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Caracterización de una colección de *Theobroma cacao* L. en Tingo María usando marcadores moleculares ISSR.

Characterization of a *Theobroma cacao* L. collection at Tingo Maria using ISSR molecular markers.

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ABSTRACT

Cacao breeding programs focus in obtaining cacao varieties with important traits to both producers and consumers. A vital step for plant breeders is the characterization of the genetic material kept in germplasm banks in order to describe its level of diversity, and find interesting traits both morphologically and molecularly. In this way, the investigator will monitor how useful the accessions could be, determine duplications, and manage the bank appropriately. In this study, we aimed to test: the potential of ISSR (Interspread Sequence Repeats) markers to differentiate 46 cacao accessions maintained in Tingo Maria - Perú, and the similarity relationships between the accessions. In this research, the results showed that ISSR, despite its dominance nature, could establish eye-catching associations, such as, the grouping of Trinitario accessions into a common cluster.

Keywords: cacao, Interspread single repeats, diversity, UPGMA grouping

INTRODUCTION

Cacao is a small tree from the tropical South American forest that has become an economical important crop for smallholders of developing countries all over the world. Furthermore, it has a great environmental value, because it contributes significantly in the recovering of deteriorated soils and constitutes an ecological habitat for important fauna (Guiltinan, 2007). Cacao products are consumed mainly by developed countries, progressively increasing their demand and diversifying their tastes. Therefore, in order to meet those needs by the consumers and to fulfill a sustainable agriculture for producing countries, it has to be emphasized the generation of technologies for a more productive and high quality cacao. One important component of this goal could be accomplished by obtaining varieties with important traits; such as, pest and disease resistance, higher yields, etc. A genebank is the starting point of a breeding program. But to find usefulness in the genetic materials the germplasm have to be appropriately characterized (Spooner *et al.*, 2005). The characterization can be performed phenotypically or molecularly as reported in many papers and there are a number of DNA markers available to researchers who ought to investigate genetic diversity among plant species. PCR-based techniques of random multilocus analysis (RAPD, AFLP, ISSR) have been successfully used in genotyping, genome mapping and phylogenetic studies in cacao (Guiltinan, 2007). The value of these molecular markers is influenced by several considerations: the method should be quick, inexpensive and technically simple, but must be sufficiently informative to distinguish between the most individuals (Charters and Wilkinson, 2000).

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Peru has a very rich biodiversity and is recognized as one of the cacao origin centers (Zhang *et al.*, 2006). Despite of this, small farmers achieve an average yield of about 551 kg/ha (DGPA, 2007). This could be due to low-technology agriculture, and poor genetic varieties. At Tingo Maria – Perú, the cacao program from the local university, Universidad Nacional Agraria de la Selva (UNAS), carried out a Bioversity International sponsored breeding program which aims to obtain improved varieties. Trait evaluation of the genetic material is ongoing, and with molecular characterization support, selection could be done faster, and crosses between suitable genotypes could be done wisely. In this approach, we present preliminary results of a molecular characterization of a Tingo Maria cacao collection with ISSR markers, in order to test the potential of these markers to differentiate individuals and to find some interesting relationships.

