Project number		M161/11
Project title		Fingerprinting of maize genotypes
Project manager		CMS Mienie
Co-worker(s)	Internal	RE Terblanche, VJ Gobiyeza, AP Fourie
	External	None
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Final abstract

One thousand and forty three maize genotypes including white and yellow maize inbred lines as well as hybrids from the public germplasm collection were characterized with 80 microsatellite markers, distributed throughout the genome. A total of 1 874 alleles were amplified and used in genetic diversity analysis. Principal coordinate analysis confirmed the geographical distribution of breeding lines. Cluster analysis using Rogers distance measures placed the breeding lines in several clusters and corresponded well with known pedigrees. Lines with mixed origin were classified in separate clusters and duplicate entries in the collection were identified. These mixed lines could not be placed in known heterotic groups, but could rather be used to identify new groups to use in the breeding programme. The genetic distances determined in the study can be applied to plan a more focused breeding programme.

Keywords: genetic distances, maize, microsatellites

Introduction

Maize is one of the most widely grown crops worldwide and certainly an important staple food in the African diet. It is used as human food, animal feed and provides an important source of income and employment for a large proportion of the population. Maize breeding is monopolized by large multi-national private companies, with little attention given to more specific needs of local markets. The public sector breeding programme is essential in breeding and providing varieties for this niche market as well as providing inbred lines to local private companies. Due to ever decreasing research funds, it is however, essential to scale down the amount of test crosses and the size of current breeding programmes.

Genetic diversity among inbred lines has traditionally been based on morphological data such as endosperm type, pedigree record and amount of heterosis expressed by the hybrid. This method has several limitations as morphological characters do not always reliably portray genetic relationships due to environmental interactions. Test cross designs with numerous testers are extremely expensive, labour intensive and require large-scale field evaluations. Molecular analyses can lower the cost involved in plant breeding. It also provides a means for determining the purity of hybrids produced in the seed industry and can save seed companies and farmers money in terms of ensuring the quality of the seed. Knowledge of the relationships among lines would also help identify a set of inbreds that have maximal diversity for the analysis of the effects of genetic background (Liu *et al*., 2003). The ideal marker system is highly polymorphic, co-dominant, accurate, reproducible, high-throughput and low cost (both in terms of capital investment and cost per assay). Until recently, Simple Sequence Repeats (SSR) or microsatellites, have been the genetic markers of choice, because they are economical to score, have high allelic diversity and are usually selectively neutral (Smith *et al*., 1997). Large numbers of single nucleotide polymorphisms (SNPs) are already available and have been used extensively in genotyping maize. However, it is still not cost effective in South Africa to genotype large numbers of inbreds for the purpose of association analysis and it is thus necessary to identify a set of lines that capture the maximum number of alleles or haplotypes and determine population structure. Hamblin *et al*. (2007) compared the use of SSR and SNP markers in maize. The different mutational properties of these two classes of markers resulted in differences in heterozygosities and allele frequencies, but SSRs performed better in clustering germplasm into populations. SSRs provided more resolution in measuring genetic distance based on allele-sharing. Their results suggested that large numbers of SNP loci would be required to replace highly polymorphic SSRs in studies of diversity and relatedness (Hamblin *et al*., 2007). The relationship between mid-parent heterosis of single-cross hybrids and the genetic distance of their parental inbreds, determined with molecular markers, were investigated

both in theory (Charcosset and Essioux 1994) and numerous experiments with maize and other crops (Brummer 1999). Xia *et al*. (2004) concluded from their study of CIMMYT sub-tropical maize inbreds that the SSR based genetic distances, calculated with a modified Roger's distance measure, in combination with field evaluations provided a solid basis for the detection of heterotic groups.

The objectives of our research were to:

i) Fingerprint local maize genotypes according to the most appropriate and cost-effective procedure for determining genetic distances of breeding lines and

ii) Compile a database of local maize breeding material according to DNA data.

Materials and methods

Plant material

Seeds from breeding lines and hybrids were obtained from plant breeders at ARC-GCI in Potchefstroom and Cedara, South Africa and planted in small pots for germination. Young leaves from approximately twenty plants from each line were harvested and two bulks made up from equal amounts of leaf material from ten plants each. We assumed that the bulk sample will capture all heterozygosity present in the genotypes. Leaf material was freeze dried and ground to a fine powder.

SSR genotyping

DNA was extracted from each bulk sample using a modified CTAB extraction technique (Saghai-Maroof et al., 1984) and diluted to 50 ng/µl. Microsatellite marker genotyping involved the use of 96 fluorescently labelled SSR selected from published markers (www.agron.missouri.edu/ssr.html) to be evenly distributed throughout the maize genome (Figure 1). The SSR primers were labelled with four different dyes (6FAM (blue), HEX (green), NED (yellow) and PET (red)) and combined in 12 multiplex groups (Table 1) with each containing seven to ten primer pairs according to colour and size avoiding overlapping of the same colour. PCR amplifications were carried out on a Techne 384-well thermal-cycler in a final reaction volume of 5-10 µl, containing 50 ng genomic DNA, 1X multiplex PCR master mix (Qiagen, Valencia, USA) containing [HotStarTaq DNA polymerase, multiplex PCR buffer, 2 mM $MgCl_2$, 250 mM of each dNTP (dATP, dCTP, dGTP, dTTP)], 0.05 µM of each multiplex fluorescent primer mix. The PCR products were diluted 1:20 and 1 µl added to HiDi-formamide (Life Technologies) containing 0.12 µl GeneScan-LIZ500 as size standard and electrophoresed on a ABIPrism3130xl sequencer. Fragments were analysed with Genemapper 4.0 (Applied Biosystems) software and allele sizes verified. A list of microsatellite loci and their chromosomal locations is given in Table 1.

Data analysis

Genetic distances of breeding lines and hybrids tested were calculated with the Powermarker ver 3.25 (Liu and Muse, 2005) using the Rogers 1972 parameter. The Polymorphic Information Content (PIC) for each marker was determined using Powermarker ver 3.25 software. Average linkage (UPGMA, Unweighted Paired Group Method using

Arithmetic Averages) clustering was calculated based on Rogers Distance (RD) estimates between pairs of inbred lines for the yellow and white lines and hybrids separately. To evaluate the robustness of the UPGMA dendrogram, the cophenetic correlation was calculated (Sneath and Sokal, 1973) utilizing NTSYS ver 2.21 software (Rohlf, 2009). The data was transformed with the dcenter module and eigenvectors calculated. Principal coordinates analysis (PCoA) was carried out on the calculated distances using NTSYS ver. 2.21 software (Rohlf, 2009).

Results

A total of 96 SSR primer sets were used to fingerprint the breeding lines and hybrid samples. The SSR markers with more than 20 % missing values were discarded and data from 80 markers were used in the final analysis. The PIC values of markers varied between 0.2 and 0.9. The average number of alleles per locus was 12.7 for a total of 1 043 genotypes tested, with a total of 1 874 alleles amplified (Table 1). The number of alleles amplified varied between 6 and 36 per locus for the total population screened. When looking at the separate populations tested, the mean number of alleles per locus was the highest for the yellow maize breeding lines, which also had the most entries in the screening programme (Table 2).

In PCoA of the RD of the breeding lines, the white and yellow groups were clearly separated in two clusters, with relatively few overlapping genotypes (Figure 2). When regarding the two localities where the breeding lines originated, it was clear that the white lines could be separated into three clearly distinguished groups, with the lines from Potchefstroom grouping in two separate clusters (Figure 3). The yellow breeding lines were much more heterogeneous between the two localities (Figure 4), with only a few lines falling in definite clusters. The lines originating from CIMMYT, Mexico, clustered together with the Cedara material for both white and yellow lines. These lines were used in the Cedara breeding programme over a long period of time and therefore the result was as expected. The white lines were compared to some anchor lines with known heterotic grouping (Figure 5 and 6). The lines with known pedigrees related to K64, clustered together (r=0.91), with only one line with larger RD from the rest of the group. A large number of white lines clustered with the Lancaster line Mo17. The rest of the groups could be separated into distinguishable clusters by the UPGMA derived dendrogram (Figure 6). PCoA and cluster analyses of yellow breeding lines revealed a small group of lines clustering with Mo17 (Figure 7 and 8) (r=0.72), with the rest of the lines with mixed origin. The cluster analysis (Figure 8) identified several related groups of lines indicating close genetic relationships. These results show the extent to which these lines were interbred in the breeding programme over several decades.

One hundred and ninety three hybrids were included in the study to explore the diversity achieved and compare the genotypes to locally cultivated maize cultivars produced by private companies. Five distinct groups of ARC hybrids could be identified. No overlap could be seen between ARC hybrids and those from the private companies tested (Figure 9).

Cluster analysis (Figure 10) revealed several identical hybrids included in the entries (r=0.88).

Discussion

Maize breeding is a never-ending challenge for developing new inbred lines and cultivars with improved yield, as well as tolerance to a diverse spectrum of biotic and abiotic stress conditions, e.g. diseases, pests, low soil fertility and drought. New cultivars must also conform to changing industry requirements such as grain quality for milling and ethanol production. Climatic changes such as global warming pose new challenges to breeders for developing cultivars that will perform well under changed conditions. Although seed companies produce their own hybrids they remain dependent on foundation-seed suppliers for inbred lines and even sometimes locally adapted hybrids.

The ARC-GCI has a large germplasm collection of inbred lines, containing both white and yellow lines, at its disposal. These lines needed to be characterized on a molecular level to determine close relationships or genetic distances to narrow down the possibilities for utilizing this material in test crosses. It also served the purpose of identifying duplicate lines, as well as genetic drift within well-known breeding lines.

During the past decade, the technology used in fingerprinting of field crops developed from restriction fragment length polymorphism (RFLP) to amplified fragment length polymorphism (AFLP), SSR and single nucleotide polymorphism (SNP). The RFLP technique was laborious and time consuming and only single data points could be analyzed per reaction. The AFLP technique was less time consuming and labour intensive and rendered up to a 100 data points per reaction, but it was difficult to duplicate results over time and between different laboratories. The SSR technique is a simple polymerase chain reaction (PCR) technique, which lends itself to high throughput of samples when multiplex reactions are used with an automatic genetic analyzer like the ABI3130xl. The technique is also easy to duplicate among different laboratories. Although the SNP technique can analyze thousands of data points per run, one has to look at the application that you need the analysis for. The SSR technique can be utilized to give low coverage of the whole genome of maize, giving an overall indication of the genetic material present in a specific population. Knowledge of the relationships among lines can help identify a set of specific inbreds that have maximal diversity for the analysis of the effects of background (Liu *et al*., 2003).

SSR markers were utilized in this study in the genotyping of the germplasm collection of the ARC-GCI from two different localities and were used at a density of 8 markers per chromosome. The 80 markers utilized amplified a total of 1 874 alleles, which were used in determining the genetic relationships among the inbred lines and hybrids. Principal coordinates analysis clearly separated white and yellow lines as expected from the pedigree data that were available from the breeding programmes. These groups were further analysed separately. In the analysis of white breeding lines based on Rogers estimates, the lines clearly separated in two diferent groups with PCoA analysis. A large group of lines were grouped together with Mo17, a Lancaster line, in accordance with pedigree data. All the white lines could not be grouped into specific heterotic groups and were of mixed origin. These lines were inbred between the different groups in the breeding programme and it is thus not possible to group them with currently known heterotic groups. The cluster analysis gave a more detailed grouping of lines (Figure 6): (1) The K-group was derived from K64 crosses. K64 is a Kansas inbred known for fairly good drought tolerance. (2) The CB group derived from inbreeding US Corn belt hybrids clustered with Mo17, a Lancaster line. (3) The 3rd group was identified as a mixed group containing combinations of Corn belt material with Sahara or I-group lines as well as the maize streak virus resistant lines. The M37W group was derived from the Australian inbred 21A. Lines originating from CIMMYT also clustered in this group in a sub-group. (4) The I137TN group is the major South African group known for its superior combining ability for grain yield, especially in combination with US Corn belt material. Several entries from the Cedara yellow inbred lines could be identified as duplicates. A large group of these inbred lines (CNO-numbers) were closely related to lines originating from CIMMYT, Mexico. The yellow corn belt related lines are clustered in a very complex pattern and could not be classified in specific heterotic groups, as was the case with the white inbred lines. It is clear that the breeding lines need to be classified in new groups according to the current genetic data that was made available in this study. Hybrid entries from the public germplasm source differed from commercial hybrids released by private companies.

