCGACAGCAAGATCG
33	10	RM25368	TATAGTTAAGGGAGCCACGCAAGC	CCACCTCGTAAGAACATGGAGAACC
34	10	RM25712	TCTCCCTATTCCCGTGTAAATCG	CCCGATGATCGATTGTACCTAGC
35	10	RM25817	GCCTCGAATCAACCAAATAGTGG	GGGCTGAGACCTTGTGGTATGG
36	11	RM26033	AAGAGGACCTCGAGGATGTACCG	CGTCAGCCTGTCTTGTTGTACTCG
37	11	RM26269	GGAGGTAGGGAAATCAGGTGAGG	GTGCACGTGACCATAAACACTCC
38	11	RM27201	GCCTACTGGTCCAAGGTGAAGC	CAAGTAGACGTCGTGGAACATGG
39	12	RM27635	CCAAACTATCAGCGACATGAACG	CTCTGTTGTTTGGGAGACTGTGC
40	12	RM27692	AAACCACCTCGTCGTCTTCTTCC	GCTGATGACGTTGAATGACTTGG
41	12	RM27955	AATGGTGCTTACTCACAGCAATGG	TTCATGAGCTGTGCCTGTTGG

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2.2 Genomic DNA extraction and SSR assay

DNA was extracted from selected mature seeds using CTAB method (Doyle and Doyle,1990). 41 SSR markers (Table -2) were selected to study the phylogenetic relationships (Temnykh *et al*., 2000). The PCR reaction mixture contained 25 mg template DNA, 0.25 mm dNTPs (Ferments), 1X PCR buffer (Biotool B & M Labs, Spain), 75mM Tris HCl (pH 9.0), 50mM KCl, 20 mM $(NH_4)_2$ SO_4, 2 mM $MgCl_2$ and 1 unit of Taq DNA polymerase (Biotool B and M Labs, Slume of 10 µl). The reaction mixture was initially denatured for 5 min at 94 °C and than subjected to 35 cycles of 30 seconds.

2.3 Data analysis

2.4 Scoring of the data for SSR analysis

The data were scored as 1 for the presence and 0 for the absence of the band for each primer-species combination for SSR analysis.

2.5 Resolving power (Rp)

Resolving power of the primer/primer combination was calculated as per Prevost and Wilkinson (1990) is:

Rp=ΣIB [IB (band informativeness) = 1- [2 × (0.5 – P)]

P is the proportion of the given species containing the band.

2.6 Marker Index

The Primer Index was calculated from the polymorphic Index. PIC (polymorphism information content) was calculated as PIC = Σ P^2i, Pi is the band frequency of the ith allele (Smith et al.,1997). Here, the PIC was considered to be 1-p2-q2, where p is the band frequency and q is no band frequency (Ghislain *et al*.,1999). PIC value was then used to calculate the SSR Primer Index. It is the sum of the PIC of all the markers amplified by the same markers.

2.7 PIC (Polymorphism Information Content)

PIC value of a marker was calculated according to a simplified version ofAnderson *et al,*.(1993), where *Pij* is the frequency of the *j*th allele for the *i*th marker, and summed over *n* alleles.

$$PIC_i = 1 - \sum_{j=1}^{n} P_{ij}^2$$

2.8 Dendrogram and Jaccard's similarity

Jaccard's coefficient of similarity(Jaccard,1908) was measured and a dendrogram based on similarity coefficients generated by unweighted pair group method using arithmetic averages (UPGMA)(Sneath and Sokal,1973), and the SHAN (Sequential Agglometric Hierarchial and nested) clustering was obtained. The entire analysis was performed using the statistical package NTSYS-pc 2.02e (Ludwig and Reynolds,1988)**.**

2.9 Principal Co-ordinate Analysis (PCA)

PCA was done followed by Ludwig and Reynolds, (1988)**.** Principal Co-ordinate Analysis was done by the NTSYS-pc 2.02C Software. The Mantel 'Z' test was done for efficiency of marker technique (Mantel,1967).

3. Results and Discussion

The quantification of DNA reveals (Table-3) that good for optimization of selected experimental *Oryza* species. The SSR marker demarcated 30 rice accessions of wild relatives, cultivars and landraces into several groups, clustering of accessions were found to be different in the marker used (Fig-2). A possible explanation for the difference in resolution SSR is that the marker technique targeted different portions of the genome. The amplification loci of SSR are mainly in the gene expression region (Zietkiewicz *et al*.,1994; Jarne and Lagoda,1996).