MATERIALS AND METHODS

Forty six cacao trees from different genetic and geographical sources (Table 1) were chosen from the UNAS germplasm bank or genetic breeding trials at Tulumayo agriculture station, located both in Tingo Maria (Huánuco). Two or three insect unbitten and healthy leafs were placed in a clean plastic bag with some dried silical gel. DNA was isolated after grinding in a bead mill approximately 100 mg of complete dried tissue of each sample in a 1.5 ml microtube. No liquid nitrogen was used. DNA extraction and purification was carried out as described by Doyle and Doyle (1987), with minor modifications. After addition of 2 ul RNAse, DNA concentration and purity (A_{260}/A_{280}) were determined in a Eppendorf biophotometer. DNA concentration (76-1802 ng/uL) and purity (1.2-2.4) were adequate for PCR reactions. The purified DNA was diluted to 25 ng/uL TE and stored at - 20 °C until use. In order to select the best ISSR primers from a pool of fifty nine anchored and non-anchored primers (Table 2), previous tests were performed with the following four cacao genotypes: ICS – 1, CAT – 4, U – 70, EET – 223; which were chosen on their very divergent morphological characteristics. A biological replication for each sample was used (DNA from another leave of the same tree). PCR amplification was performed in a Eppendorf Thermocycler. The reaction program was the same for all primers, since the goal was to pre-select the primers which yield a reproducible and well-formed pattern of bands. The PCR reaction mix of 20 l consisted in 15 ng total DNA, 1X PCR buffer (10mM Tris-HCl, pH 8.3, 50mM KCl), 0.2 mM dNTPs, 3 mM $MgCl_2$, 0.8 uM primer, 1 U Taq polimerase (Fermentas). The PCR program consisted in the following: first step: 94°C for 90 s; second to seventh cycle: denaturing step: 94° C for 40 s, annealing step for 45 s and decreasing the temperature from 55 °C to 50 °C cycle by cycle for 6 cycles, elongation step: 72 °C for 90 s; finally 30 cycles 94 °C for 40 s, 49 °C for 45 s, 72 °C for 90 s. PCR products were analyzed by PAGE using 6% poliacrylamide in a 19:1 ratio (acrylamide/bisacrylamide) and carried out at a constant voltage of 500 V for 6 h. A O'generuler 100bp DNA ladder plus (Fermentas) was used. DNA fragments were detected with $AgNO_3$ and NaOH. All runs were performed in a home-made PAGE chamber. The band patterns were scored using the Cross Checker software (Buntjer, 1999) and only clearly scorable and reproducible bands were considered. Data were assigned in a MS-excel datasheet as "1" if there were a band present, assigned a "0" if bands were absent or "2" (missing data) if the band or fragment has slight brightness. Loci with more than 10% missing data were excluded from the analysis. To generate the respective dendogram, a DICE similarity matrix and UPGMA algorithm were used from NTSYSpc v2.1 (Rohlf 2000). Finally, a matrix correlation test was carried out using the 2-way Mantel test from the same software.

Table 1. Cacao genotypes used in this study.