The results from the genetic distances as calculated in study led to planning of a more focused breeding programme, conserving manpower and other resources, with better outputs. Lines with mixed origin could be classified and more informative decisions made on the crosses to be tested. A subset of these crosses has been tested in field trials and high yields (data not shown) confirmed the genetic distances calculated with the molecular data.

Acknowledgements

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Table 1 Multiplex grouping of SSR markers.

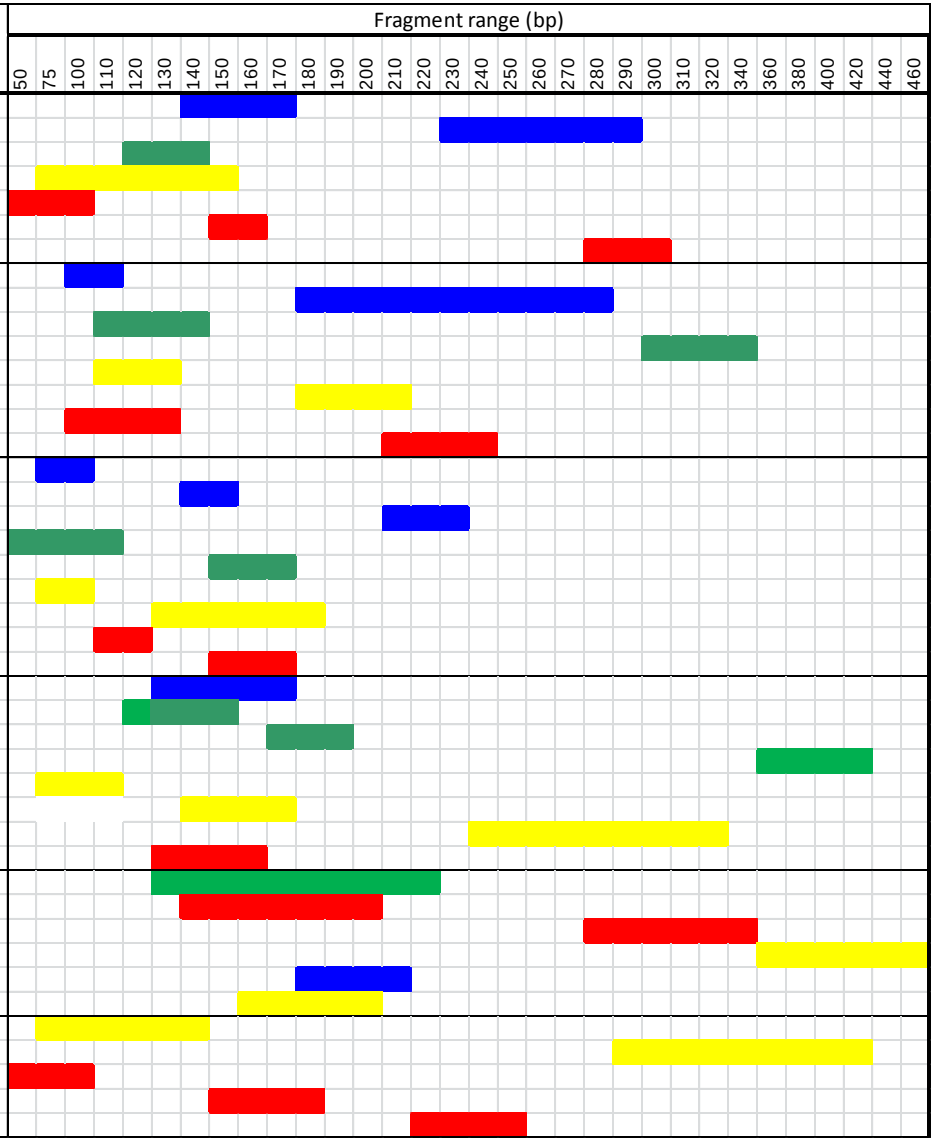
(See next page)

Table 2 Average and range of number of alleles per locus for 1043 maize genotypes.

Population	**Number of genotypes**	**Number of alleles**	**Mean alleles / locus**	**Range alleles**
White breeding lines	207	575	7.2	2-20
Yellow breeding lines	643	770	9.5	2-24
Hybrids	193	529	6.61	2-19
Total	1043	1874	12.7	6-36

Panel	Marker	Dye	Bin	Repeat	Fragment sizes (bp)	Fragment range (bp)
M1	umc2162	PET	6.06		135-162	
	umc1993	6-FAM	10.06	(CT)17	73, 75, 78, 79, 89, 90, 92, 94, 97	
	phi233376	6-FAM	8.03	CCG	(142-154) 137, 140, 149, 153, 157	
	phi046	VIC	3.08	ACGC	(62-66) 62, 65	
	phi331888	VIC	5.04	AAG	127, 130, 133, 136	
	phi062	VIC	10.04	ACG	158, 161, 164	
	phi089	NED	6.08	ATGC	(87-95) 84, 92	
	phi339017	NED	1.03	ACC	148, 151, 154, 157, 160, 163	
M2	umc1256	VIC	2.09		103, 117, 126, 133, 142, 145, 164, 180, 182	
	phi101049	VIC	2.1	AGAT	226, 230, 234, 238, 266, 270, 274	
	phi108411	NED	9.05	AGCT	113, 121, 125	
	phi96342	NED	10.02	ATCC	234, 238, 242, 246, 250	
	umc2217	6-FAM	1.04		146-167	
	umc1279	PET	9	(CCT)6	92, 85, 98, 101	
	phi104127	PET	3	ACCG	(157-169)150, 153, 158, 160, 164, 168, 172	
	phi96100	PET	2	ACCT	(269-297) 269, 277, 281, 283, 285, 290,	
M3	bnlg118	6-FAM	5.07	di	89-122	
	phi076	6-FAM	4.11	AGCGGG	(161-179) 160, 166, 169, 171	
	phi032	6-FAM	9.04	AAAG	(233-241) 232, 238	
	phi114	VIC	7.03	GCCT	(137-169) 135, 160, 165, 168, 171	
	umc1196	NED	10.07	CACACG	(137-161)138, 139, 142, 147, 151, 153,	
	phi050	PET	10.03	AAGC	(80-86) 75, 80, 82, 86, 88	
	phi123	PET	6.07	AAAG	146, 150, 154	
M4	umc1304	6-FAM	8.02	(TCGA)4	(129-137)119, 124, 128, 134	
	phi053	6-FAM	3.05	ATAC	(169-195) 157, 168, 171, 173, 175, 190,	
	umc1295	VIC	7		78, 83, 84, 87, 90, 92, 97	
	umc1657	VIC	9		144, 149, 153, 158, 160, 163	
	phi396160	VIC				
	umc1757	NED	4.01	(TCC)7	135, 143, 146, 154, 157	
	umc1109	PET	4.1	(ACG)4	104, 110, 113, 116	
	phi059	PET	10.02	ACC	147, 153, 156, 159	
M5	phi308707	6-FAM	1.1	AGC	116, 119, 122, 125, 128, 131, 134	
	umc1644	6-FAM	3.06	(TTG)8	149, 153, 159, 160, 162	
	phi072	VIC	4	AAAC	(143-167) 138, 141, 150, 155, 162	
	phi011	VIC	1.09	AGC	206, 209, 212, 215, 218, 221, 224, 227,	
	phi008	NED	5.03	GGC	100, 103, 106	
	phi079	NED	4.05	AGATG	(180-195) 161, 179, 184, 187, 189, 194	
	phi093	NED	4.08	AGCT	(274-294) 283, 284, 286, 288, 290, 292,	
	bnlg391	PET	6.01	-	80-104	
	phi112	PET	7.01	AG	151, 153, 155, 157, 159, 161	
M6	phi109275	6-FAM	1.03	AGCT	120-140	
	phi041	6-FAM	10	(CAT)5	(196-216) 182, 185, 190, 195, 196, 199,	
	umc1399	VIC	3.07	9CTAG)5	111, 115, 119, 123, 127	
	umc1122	VIC	1.06	(CGT)7	151, 154, 157, 160, 163, 166, 169	
	phi034	NED	7.02	CCT	(122-146) 121, 127, 129, 132, 136, 138,	
	phi031	NED	6.04	GTAC	187, 191, 195, 219, 223, 227, 231	
	umc1126	PET	2	ACC	153-200	
	phi127	PET	2.08	AGAC	(112-126) 114, 116, 126, 128	

Panel	Marker	Dye	Bin	Repeat	Fragment sizes (bp)
	umc1161	6-FAM	8.06	(GCTGGG)5	140, 146, 152, 164, 170
	bnlg1057	6-FAM	1		233, 235, 240, 243, 261, 263, 265, 267,
	phi083	VIC	2.04	AGCT	(125-137)124, 126, 130, 132, 133, 136
	phi070	NED	6		75, 77, 81, 83
	umc1545	PET	7	(AAGA)4	(70-86) 70, 78, 81, 83, 85
	phi029	PET	3.04	AG/AGCG	(148-162) 152, 155, 157, 159, 162
	phi213984	PET	4.01	ACC	287, 299, 302, 305
M8	umc1061	6-FAM	10.06	(TCG)6	101, 104, 107, 110
	bnlg1792k8	6FAM	7		192, 250
	phi452693	VIC	6.04	AGCC	113, 125, 129, 133, 137, 141, 145
	phi227562	VIC	1.11	ACC	307, 310, 316, 322, 325, 334
	phi299852	NED	6.07	AGC	111, 114, 117, 120, 123, 132, 135, 138
	phi073	NED	3.05	AGC	181, 184, 187, 190, 193, 202, 208, 214
	phi328175	PET	7.04	AGG	100, 112, 118, 121, 125, 127, 130
	phi080k15	PET	8		203, 233
M9	umc1407	6-FAM	7		82, 85, 88, 91, 94, 98
	nc130	6-FAM	5	AGC	(140-148) 131, 138, 142
	phi374118	6-FAM	3.02	ACC	217, 220, 223, 226, 229, 232
	phi064	VIC	1.11	ATCC	(73-105) 76, 82, 84, 87,89, 93, 95, 97, 101,
	phi116	VIC	7.06	ACTG/ACG	152, 158, 162, 164, 168, 173
	umc1143	NED	6	AAAAT	(77-87) 68, 70, 72, 74, 76, 79, 81
	umc1136	NED	3.1	(GCA)5	132, 135, 138, 141, 144, 150, 153, 156,
	umc1777	PET	8	AGCC	113, 116, 119
	phi084	PET	10.04	GAA	(150-156) 152, 157, 160, 163
M10	phi100175	6-FAM	8.03	AAGC	133, 137, 141, 175
	phi109642	VIC	2	ACGG	(136-144)129, 133, 138, 142
	phi448880	VIC	9.06	AAG	173, 176, 179, 185, 188
	bnlg2291k4	VIC	4.06	(AG)17	384, 400
	umc1555	NED	2.03	(TTCA)7	71, 83, 87, 91, 95, 99, 103, 111, 115, 119
	phi014	NED			
	bnlg2305k4	NED	5.06	(GA)6	272, 310
	phi065	PET	9.03	CACTT	(131-151)132, 133, 134, 142, 143, 147, 153
M11	bnlg161k8	VIC	6		152, 208
	bnlg1754w3	PET	3.09	(AG)20	145, 160
	umc2105k3	PET	3	(CCAT)4	286, 330
	bnlg1940k9	NED	2.08	(AG)18	407, 420
	bnlg2277	6-FAM	2.02		180-211
	umc1932	NED	7	(GA)8	157-200
M12	bnlg1953	NED	1.02		86-136
	umc1586	NED	9.03	(ATC)5	290-427
	umc1955	PET	1.08		68-80
	umc1603	PET	1	(GCC)4	154-180
	umc1230	PET	2	(TAA)8	228-250