The SSR markers generated 161 polymorphic alleles (70.03%) out of 209 bands. The study also revealed that primer RM24260 on chromosome 9 generated maximum of 11 bands, while primers RM13357 and RM17600 in chromosome 2 and 4 had a minimum of 1 band. These results suggest that the manner of polymorphism differs because of marker specificity (Table-3). In addition, the relation is assumed to depend on the genome coverage and sequence type recognized by each marker system [22] . The markers showed an average PIC value of 0.999, which confirms that SSR markers used in this study were highly informative, because PIC values higher than 0.5 indicate high polymorphism. Markers with PIC values of 0.5 or higher are highly informative for genetic studies and are extremely useful in distinguishing the polymorphism rate of a marker at a specific locus**.** The mean value observed in this study was higher than the PIC value of 0.578 recorded in an earlier study among wild relatives, elite rice cultivars and landraces (Ravi *et al.*, 2003). This could indicate that the genotypes used in the present study were more diverse due to differences in origin, ecotype, and speciation. Microsatellite markers exhibit high PIC values because of their codominant expression and multiallelism. Nevertheless, 41 microsatellite markers were able to discriminate between the wild relatives/ cultivars/ landraces and demonstrated a maximum genetic similarity value of 0.830 between the wild rice species *O. coarctata* having HHKK genome. UPGMA cluster analysis of the SSR marker-based genetic similarity matrix resulted in the classification of wild relatives, cultivars, landraces and separate clusters. Moreover, varietal profiling based on SSR markers will be more reliable as compared to profiling based on other markers, since SSR markers detect finer levels of variations among closely related lines. A comparison of values of allelic diversity among the cultivars, as compared to landraces and wild relatives, clearly emphasize the scope for introgression of genes through hybridization of landraces and wild relatives with the cultivars for increasing genetic diversity in the cultivated rice gene pool (Vaughan,1991) .

This also reiterates the need for genetic diversity evaluation among the principal genotype classes and cataloguing them for the benefit of the future.

On the basis of markers used in this study, Jaccard's coefficient revealed the similarity among the wild and cultivated rice **(Table-4).** It is also indicated in Dendrogram **(Fig-3),** Principal Coordinate Analysis **(Fig-4)** and Mantel Z Correlation **(Fig-5).** It can be concluded that SSRs have the discriminatory power to reflect genetic diversity between and among 30 rice accessions of wild relatives, cultivars and landraces collected from different places of country with good certainty.

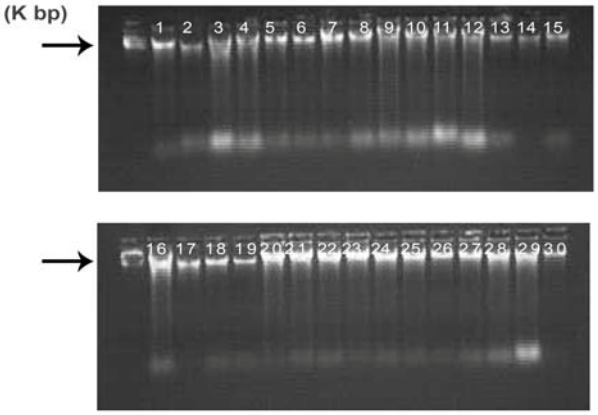

Fig 2 : Quantification of DNA

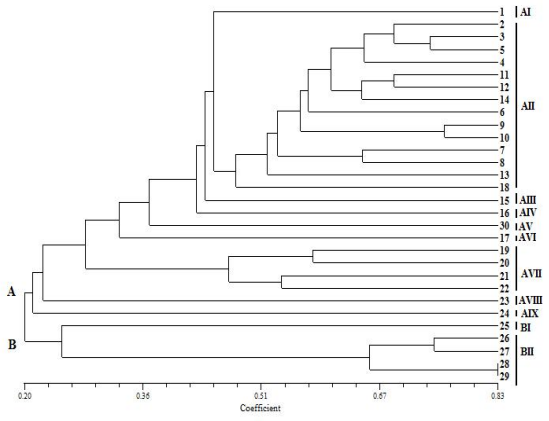

Fig 3: Dendrgram of the present study

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Table 3: Details of SSR analysis for the experiment