	genotype code	Country	group	hybrid detail
1	Pandora[1]	Brasil	Unknown	
2	CAT - 4[6]	Brasil	Lower Amazon Forastero	
3	ICS - 95[2,6]	Brasil	Trinitario	
4	EET - 400[2,6]	Ecuador	Upper Amazon Forastero	
5	EET - 228[2,6]	Ecuador	Hybrid	Amazon hybrid
6	EET - 233[1]	Ecuador	Hybrid	
7	EET - 62[3,6]	Ecuador	Hybrid	Nacional x unknown
8	CCN - 51[1]	Ecuador	Hybrid	Complex x Forastero
9	Silvestre Huallaga[1]	Peru	Upper Amazon Forastero	
10	PA - 150[1]	Peru	Upper Amazon Forastero	
11	U - 1[1]	Peru	Upper Amazon Forastero	
12	U - 9[1]	Peru	Upper Amazon Forastero	
13	U - 12[1]	Peru	Upper Amazon Forastero	
14	U - 15[1]	Peru	Upper Amazon Forastero	
15	U - 26[1]	Peru	Upper Amazon Forastero	
16	U - 43[1]	Peru	Upper Amazon Forastero	
17	U - 48[1]	Peru	Upper Amazon Forastero	
18	U - 54[1]	Peru	Upper Amazon Forastero	
19	U - 60[1]	Peru	Upper Amazon Forastero	
20	U - 68[1]	Peru	Upper Amazon Forastero	
21	U - 70[1]	Peru	Upper Amazon Forastero	
22	SCA - 6[2,3,4,5]	Peru	Upper Amazon Forastero	
23	IMC - 67[2,3,5]	Peru	Upper Amazon Forastero	
24	P - 7[1,2,3]	Peru	Upper Amazon Forastero	
25	CHUN - C[1]	Peru	Upper Amazon Forastero	
26	CHUN - S[1]	Peru	Upper Amazon Forastero	
27	Señorita Achoccha[1]	Peru	Upper Amazon Forastero	
28	M - 18,16[1]	Peru	Hybrid	IMC-67 x U-68
29	M - 1,7[1]	Peru	Hybrid	H-12 x ICS-6
30	C - 42,1[1]	Peru	Hybrid	Forastero x Trinitario
31	C - 53,10[1]	Peru	Hybrid	Forastero x Trinitario
32	C - 65,3[1]	Peru	Hybrid	Forastero x Trinitario
33	CCN - 51 x EET - 233[1]	Peru	Hybrid	Complex
34	I - 14,20[1]	Peru	Hybrid	P-7 x ICS-95
35	I - 16,20[1]	Peru	Hybrid	ICS-1 x SCA-6
36	C - 69,3[1]	Peru	Hybrid	Forastero x Trinitario
37	C - 81,4[1]	Peru	Hybrid	Forastero x Trinitario
38	C - 85,8[1]	Peru	Hybrid	Forastero x Trinitario
39	H - 12[1]	Peru	Hybrid	Forastero x Trinitario
40	H - 61[1]	Peru	Hybrid	Forastero x Trinitario
41	I - 12,12[1]	Peru	Trinitario	IMC-67 x EET-228
42	ICS - 6[3,6]	Trinidad & Tobago	Trinitario	
43	ICS - 78[2]	Trinidad & Tobago	Trinitario	
44	ICS - 1[3,6]	Trinidad & Tobago	Trinitario	
45	ICS - 39[2,3]	Trinidad & Tobago	Trinitario	
46	UF - 667[2,6]	Trinidad & Tobago	Trinitario	

Sources:

1 Garcia (2008) pers.comm.
2 Gonzales (1996)
3 Marita et al. (2001)
4 N'Goran et al. (1994)
5 Piñan (1993)
6 Soria et al. (1981)

Table 2. ISSR primers used in this research. The bold ones were the primers that showed better results. **Note:** Degenerate primers are mixed PCR primers where symbols in the primer formula represent: **B** = G, T, or C; **D** = G, A, or T; **H** = A, T, or C; **V** = G, A, or C; **R** = A, G and **Y** = C, T.

Anchored primers				Non-anchored primers	
#	Sequence	#	Sequence	#	Sequence
1	$GAGC(CAA)_5$	21	$(AC)_8C$	**41**	**$(AG)_8$**
2	$CTG(AG)_8$	22	$(AC)_8G$	42	$(CCA)_5$
3	$(AG)_8TG$	**23**	**$(TG)_8C$**	43	$(AC)_8$
4	$(AG)_8T$	**24**	**$(TG)_8G$**	**44**	**$(ACTG)_4$**
5	$(AG)_8C$	**25**	**$(AG)_8YT$**	**45**	**$(GACA)_4$**
6	$(AG)_8G$	**26**	**$(AG)_8YC$**	46	$(GATA)_4$
7	$(GA)_8T$	**27**	**$(GA)_8YC$**	47	$(GACAC)_4$
8	$(GA)_8C$	**28**	**$(GA)_8YG$**	48	$(ACC)_6$
9	$(GA)_8A$	29	$(CT)_8RC$	49	$(ACG)_6$
10	$(CT)_8T$	30	$(CA)_8RG$	**50**	**$(ATG)_6$**
11	$(CT)_8A$	31	$(GT)_8TYC$	51	$(CCG)_6$
12	$(CT)_8G$	32	$(TG)_8RT$	52	$(CTC)_6$
13	$(CA)_8T$	33	$(TG)_8RC$	53	$(GGC)_6$
14	$(CA)_8A$	34	$(TG)_8RA$	**54**	**$(GAA)_6$**
15	$(CA)_8G$	**35**	**$HBH(AG)_7$**	55	$(GATA)_2(GACA)_2$
16	$(GT)_8C$	36	$BHB(GA)_7$	56	$(GGAT)_4$
17	$(TC)_8A$	37	$DVD(TC)_7$	57	$(CTTCA)_3$
18	$(TC)_8G$	38	$DBD(AC)_7$	**58**	**$(GGAGA)_3$**
19	$(TC)_8G$	39	$VHV(GT)_8$	59	$(GGGTG)_3$
20	$(AC)_8T$	40	$HVH(TG)_9$		