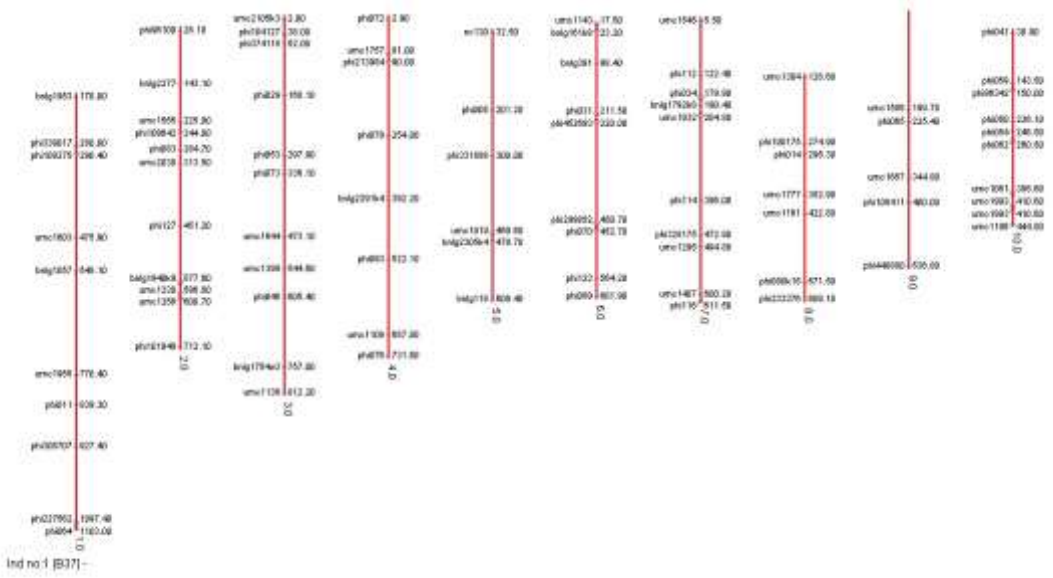


Figure 1 Maize genomic map indicating SSR markers used in the study (based on IBM2 2008 distances).

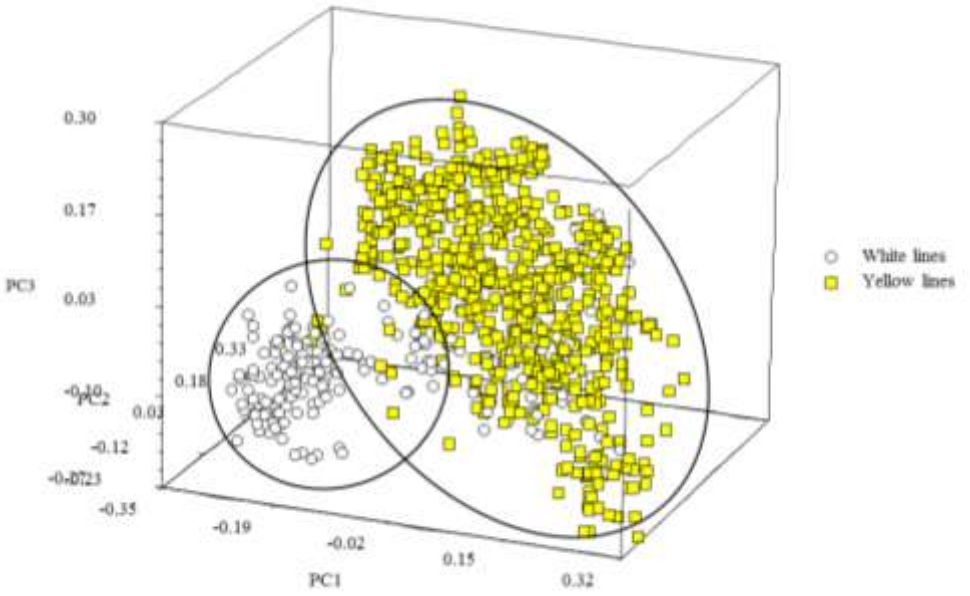


Figure 2 Principal coordinates analysis of white and yellow breeding lines from two localities.

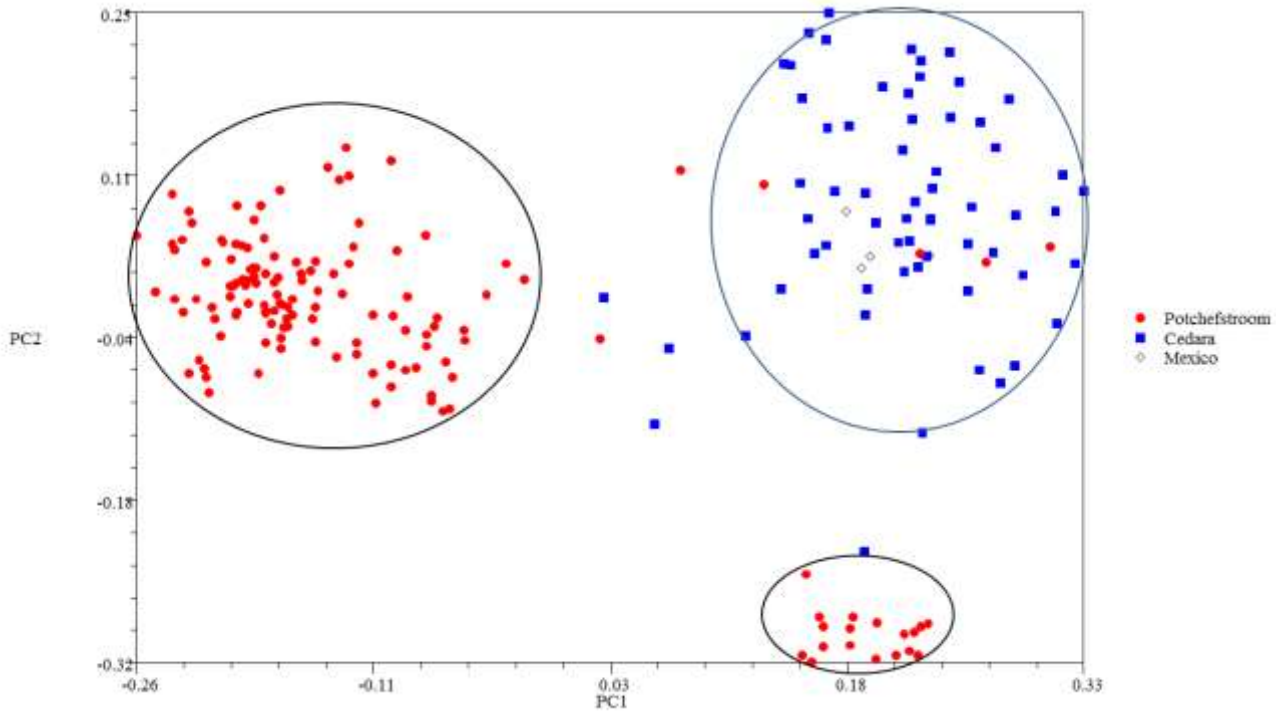


Figure 3 Principal coordinates analysis showing the geographical distribution of white breeding lines.

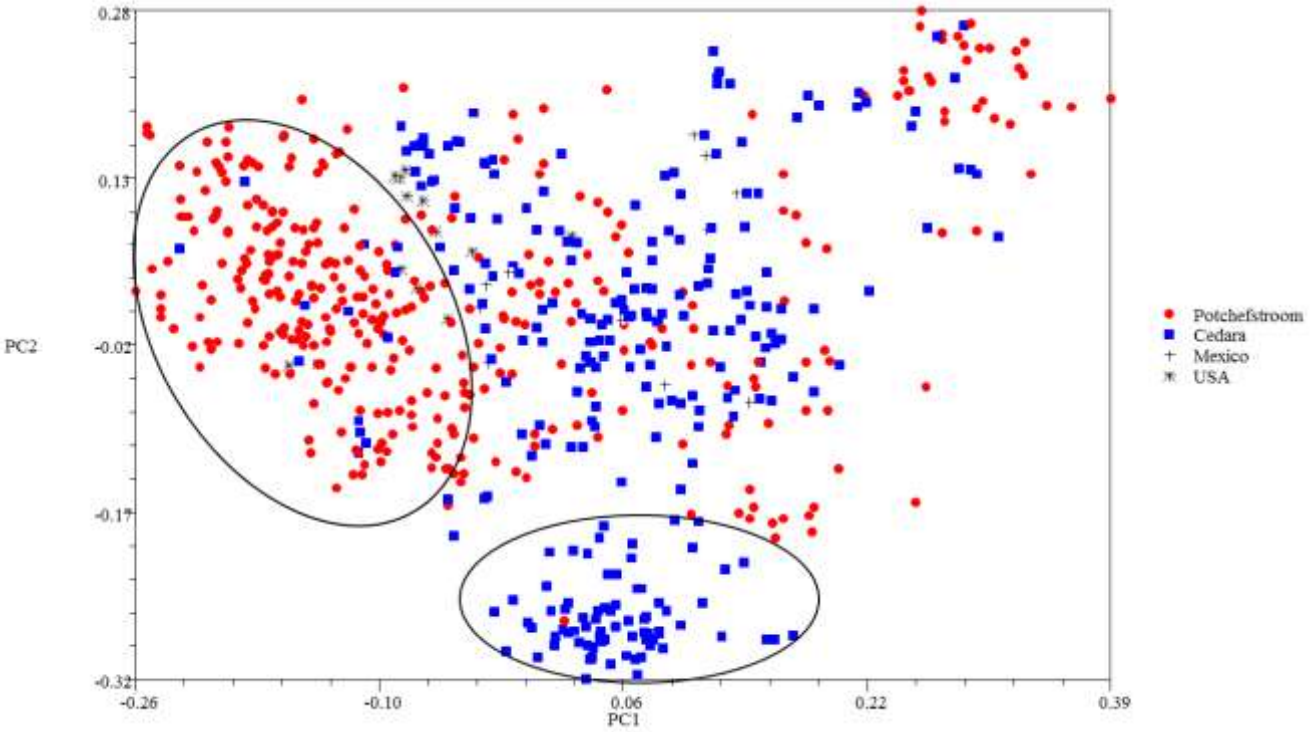


Figure 4 Principal coordinates analysis indicating relationships between yellow breeding lines from Potchefstroom and Cedara.

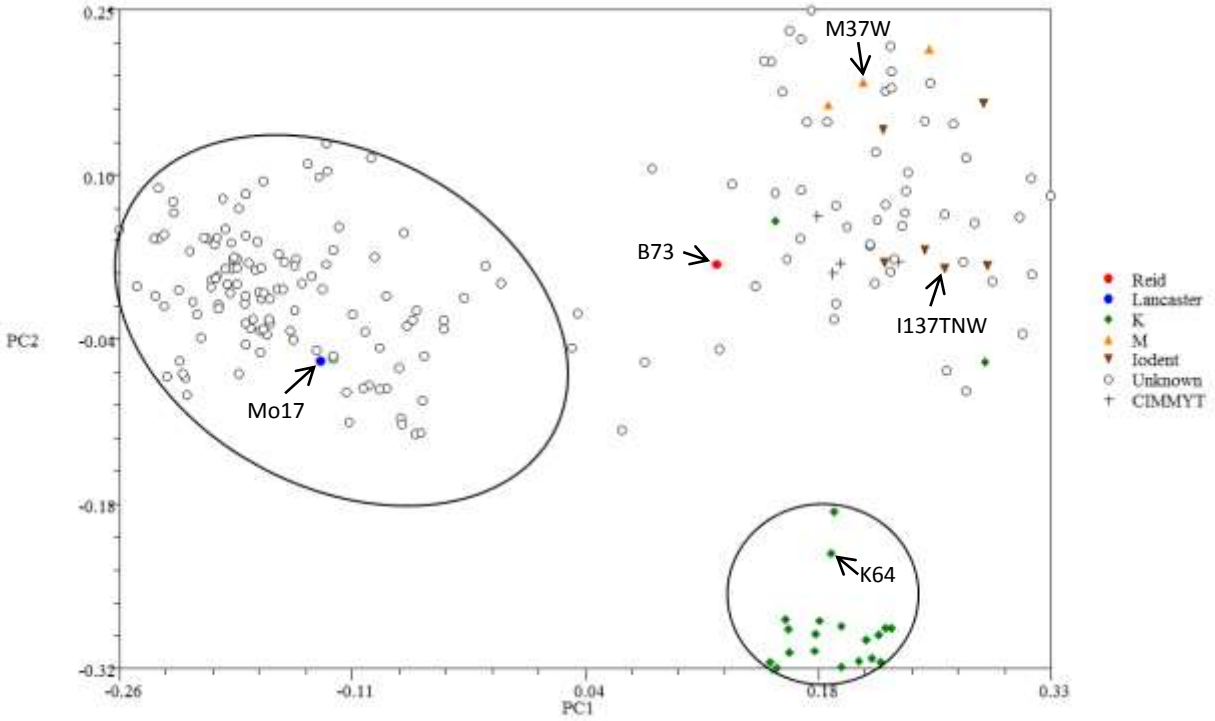


Figure 5 Principal coordinates analysis of white breeding lines compared with anchor lines of known heterotic groups.

