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DNA methylation variations between mono - and dizygotic twins

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Abstract

Monozygotic twins (MZ) who have a common genotype may show phenotypic discordance. One of the possible explanations is the existence of epigenetic changes which occur as DNA methylation alterations. DNA methylation changes were studied in 16 MZ twin pairs and 8 dizygotic (DZ) twin pairs using CRED-RA technique. The most suitable primer (5'-GATGACCGCC-3') was selected out of 20, random, decamer primers. Significant DNA methylation changes were observed in either MZ or DZ twins. Each MZ twin pair share common RAPD profile, however some of them have their own methylation profile regardless their backgrounds. Environmental conditions alone should not be accounted for phenotypic discordance between MZ twins, although external factors may influence genetic and epigenetic nature and also the phenotype. Our results might be also useful for the identification of MZ twins. To our knowledge, this is the first study to use CRED-RA in human genetics.

Keywords: *Twins*, *RAPD*, *CRED-RA*, *Cytosine methylation*, *Epigenetics*

Introduction

Human monozygotic (MZ) twins, originated from a single zygote, account for 1 in 250 live births (Hall & Lopez-Rangel [1]). Although they are considered genetically identical, significant phenotypic discordance between them may exist. This quality is particularly noticeable for psychiatric diseases, such as schizophrenia and bipolar disorder (Cardno & al. [2]). Studies on MZ and dizygotic (DZ) twins had a significant impact on understanding of many diseases and syndromes, especially psychiatric ones, such as schizophrenia, autism, bipolar disease, etc, (Petronis & al. [3]). MZ twins have been used to demonstrate the role of environmental factors in determining complex diseases and phenotypes, but the true nature of the phenotypic discordance nevertheless remains extremely poorly understood (Fraga & al. [4]). It has been showed that epigenetic modifications of DNA and histones can have a primary role in phenotypic outcomes, including human diseases (Robertson & Wolffe [5]). "Epigenetics is a new development in human morbid biology which suggests that inherited and acquired epigenetic misregulation of genes and genomes (rather than DNA sequence changes and environment) can play the primary role in the origin of complex non-Mendelian diseases such as autism, schizophrenia, addictions, among numerous others." says Dr. Arturas Petronis from University of Toronto (http://www.neurodevnet.ca/ContactUs/Team.aspx), who investigates the role of epigenetic factors in complex diseases. Numerous studies in MZ twins have been conducted, looking beyond the primary DNA sequence and differences in epigenetic patterns some of which are believed to result in discordant phenotypes, have been identified (Petronis et. al., 2003; Fraga et al., 2005; Heijmans & al. [6]). Epigenetic modifications consist of cytosine methylation, as well as histone modification including methylation, phosphorylation, acetylation, and ubiquitination (Li [7]). In mammals, DNA methylation occurs most commonly where cytosine is directly followed by guanine, forming a CpG dinucleotide. Clusters of CpG dinucleotides are referred to as CpG islands (Takai &

Jones [8]). Many CpG islands across the genome are located proximal to gene promoters and their DNA methylation status can affect gene transcription (Holliday & al. [9]). Conversely, unmethylated DNA is associated with an open chromatin conformation, allowing for access of DNA binding elements and transcriptional activation (Nemeth & Langst [10]).

RAPD (Random Amplified Polymorphic DNA) allows the amplification of genomic DNA at low annealing temperatures (Williams & al. [11]). CRED-RA (Coupled Restriction Enzyme Digestion-Random Amplification) is based on the amplification of genomic DNA after digestion with isoschizomers which have different methylation sensitivity. Genomic DNA sample is divided into 2 groups and each group is digested with a different enzyme. *Hpa*II (EC= 3.1.21.4) and *Msp*I are usually preferred in CRED-RA (Prakash & Kumar [12]; Leljak-Levanic & al. [13]). Although both recognize the same sequence 5'-CCGG-3', their restriction abilities depend on the methylation status of cytosines (Temel & al. [14], Table 1). The differences in cleavage patterns result in DNA fragments of different sizes and therefore serve to distinguish methylation levels of DNA samples (Ferrucci & al. [15]).

Table 1. Methylcytosine sensitivity and restriction patterns of the enzymes (*Hpa*II, *Msp*I).

Type	Methylation pattern	*Hpa*II	*Msp*I
Type I	CCGG C<u>C</u>GG GGCC GGCC	Active	Active
Type II	<u>C</u>CGG GGCC	Active	Inactive
Type III	C<u>C</u>GG GG<u>C</u>C	Inactive	Active
Type IV	<u>C</u>CGG GGC<u>C</u>	Inactive	Inactive

We aimed to investigate methylation profiles of 16 MZ and 8 DZ twin pairs with CRED-RA technique and were able to detect methylation variations and achieve identification of some MZ twins according to their unique methylation profiles.