RESULTS & DISCUSSION

Thirteen primers showed that were potential for further assays according to the intensity and sharpness of the DNA pattern bands (Table 2, highlighted in bold). Those were comprised by both anchored and non-anchored primers. Some of these primers share the AG or GA motif (primers 25, 26, 27, 28, 35, 58). Due to time and funding availability, five ISSR primers were selected to amplify all forty six genotypes (Table 3). This table also shows the marker index for each ISSR primer, calculated from the "Polymorphic Index Content" (PIC) values according to Ghislain et al. (1997). The last two primers exhibited the higher indexes, and demonstrate the potential for using them in future ISSR characterization assays. Primers 25 and 27 coincide with the two ones (834 and 841) used by Charters and Wilkinson (2000) in a genetic characterization of cocoa germplasm. They found that 6 ISSR primers could distinguish 56 out of 62 genotypes. The dendogram in Figure 1 shows similarity relationships between the accessions of cacao obtained with ISSR markers. We found that UNAS cacao hybrids (codes in rectangular boxes) as well as cacao recently collected accessions (codes in elyptical boxes) are dispersed all over the different branches of the tree. In the case of the hybrids from the UNAS, it failed to group them with their respective parentals, for example, M-18,16 (progeny tree from IMC-67 x U-68) is grouped together with EET accessions (cluster 1), despite the fact that they share neither geographic nor genetic origin. We could infer that some similarity between these individuals were detected by ISSR markers. And since there were not used a considerable quantity of markers in this study, the differentiation could not be a broad one. A notable characteristic is that these markers could detect some functional variability, since the amplified fragment might comprise genes between the two microsatellites which serves as annealing point for

the primer. An interesting grouping (cluster 1, bottom branch) is made up by Trinitario accessions (ICS and UF). This branch includes the hybrid I-16,20 which is a progeny tree from (ICS-1 x SCA-6). Ucayali individuals in this study (clusters 5, 8, 9 and 10) are scattered but show a considerable variability as demonstrated by Zhang et al. (2006) with SSRs. The Matrix correlation is r = 0.88982 (normalized Mantel statistic Z), therefore, a good relationship between similarity and cophenetic matrices was achieved. The Matrix comparison graph is shown in Figure 2.

Table 3. Some important features from the ISSR primers used in this approach.

	# loci	# polymorphic loci	marker index
$(AG)_8YT$	12	12	0.76
$(AG)_8YC$	14	14	1.63
$(GA)_8YC$	22	20	1.90
$(GA)_8YG$	17	15	3.33
$HBH(AG)_7$	13	12	2.66

CONCLUSIONS

Phenetic dendogram showed that ISSR can establish a similarity cluster between Trinitario genotypes in cacao, regretfully, in the case of UNAS cacao hybrid trees, they were not grouped with their respective parental genotypes. All of this could mean that more ISSR primers should be tested in order to provide more insights of the meaningful of Peruvian cacao diversity. Two out of five chosen anchored ISSR primers showed to have more potential in determining the polymorphism through fourty six cacao genotypes characterized from germplasm bank and genetic trials at Tingo Maria – Peru. These ISSR primers were $(GA)_8YG$ and $HBH(AG)_7$. Similarity matrices were shown to be in fine correlation with cophenetic ones, indicated by the normalized Mantel test. Finally, we propose that the ISSR markers have a great potential for practical genotyping and encourage further exploration with freshly cacao accessions in Peru, while this country bears great cacao diversity.