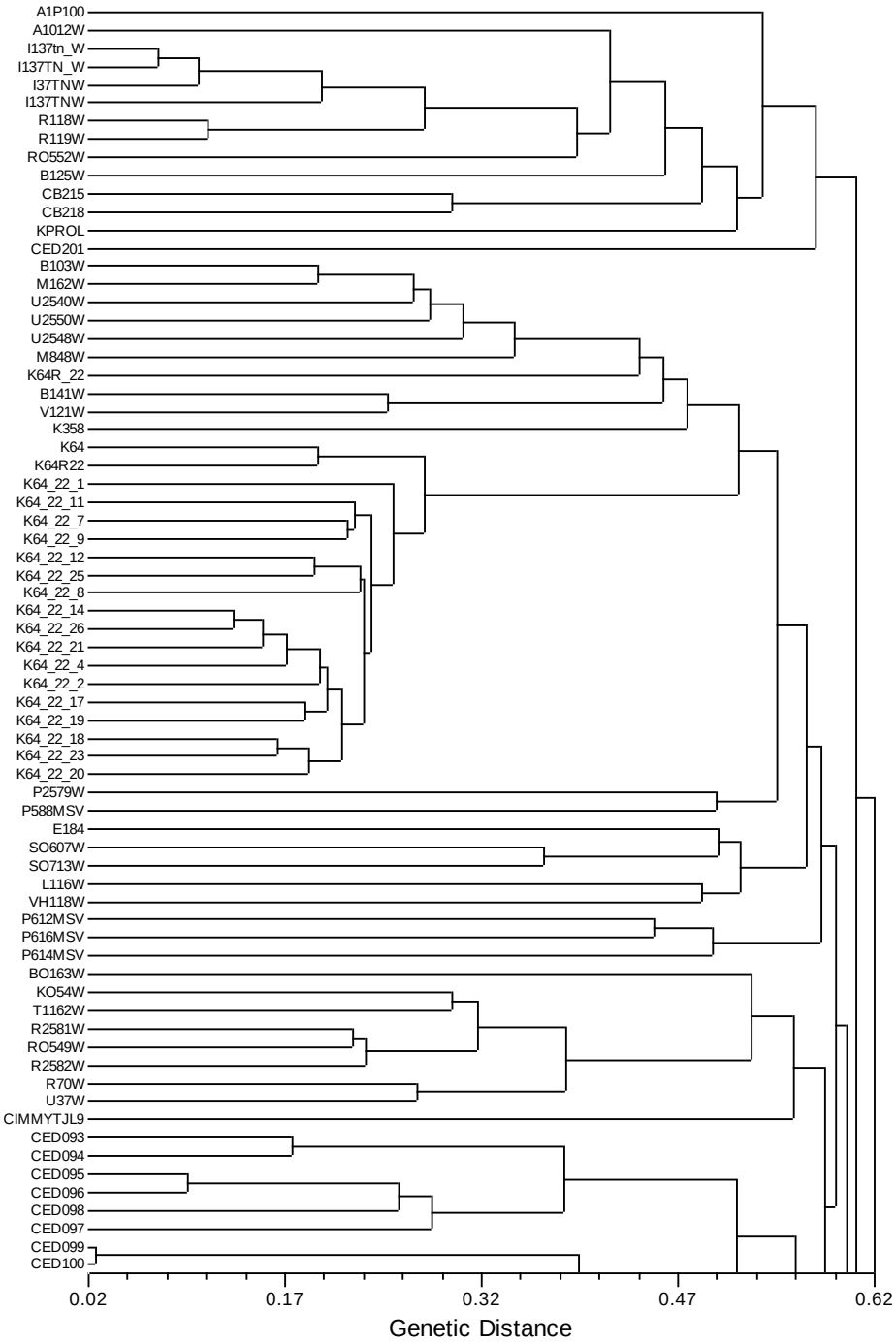


Figure 6 (1) Associations among white inbred lines revealed by average linkage (UPGMA) cluster analysis based on Rogers distance.

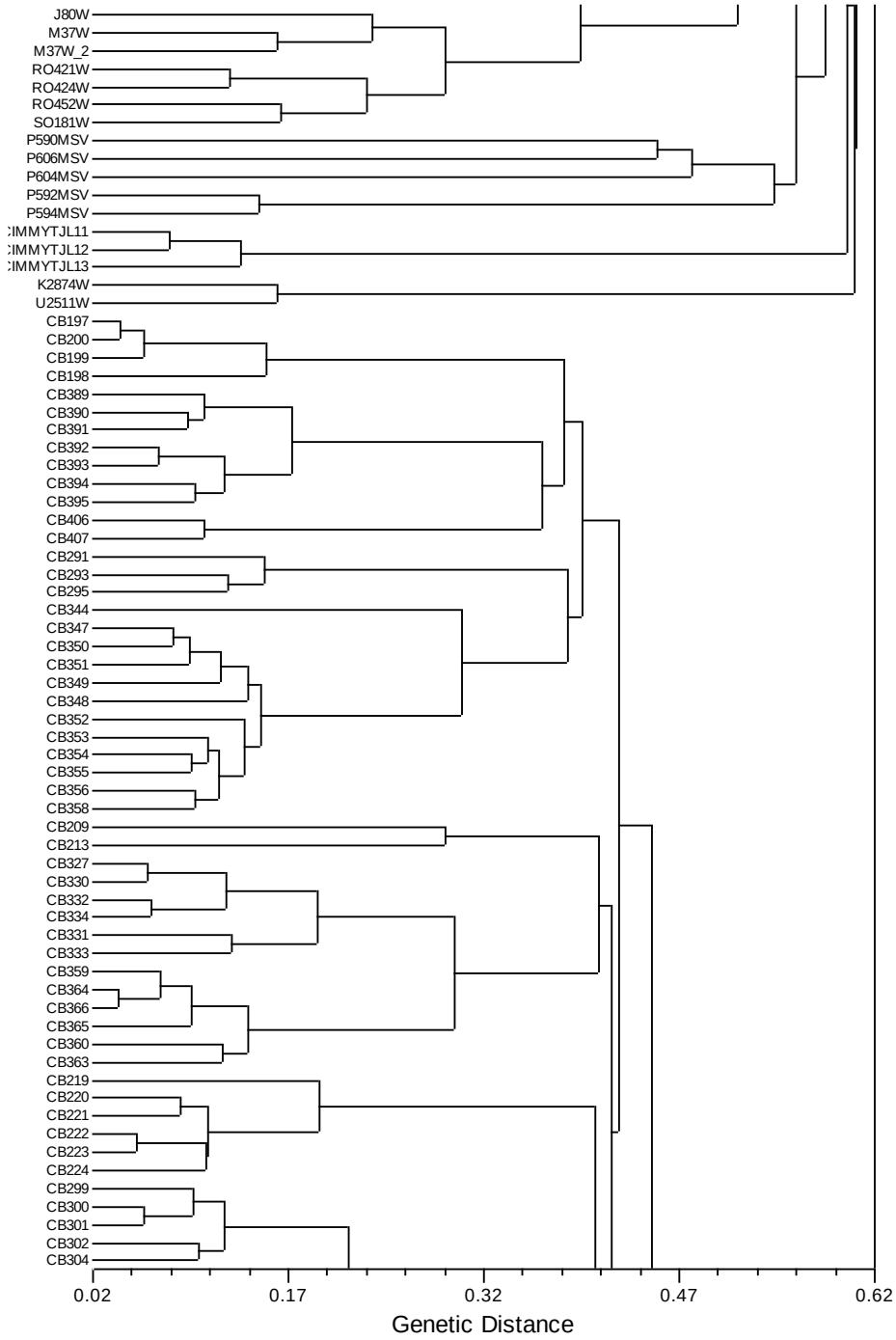


Figure 6 (continued (2)) Associations among white inbred lines revealed by average linkage (UPGMA) cluster analysis based on Rogers distance.

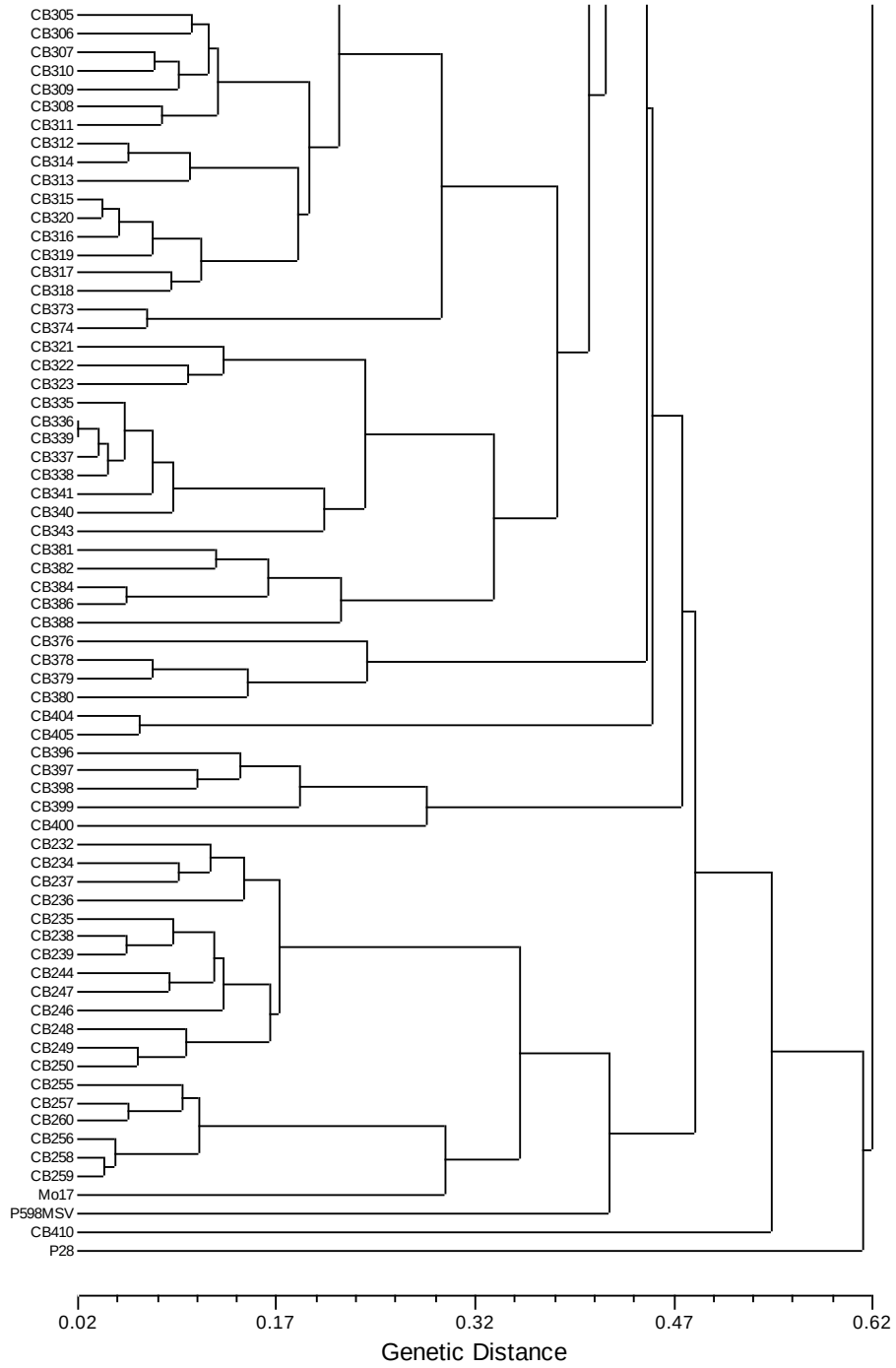


Figure 6 (continued (3)) Associations among white inbred lines revealed by average linkage (UPGMA) cluster analysis based on Rogers distance.