Materials and Methods

Twin samples

Written informed consent was obtained from all participants and studies were approved by the local institutional review boards at participating institutions. Venous blood samples (5 mL) of 16 MZ and 8 DZ twin pairs from Turkish twin population were collected in EDTA vacutainers. Subjects consisted of 25 females and 23 males of 27.1 ± 12.53 [46%] years old (mean ± s.d. [CV]).

Genomic DNA Isolation

Genomic DNAs were extracted from blood samples using "SpinKlean Genomic DNA Miniprep Kit" (K5125, Biomatic Corporation). The DNA concentrations were measured using a spectrophotometer and genomic DNA samples were separated on an EtBr-stained, 0.7% agarose gel to estimate the quality of the DNA. For RAPD analyses 10-mer, random primers (Table 2) with a GC content of 60-80% were used.

Table 2. Primers used in RAPD analysis.

No	Name	Sequence	No	Name	Sequence
1	OPA11	CAGGCCCTTC	11	OPC18	TGAGTGGGTG
2	OPA15	CAAACGTCGG	12	OPC20	ACTTCGCCAG
3	OPA22	TGCCGAGCTG	13	GA05	AGGGGTCTTG
4	OPB12	CCTTGACGCA	14	GA09	GGGTAACGCC
5	OPB22	TGATCCCTGG	15	GA11	CAATCGCCGT

6	OPC03	GGGGGTCTTT	16	GA17	GACCGCTTGT
7	OPC05	GATGACCGCC	17	GA18	AGGTGACCGT
8	OPC08	TGGACCGGTG	18	GB07	GGTGACGCAG
9	OPC12	TGTCATCCCC	19	GB08	GTCCACACGG
10	OPC13	AAGCCTCGTC	20	GB09	TGGGGGACTC

RAPD

RAPD-PCR mix consisted of 1X enzyme buffer, 2.5 mM $MgCl_2$, 0.2 mM each dNTP, 10 µM primer (Table 2), 25 ng genomic DNA and 0.5 U *Taq* polymerase (GoTaq® Flexi, Promega) in a total volume of 25 µl. Amplification was achieved using a thermocycler program as follows: 1 cycle of 2 min at 94°C; 40 cycles of 1 min at 94°C, 1 min at 34°C, 2 min at 72°C and 1 cycle at 72°C for 10 min. Amplification products were electrophoresed in 1.7% agarose gel, stained with 0.5 µg/ml EtBr and visualized under UV light.

CRED-RA

Five microgram genomic DNA from each individual were separately digested with 10 U *Hpa*II and *Msp*I enzymes (Fermentas) separately, according to manufacturer's recommendations. After checking on agarose gel, 5 micro litres of each digestion mixture were amplified with primer OPC05 (Table 2). Amplification and visualization conditions for CRED-RA are the same as described for RAPD. Data presented as figures and the positions of RAPD and CRED-RA bands were scored and compared.

Results and Discussions

All subjects were requested to answer a few questions. Answers and detailed information about subjects were presented in Table 3 and 4.

Table 3. Evaluation forms of DZ twins.

Subjects		Sex	Age	Any relatives with any genetic disorder?		Any relatives with any type of cancer?		Exposed to radioactive/ chemical agents?		Drug treatment?		Smoking?	
Pair	**Individual**			**Y**	**N**	**Y**	**N**	**Y**	**N**	**Y**	**N**	**Y**	**N**
1	01	F	11		X	X			X		X		X
	02	F	11		X	X			X		X		X
2	03	M	36		X		X		X		X	X	
	04	M	36		X		X	X			X	X	
3	05	M	23		X		X		X		X	X	
	06	M	23		X		X		X		X	X	
4	07	M	12	X		X			X		X		X
	08	M	12	X		X			X		X		X
5	09	F	20		X		X		X		X		X
	10	M	20		X		X		X		X	X	
6	11	F	25		X		X		X		X		X
	12	M	25		X		X		X		X		X
7	13	F	46		X		X		X		X		X
	14	M	46		X		X	X			X	X	
8	15	F	35		X	X		X			X		X
	16	F	35		X	X		X		X			X

Y: Yes, N: No; F: Female, M: Male.

Table 4. Evaluation forms of MZ twins.