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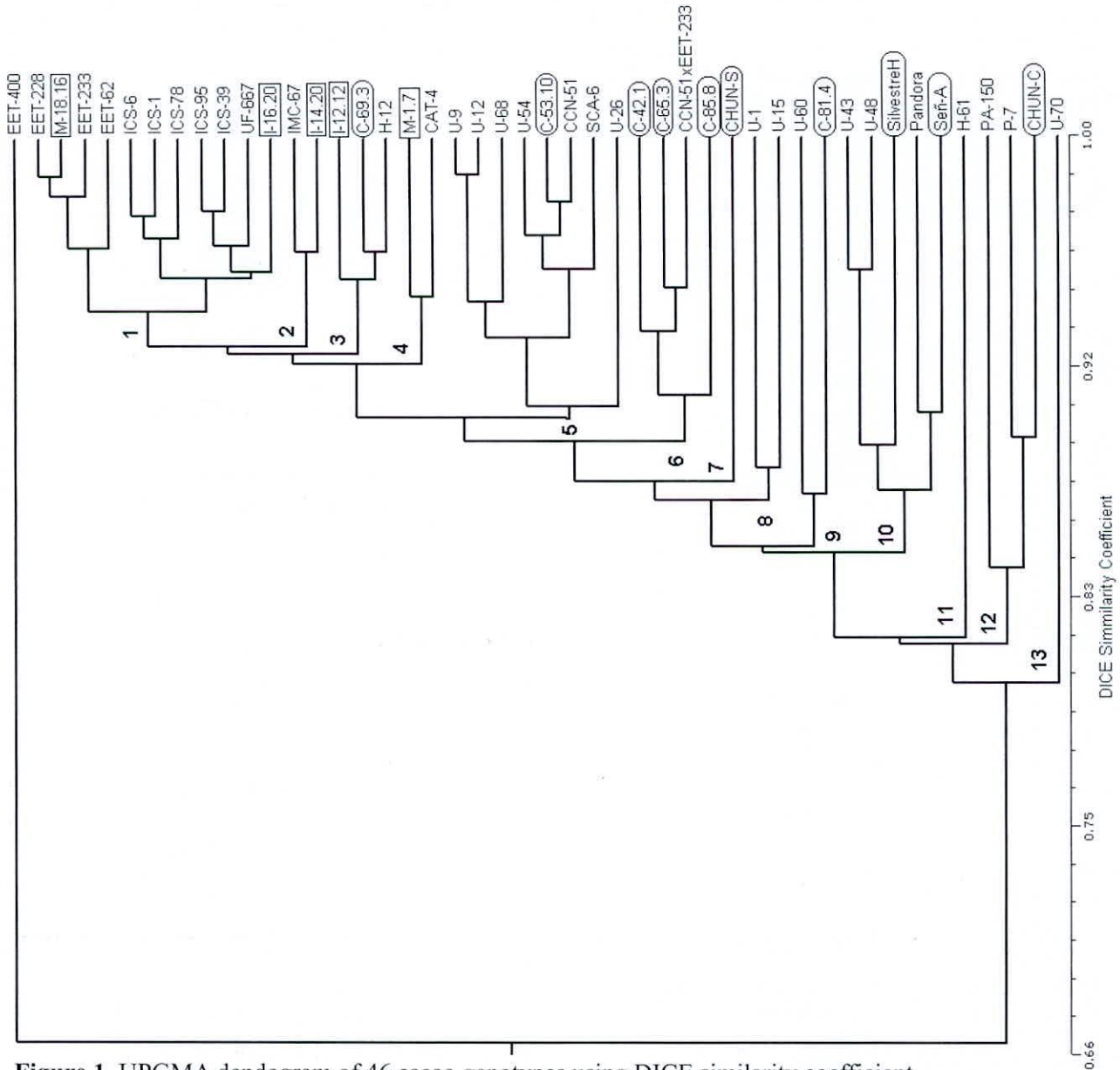

Figure 1. UPGMA dendogram of 46 cacao genotypes using DICE similarity coefficient.