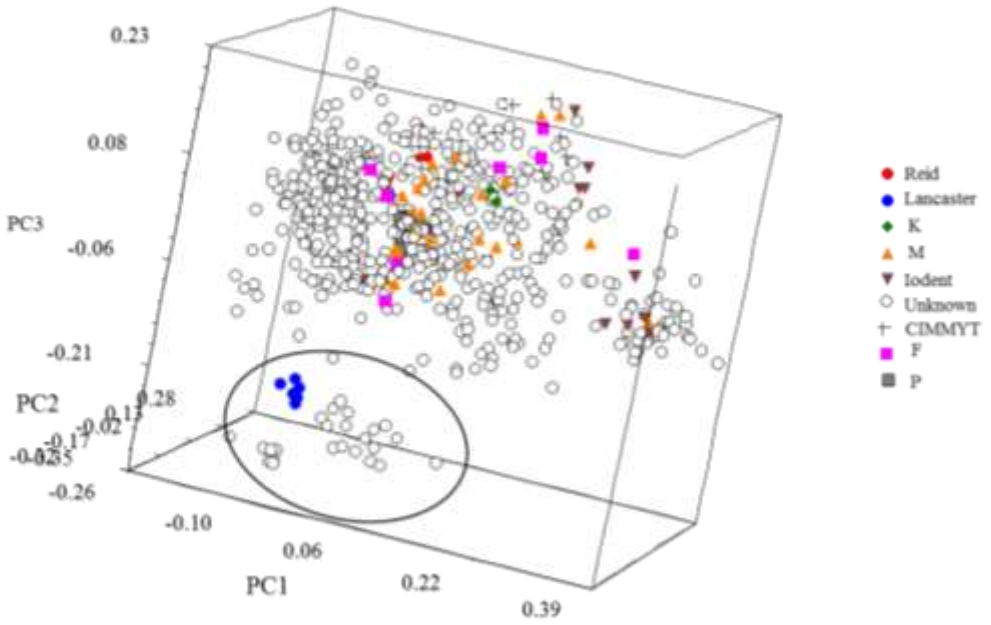


Figure 7 Principal coordinates analysis of yellow breeding lines compared with anchor lines of known heterotic groups.

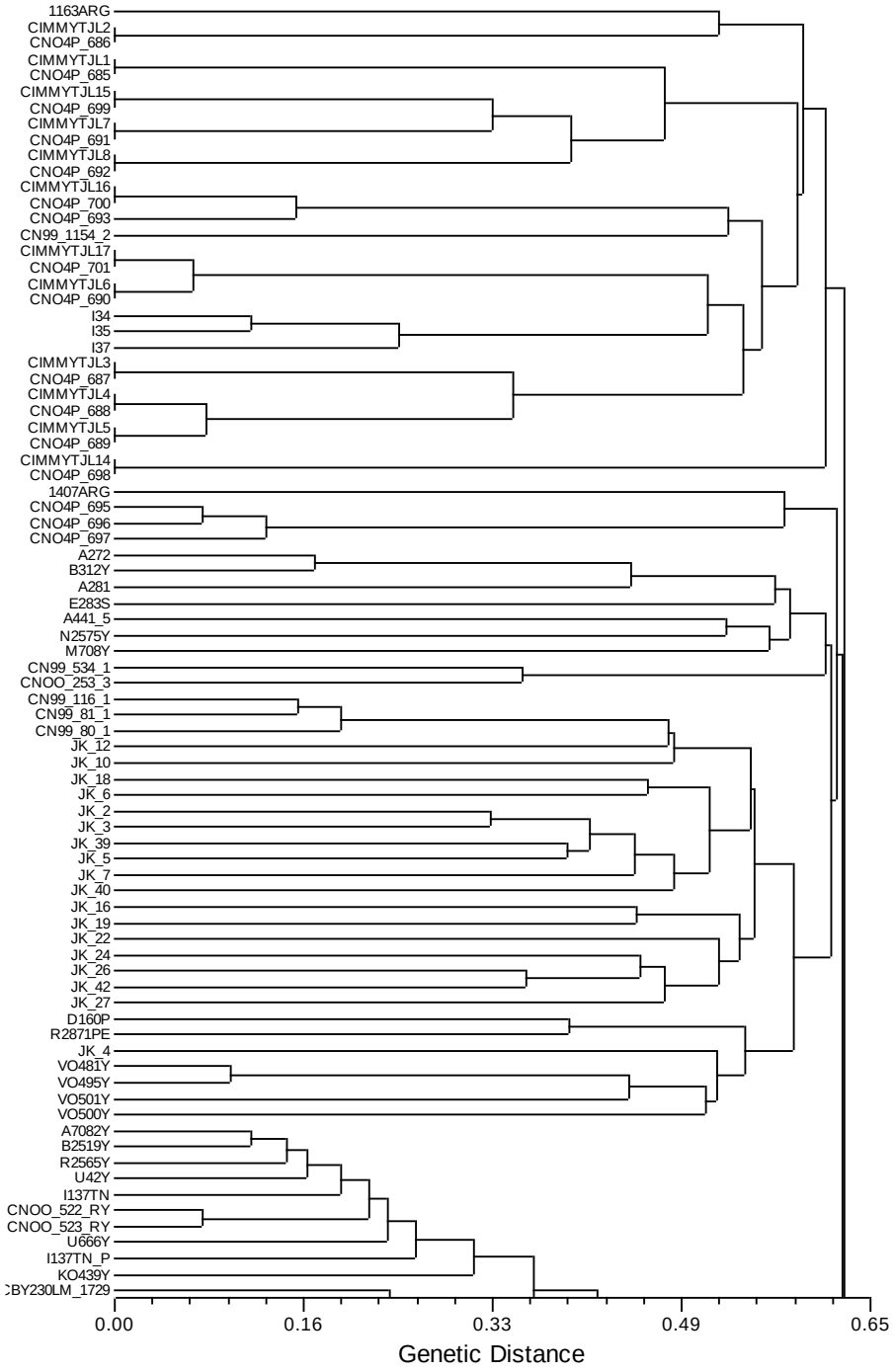


Figure 8 (1) Associations among yellow inbred lines revealed by average linkage (UPGMA) cluster analysis based on Rogers distance.

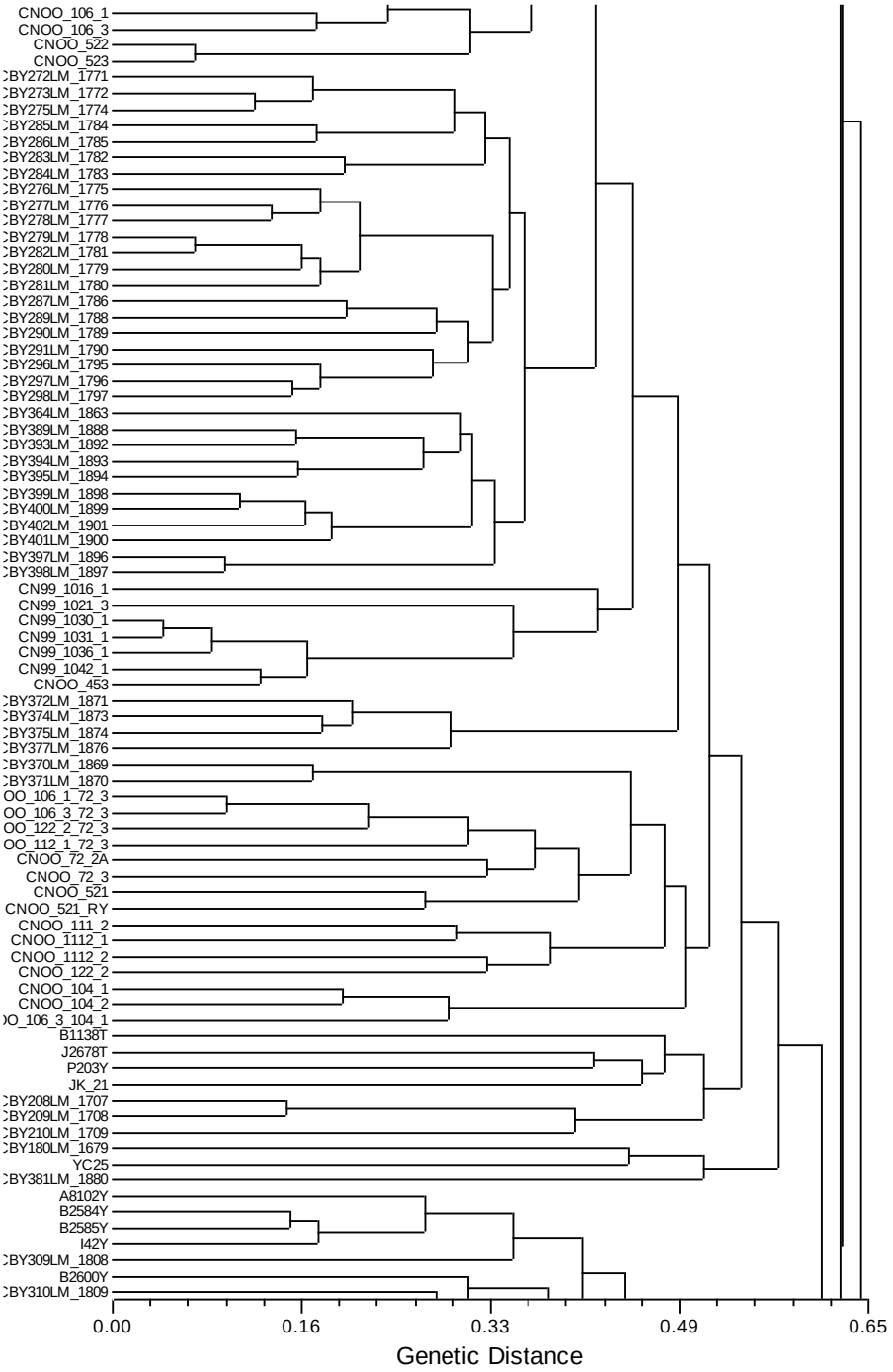


Figure 8 (continued(2)) Associations among yellow inbred lines revealed by average linkage (UPGMA) cluster analysis based on Rogers distance.