SL. No.	Primer	TB	PB	MB	UB	RP	MI	PIC	Approx. Fragment size (bp)
1	RM5443	7	5	0	2	2.000	0.245	0.964	120-1200bp
2	RM11229	6	5	0	1	2.333	0.313	0.954	250-750bp
3	RM3148	4	3	0	1	3.267	0.483	0.725	145-190bp
4	RM11278	7	6	0	1	2.267	0.271	0.956	200-500bp
5	RM11258	7	5	0	2	2.333	0.278	0.950	80-150bp
6	RM12349	7	5	0	2	2.067	0.252	0.971	180-410bp
7	RM13097	4	3	0	1	2.400	0.420	0.801	100-300bp
8	RM13260	9	7	0	2	3.933	0.342	0.898	200-500bp
9	RM13357	1	0	0	1	0.067	0.064	0.999	550bp
10	RM13679	3	3	0	0	1.933	0.437	0.866	200-290bp
11	RM14860	4	3	0	1	3.200	0.480	0.694	150-200bp
12	RM14981	2	1	0	1	1.600	0.480	0.706	500-900bp
13	RM15809	5	5	0	0	4.600	0.497	0.730	400-1000bp
14	RM15981	8	6	0	2	5.733	0.460	0.806	150-240bp
15	RM16770	3	2	0	1	2.133	0.458	0.687	250-500bp
16	RM17201	3	3	0	0	3.533	0.484	0.590	100-200bp
17	RM17480	2	2	0	0	2.000	0.500	0.740	180-200bp
18	RM17600	1	0	0	1	0.067	0.064	0.999	200bp
19	RM18065	6	5	0	1	3.333	0.401	0.877	300-800bp
20	RM18691	7	5	0	2	2.000	0.245	0.969	380-480bp
21	RM19029	3	3	0	0	1.600	0.391	0.873	100-340bp
22	RM19255	2	1	0	1	2.000	0.500	0.532	80-120bp
23	RM19844	6	5	0	1	3.667	0.424	0.839	220-480bp
24	RM20615	4	3	0	1	2.000	0.375	0.868	280-480bp
25	RM21024	6	5	0	1	1.933	0.270	0.959	280-300bp
26	RM21258	7	4	0	3	3.333	0.363	0.878	200-1000bp
27	RM21559	8	7	0	1	4.533	0.406	0.868	200-1000bp
28	RM22252	5	3	0	2	1.467	0.250	0.967	190-400bp
29	RM22571	9	7	0	2	4.667	0.384	0.888	95-380bp
30	RM22914	5	4	0	1	2.200	0.343	0.928	120-180bp
31	RM24260	11	7	0	4	2.067	0.170	0.987	180-280bp
32	RM24683	4	4	0	0	2.800	0.455	0.851	140-180bp
33	RM25368	4	3	0	1	2.400	0.420	0.866	290-550bp
34	RM25712	2	2	0	0	1.667	0.486	0.779	180-200bp
35	RM 25817	5	4	0	1	1.800	0.295	0.933	250-480bp
36	RM26033	6	4	0	2	3.333	0.401	0.816	280-700bp
37	RM26269	7	7	0	0	1.867	0.231	0.977	90-120bp
38	RM27201	4	3	0	1	3.267	0.483	0.724	140-250bp
39	RM27635	6	5	0	1	5.733	0.499	0.641	105-250bp
40	RM27692	5	4	0	1	3.867	0.474	0.750	140-500bp
41	RM27955	4	2	0	2	1.667	0.330	0.903	115-140 bp
Total		209	161	0	48	*2.650	*0.369	*0.847	

(TB- Total no. of Bands, PIC -Polymorphism Information Content, PB- Polymorphic bands, RP- Resolving power, MB-Monomorphic bands, MI- Marker Index, UB- Unique bands, * Average of the column)

Table 4: Jaccard's coefficient similarity

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	1.000														
2	0.467	1.000													
3	0.486	0.703	1.000												
4	0.565	0.646	0.653	1.000											
5	0.444	0.680	0.739	0.653	1.000										
6	0.458	0.608	0.658	0.582	0.571	1.000									
7	0.528	0.517	0.500	0.625	0.518	0.494	1.000								
8	0.493	0.525	0.568	0.622	0.526	0.519	0.649	1.000							
9	0.380	0.558	0.583	0.532	0.562	0.533	0.412	0.507	1.000						
10	0.438	0.520	0.588	0.514	0.521	0.535	0.481	0.529	0.759	1.000					
11	0.403	0.640	0.625	0.635	0.648	0.532	0.557	0.548	0.734	0.641	1.000				
12	0.437	0.531	0.595	0.605	0.553	0.526	0.610	0.629	0.577	0.514	0.691	1.000			
13	0.423	0.538	0.539	0.494	0.481	0.513	0.538	0.468	0.521	0.522	0.562	0.554	1.000		
14	0.413	0.641	0.627	0.595	0.584	0.557	0.506	0.513	0.568	0.571	0.653	0.644	0.526	1.000	
15	0.347	0.398	0.444	0.476	0.481	0.372	0.464	0.430	0.405	0.479	0.443	0.474	0.425	0.545	1.000
16	0.307	0.500	0.425	0.439	0.425	0.474	0.379	0.358	0.421	0.417	0.461	0.474	0.337	0.589	0.370
17	0.352	0.388	0.366	0.365	0.383	0.314	0.326	0.286	0.293	0.250	0.346	0.310	0.363	0.357	0.298
18	0.368	0.556	0.500	0.459	0.558	0.407	0.448	0.468	0.462	0.421	0.481	0.494	0.427	0.544	0.393
19	0.207	0.315	0.307	0.337	0.337	0.303	0.260	0.337	0.313	0.272	0.333	0.270	0.287	0.330	0.244
20	0.188	0.345	0.321	0.307	0.321	0.302	0.258	0.305	0.313	0.269	0.317	0.282	0.301	0.314	0.256
21	0.146	0.319	0.311	0.298	0.297	0.308	0.240	0.295	0.273	0.247	0.307	0.261	0.278	0.290	0.223
22	0.190	0.312	0.303	0.304	0.289	0.300	0.298	0.349	0.310	0.284	0.299	0.310	0.299	0.326	0.284
23	0.189	0.240	0.230	0.245	0.218	0.240	0.240	0.240	0.206	0.207	0.250	0.222	0.237	0.250	0.188
24	0.145	0.232	0.220	0.198	0.220	0.204	0.182	0.202	0.193	0.207	0.213	0.185	0.213	0.270	0.187
25	0.145	0.217	0.234	0.193	0.203	0.231	0.217	0.213	0.254	0.297	0.211	0.224	0.227	0.244	0.243
26	0.194	0.161	0.172	0.152	0.172	0.157	0.161	0.181	0.185	0.184	0.179	0.205	0.193	0.195	0.238
27	0.183	0.152	0.163	0.143	0.163	0.148	0.140	0.171	0.175	0.173	0.155	0.181	0.169	0.172	0.228
28	0.163	0.186	0.185	0.189	0.211	0.183	0.198	0.221	0.184	0.183	0.218	0.230	0.191	0.220	0.262
29	0.150	0.175	0.187	0.179	0.200	0.198	0.175	0.195	0.146	0.143	0.180	0.205	0.167	0.196	0.207
30	0.324	0.302	0.359	0.410	0.359	0.338	0.400	0.360	0.316	0.343	0.355	0.425	0.288	0.440	0.373