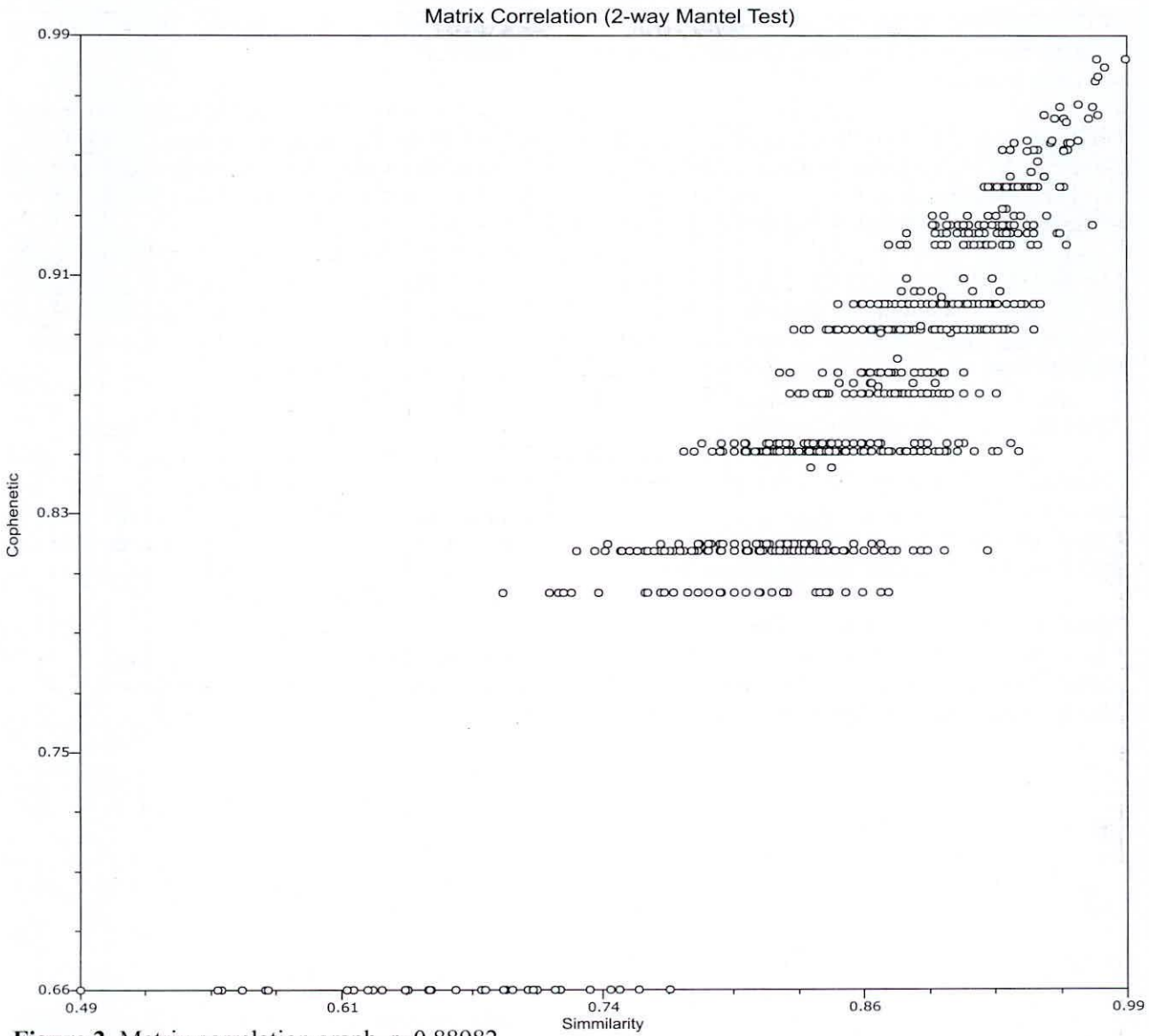

Figure 2. Matrix correlation graph. r=0.88982.

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