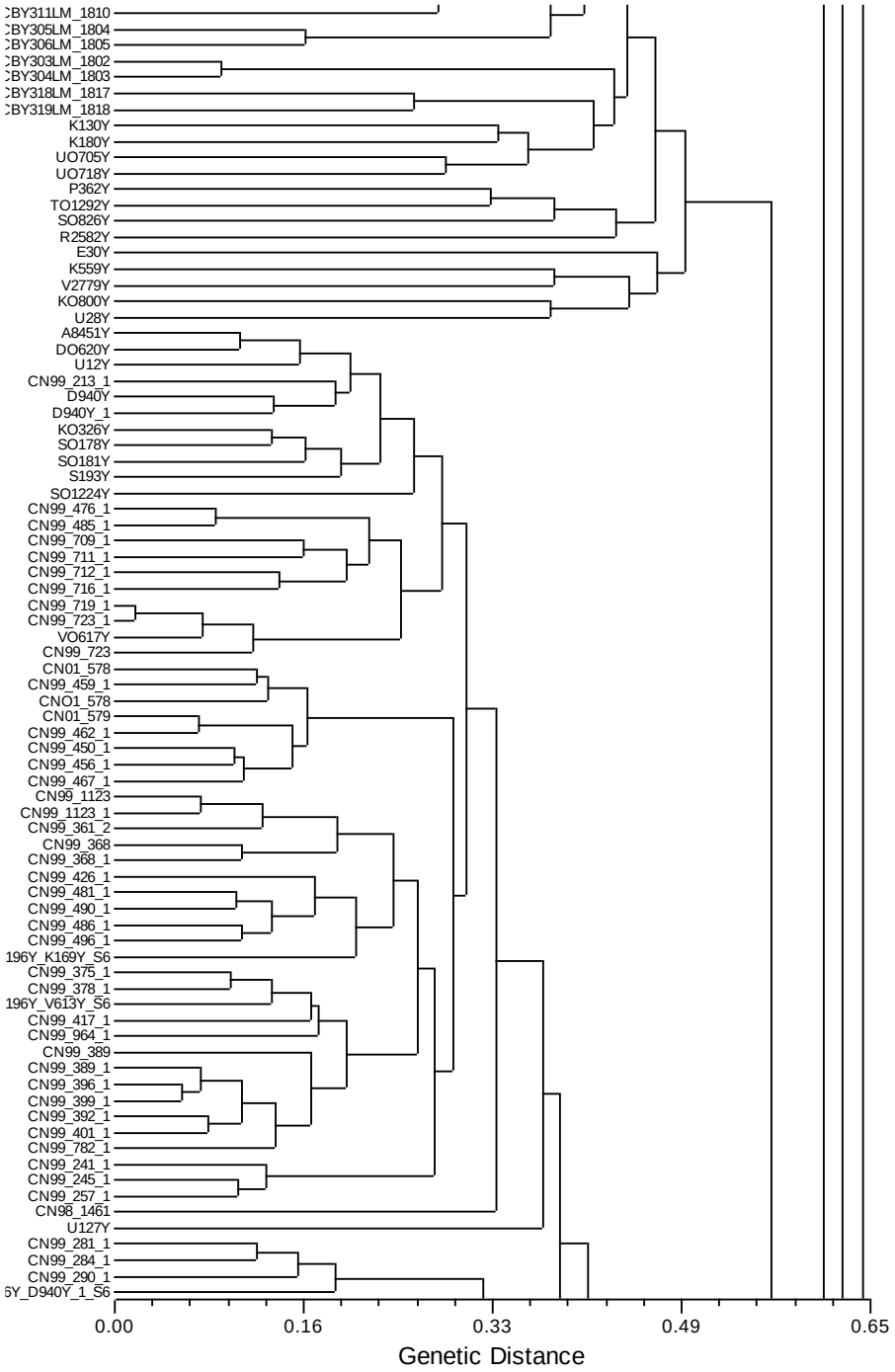


Figure 8 (continued (3)) Associations among yellow inbred lines revealed by average linkage (UPGMA) cluster analysis based on Rogers distance.

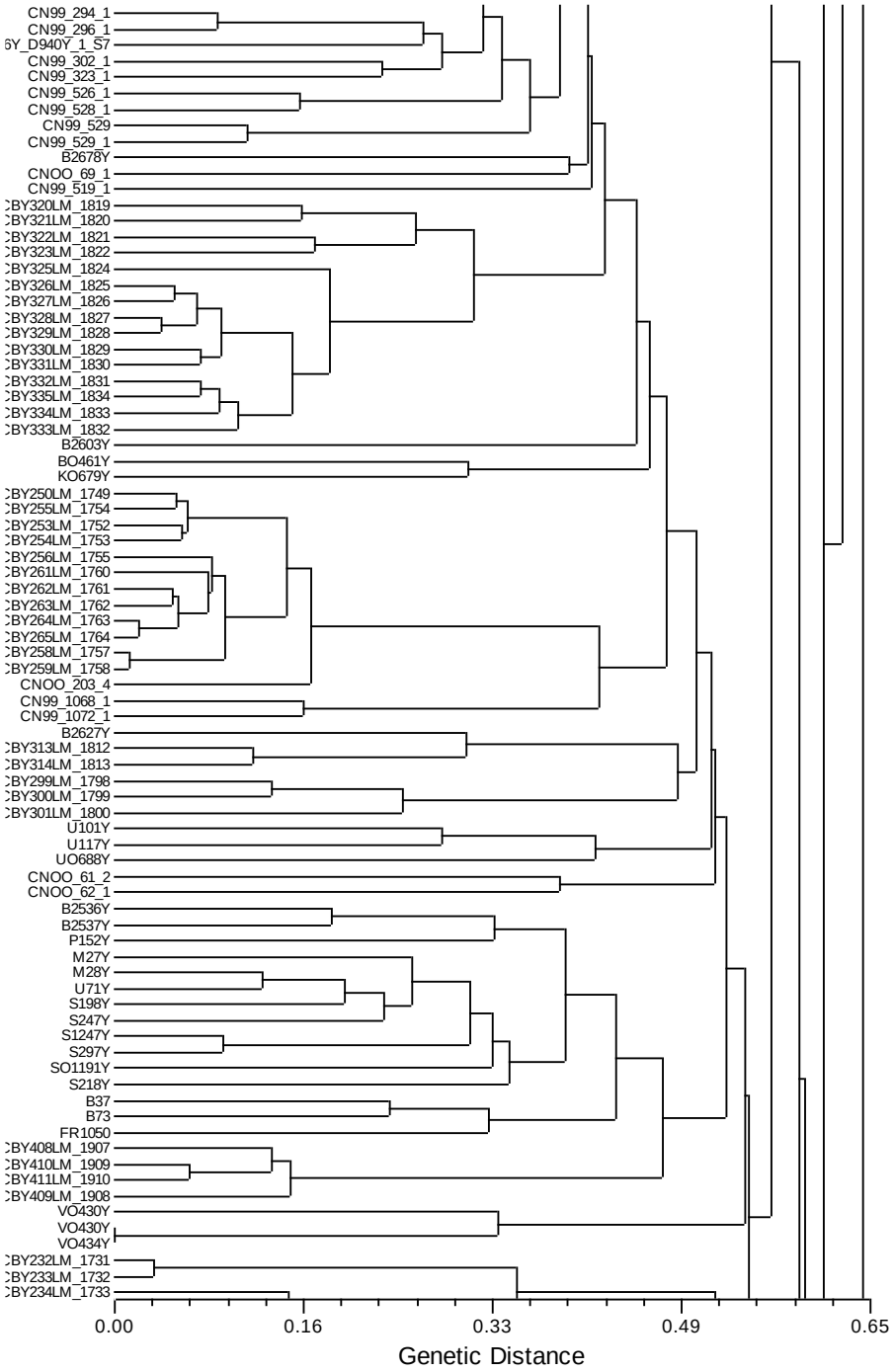


Figure 8 (continued (4)) Associations among yellow inbred lines revealed by average linkage (UPGMA) cluster analysis based on Rogers distance.

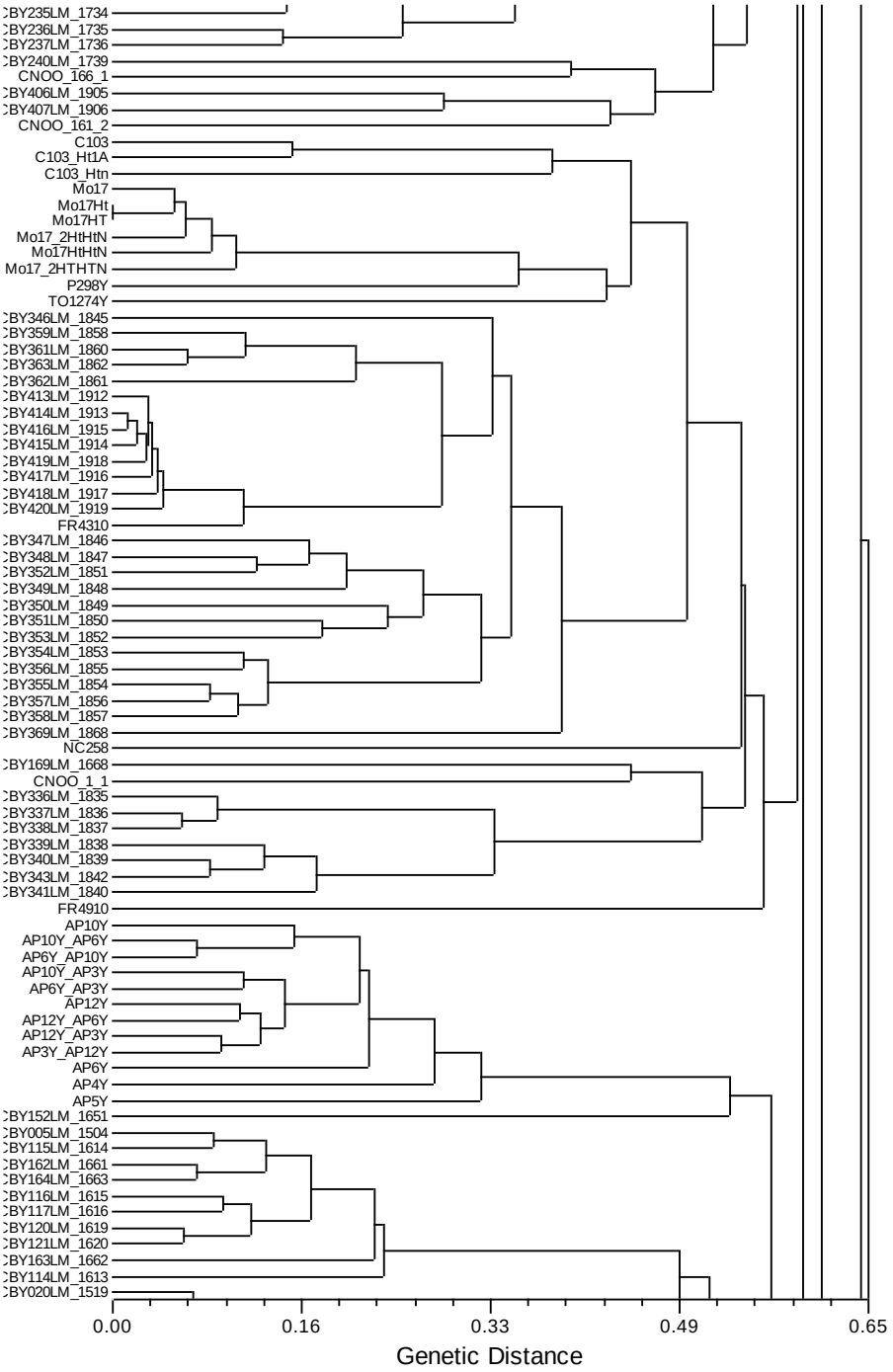


Figure 8 (continued (5)) Associations among yellow inbred lines revealed by average linkage (UPGMA) cluster analysis based on Rogers distance.