	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
16	1.000														
17	0.309	1.000													
18	0.443	0.318	1.000												
19	0.225	0.274	0.307	1.000											
20	0.221	0.212	0.291	0.582	1.000										
21	0.244	0.183	0.229	0.395	0.453	1.000									
22	0.236	0.213	0.247	0.480	0.551	0.541	1.000								
23	0.158	0.150	0.183	0.242	0.226	0.274	0.266	1.000							
24	0.193	0.144	0.261	0.233	0.244	0.198	0.244	0.175	1.000						
25	0.219	0.145	0.218	0.169	0.265	0.208	0.264	0.195	0.229	1.000					
26	0.171	0.221	0.244	0.213	0.208	0.136	0.210	0.154	0.208	0.262	1.000				
27	0.160	0.195	0.220	0.188	0.197	0.140	0.200	0.157	0.167	0.230	0.745	1.000			
28	0.170	0.188	0.239	0.195	0.235	0.216	0.280	0.204	0.220	0.254	0.685	0.712	1.000		
29	0.135	0.205	0.241	0.184	0.238	0.218	0.253	0.181	0.207	0.239	0.636	0.600	0.830	1.000	
30	0.389	0.195	0.395	0.188	0.228	0.182	0.214	0.172	0.213	0.286	0.189	0.194	0.234	0.253	1.000

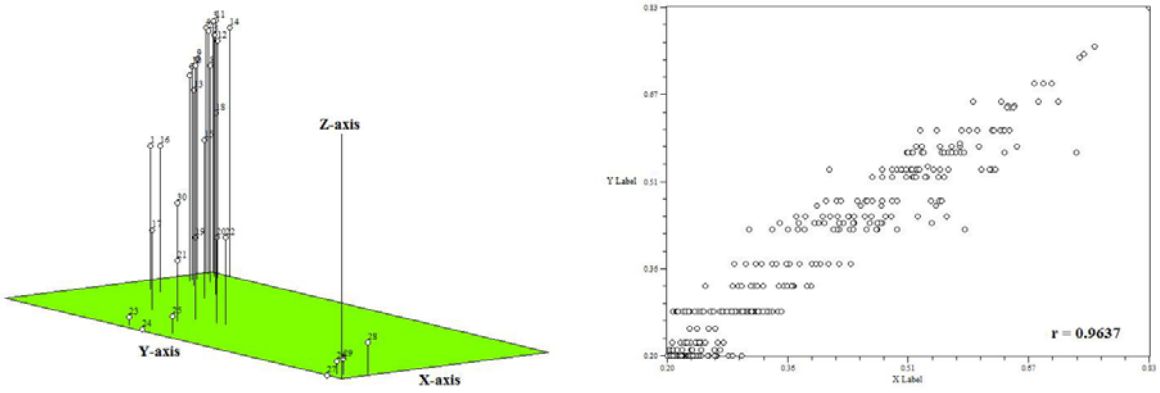

Fig 4 & 5: Principle coordinate analysis & Mantel Z correlation of the study

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