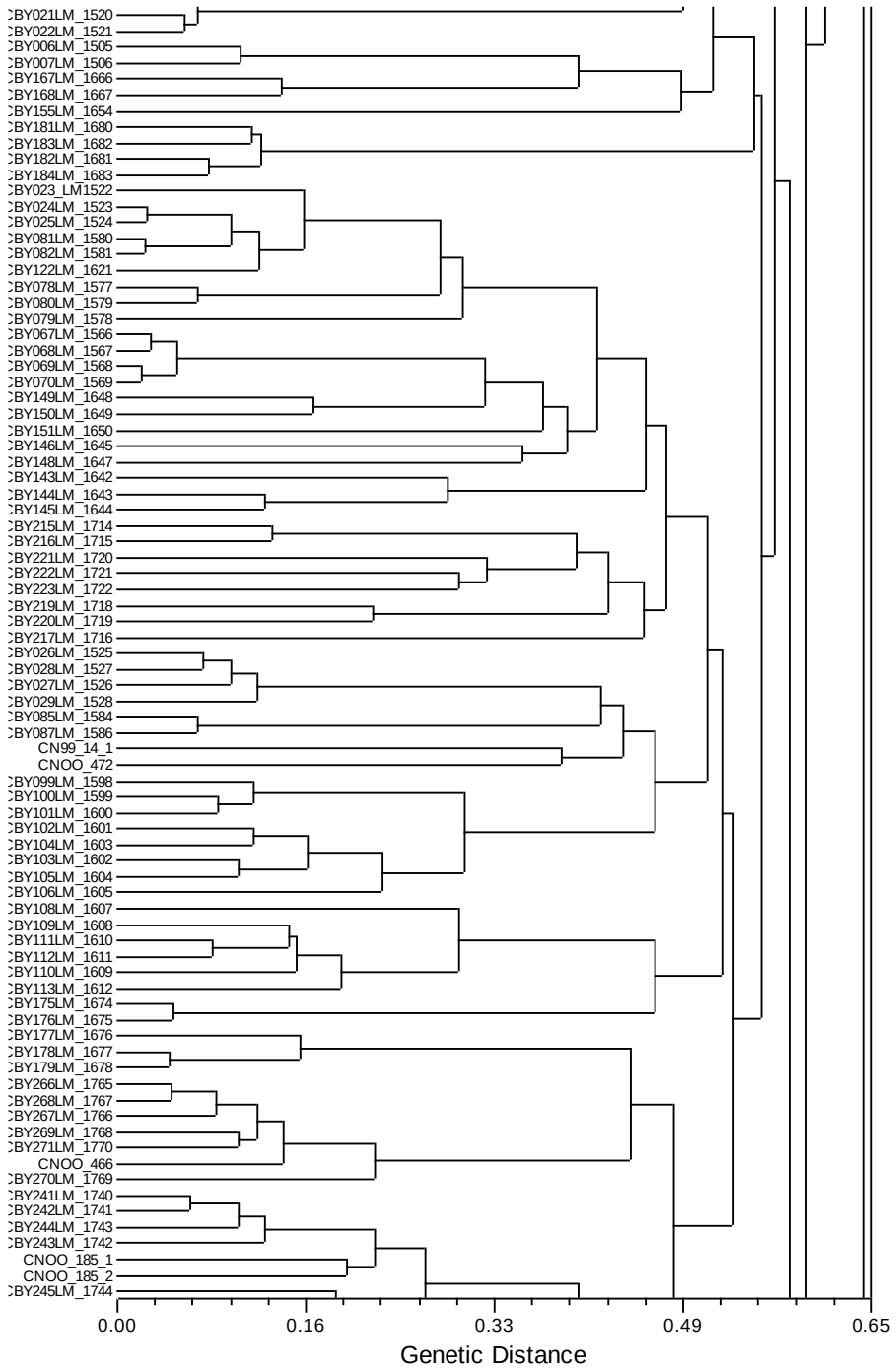


Figure 8 (continued (6)) Associations among yellow inbred lines revealed by average linkage (UPGMA) cluster analysis based on Rogers distance.

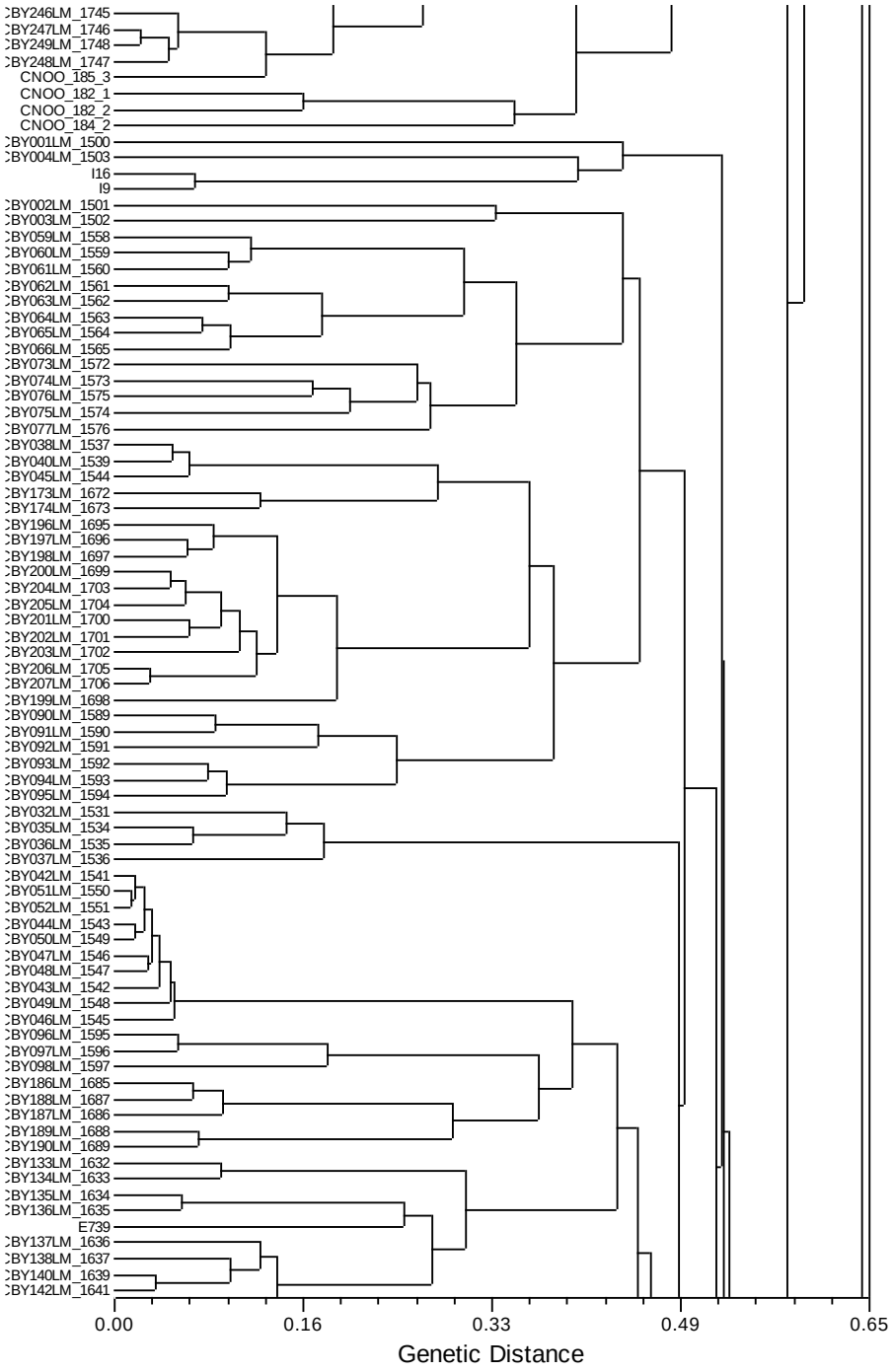


Figure 8 (continued (7)) Associations among yellow inbred lines revealed by average linkage (UPGMA) cluster analysis based on Rogers distance.

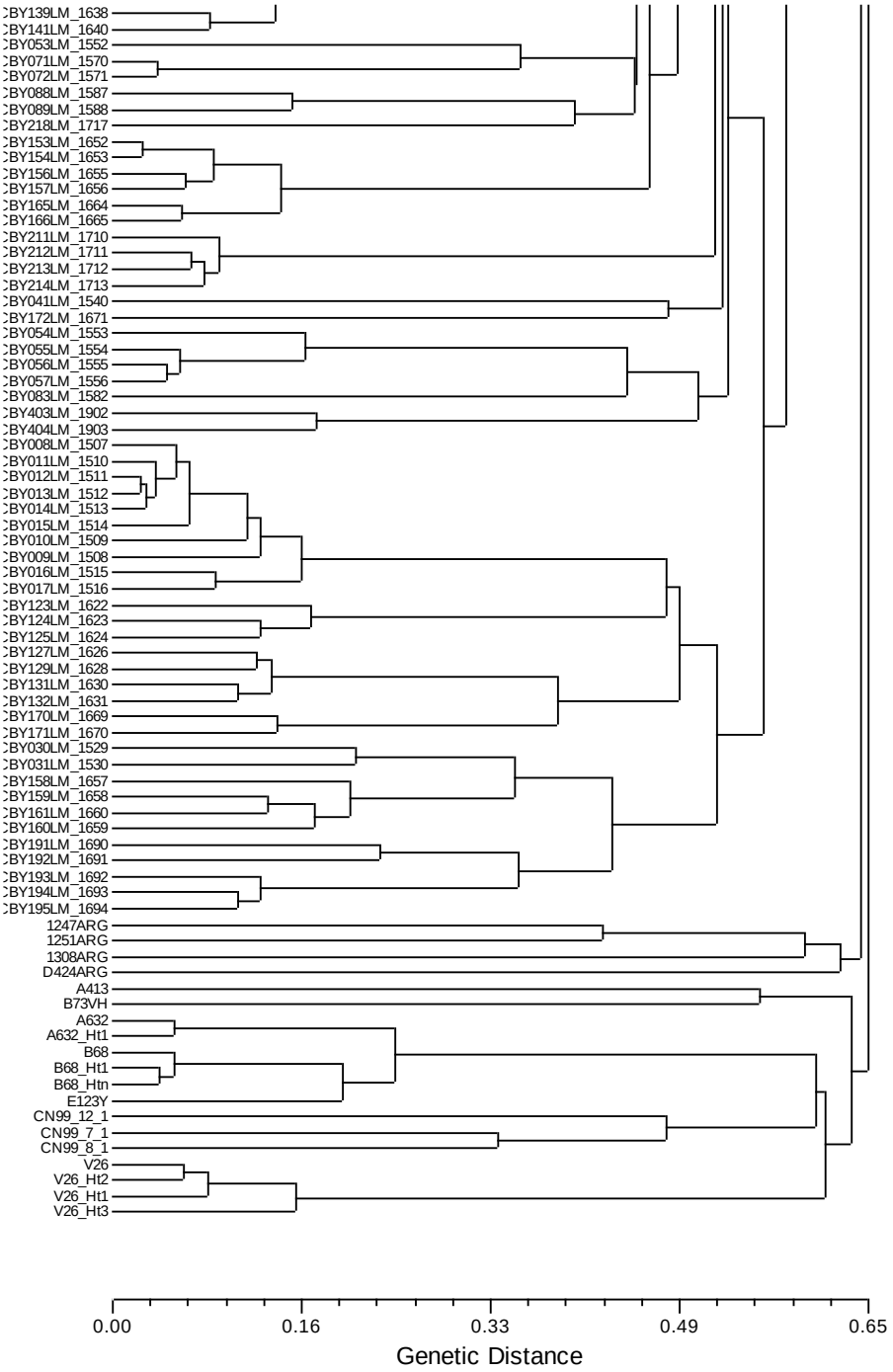


Figure 8 (continued (8)) Associations among yellow inbred lines revealed by average linkage (UPGMA) cluster analysis based on Rogers distance.

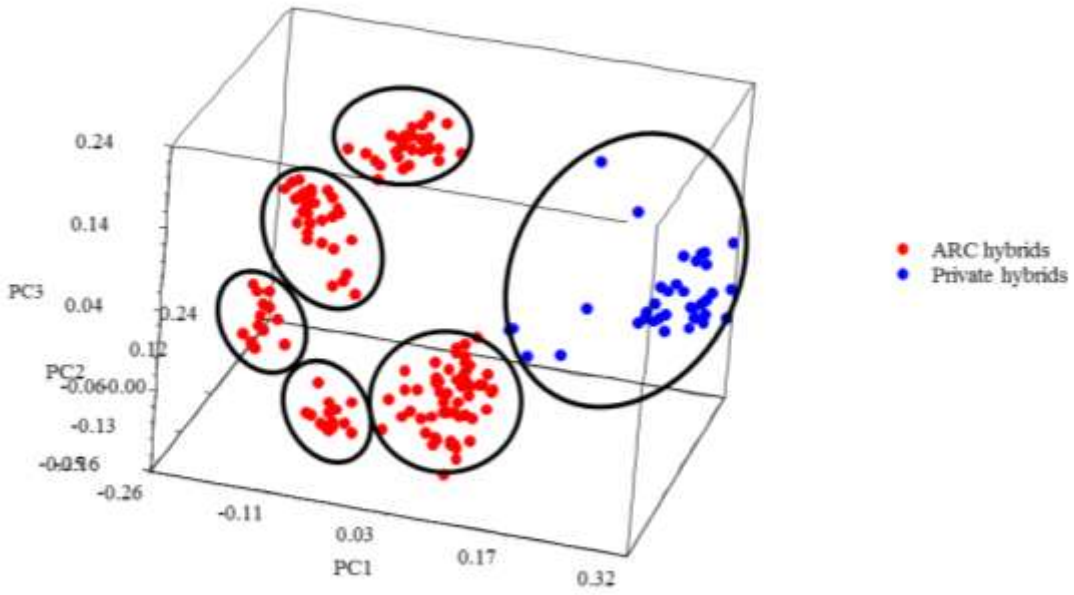


Figure 9 Principal coordinates analysis of hybrids tested.

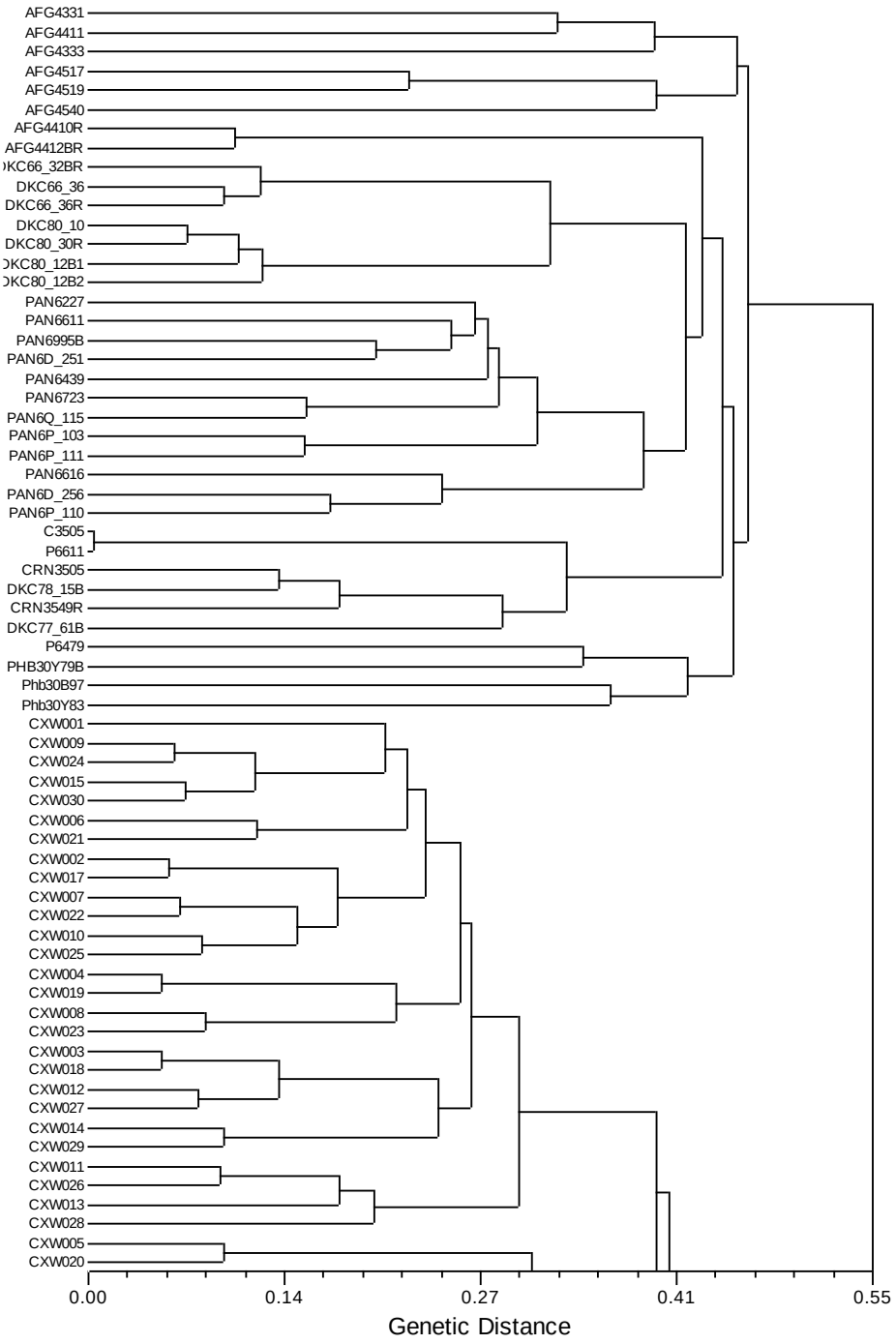


Figure 10 (1) Associations among hybrid samples revealed by average linkage (UPGMA) cluster analysis based on Rogers distance.

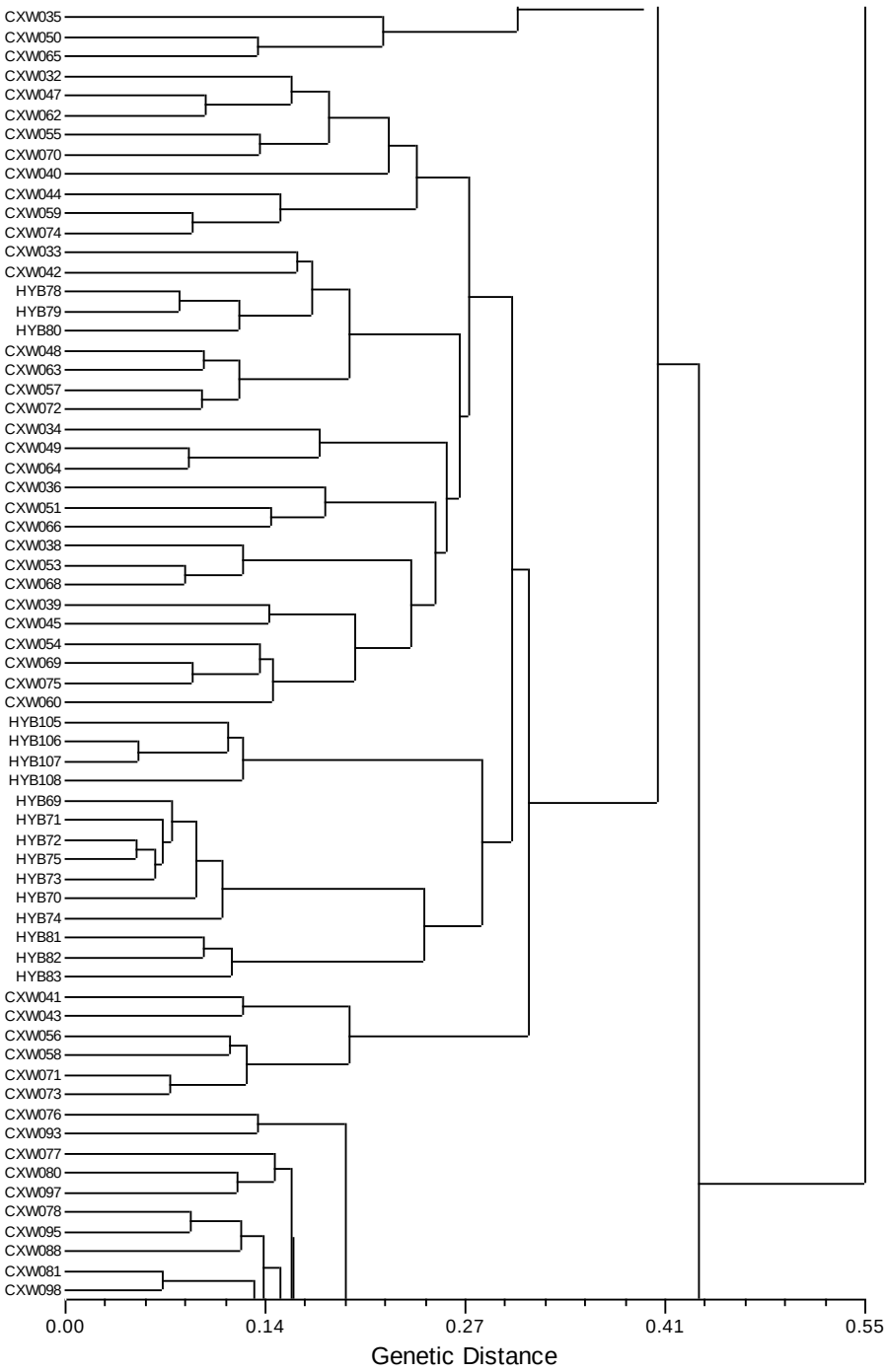


Figure 10 (continued (2)) Associations among hybrid samples revealed by average linkage (UPGMA) cluster analysis based on Rogers distance.

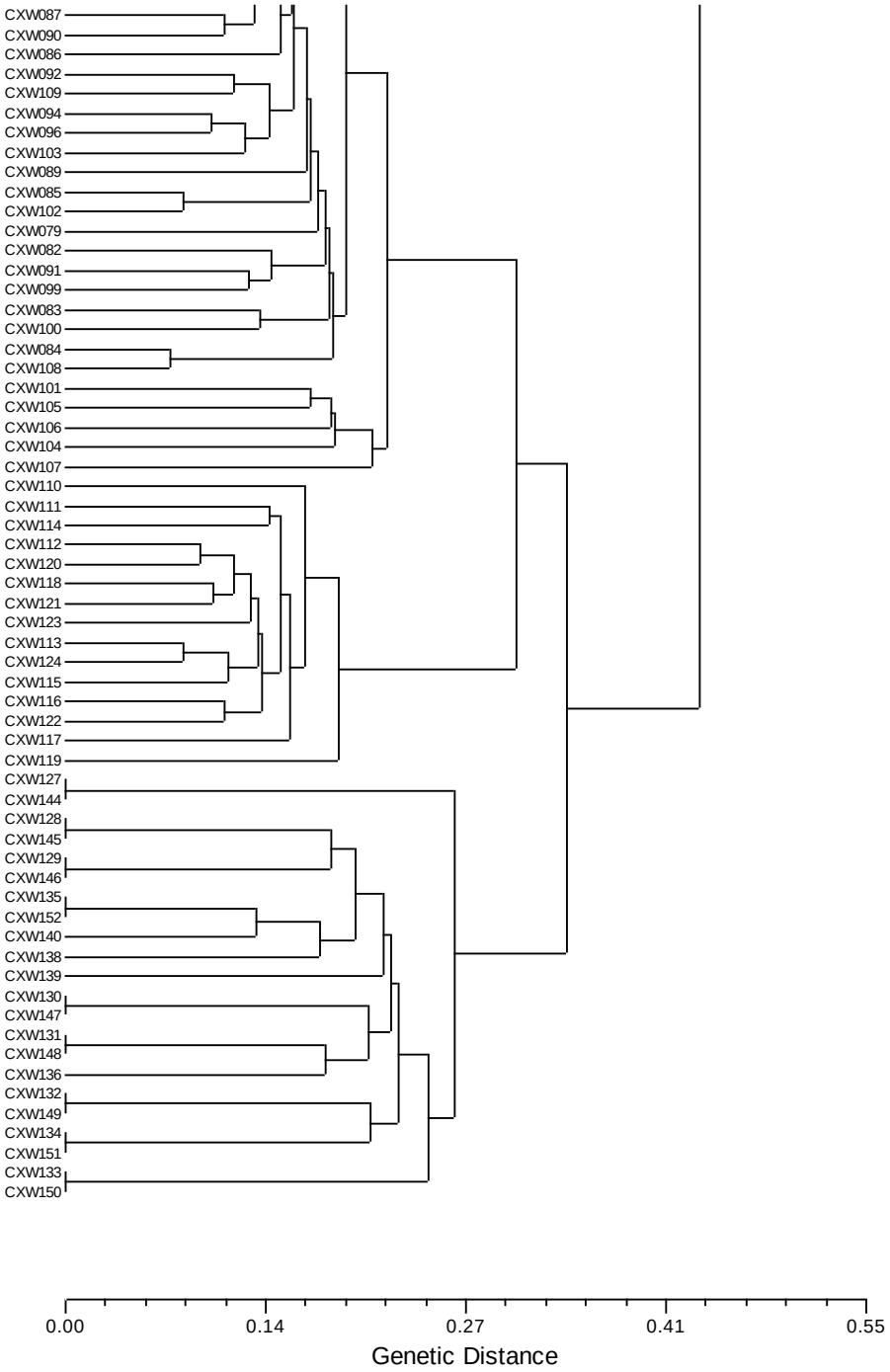


Figure 10 (continued (3)) Associations among hybrid samples revealed by average linkage (UPGMA) cluster analysis based on modified Roger's distance.