Subjects		Sex	Age	Any relatives with any genetic disorder?		Any relatives with any type of cancer?		Exposed to radioactive/ chemical agents?		Drug treatment?		Smoking?	
Twin	**Individual**			**Y**	**N**	**Y**	**N**	**Y**	**N**	**Y**	**N**	**Y**	**N**
1	01	F	24		X	X			X		X		X
	02	F	24		X	X			X		X		X
2	03	F	6		X		X		X		X		X
	04	F	6		X		X		X		X		X
3	05	F	20		X	X			X		X		X
	06	F	20		X	X			X		X		X
4	07	F	39		X	X		X		X			X
	08	F	39		X	X		X			X		X
5	09	M	28		X		X		X		X	X	
	10	M	28		X		X		X	X		X	
6	11	M	40		X		X		X		X	X	
	12	M	40		X		X		X		X	X	
7	13	F	38	X			X	X			X		X
	14	F	38	X			X		X		X		X
8	15	F	17	X		X			X		X		X
	16	F	17	X		X			X		X		X
9	17	M	40		X	X		X			X	X	
	18	M	40		X	X			X		X	X	
10	19	F	57		X	X			X	X			X
	20	F	57		X	X			X		X		X
11	21	M	32		X	X			X		X	X	
	22	M	32		X	X			X		X	X	
12	23	M	25		X		X		X		X		X
	24	M	25		X		X		X		X		X
13	25	F	25		X		X		X		X		X
	26	F	25		X		X		X		X	X	
14	27	F	14		X		X		X		X		X
	28	F	14		X		X		X		X		X
15	29	M	9		X	X			X	X			X
	30	M	9		X	X			X	X			X
16	31	M	30		X		X		X	X		X	
	32	M	30		X		X		X	X		X	

Y: Yes, N: No; F: Female, M: Male.

In this study, methylation variations between DZ and especially MZ twins were investigated by means of CRED-RA. First, 20 random primers were tested in some genomic DNA samples. Of the tested 20 primers, OPC05 primer (5'-GATGACCGCC-3') gave the best amplification pattern (Figure 1). Therefore, all CRED-RA experiments were conducted with OPC05 primer. RAPD profiles of MZ twins were quite monomorphic.

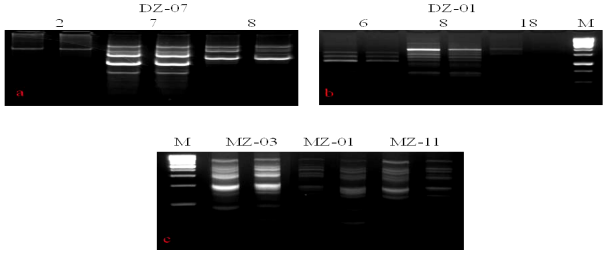

Figure 1. RAPD optimization results. (a) Primers 2, 7, 8 in DZ-07; (b) Primers 6, 8, 18 in DZ-01; (c) Primer 7 (OPC05) in MZ-03, 01, 11. M. 1 Kb Marker (Fermentas).

All genomic DNA samples were digested with *Hpa*II and *Msp*I enzymes, separately. *Hpa*II-digested samples gave an undigested, heavy, single band; however all *Msp*I-digested samples appeared as a smear ranging between 1-10 Kb. A narrower smear (not exactly a single band) was observed in *Hpa*II-digested MZ samples (Figure 2). Therefore methylation profiles of MZ twins are more similar rather than DZ twins.

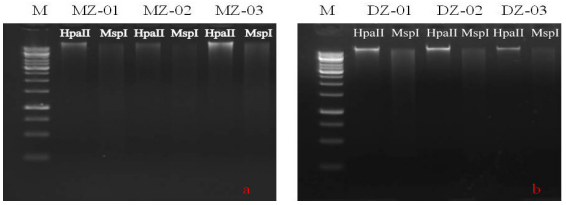

Figure 2. Digestion patterns of MZ (a) and DZ (b) twins. M. 1 Kb DNA Marker (Fermentas).

Five microliter aliquots of each digestion product was amplified with OPC05 primer under RAPD conditions. As expected, amplification patterns of MZ twins were more monomorphic than of DZ twins. Polymorphism was observed not only between siblings but also *Hpa*II and *Msp*I digestions of the individual (Figure 3).

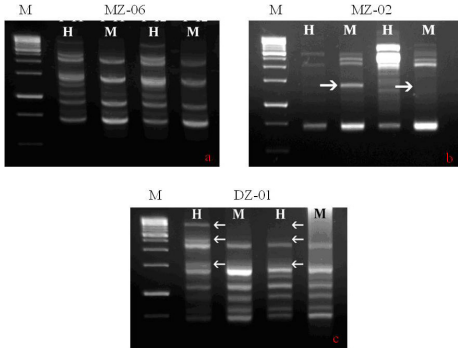

Figure 3. CRED-RA patterns of some MZ and DZ twins. (a) MZ-06; (b) MZ-02; (c) DZ-01. Arrows indicate polymorphic bands. M. 1 Kb DNA Marker (Fermentas); H. *Hpa*II; M. *Msp*I.

CRED-RA analysis in 8 DZ twin pairs gave a total of 233 bands. One hundred thirty-seven bands were *Hpa*II-specific; 96 bands were *Msp*I-specific. *Hpa*II polymorphism ranged between 0%-30%; *Msp*I polymorphism ranged between 0%-14.3%. A total of 466 bands were amplified in 16 MZ twin pairs. Two hundred-eighty bands were *Hpa*II-specific; 186 bands were *Msp*I-specific. *Hpa*II polymorphism ranged between 0%-50%; *Msp*I polymorphism ranged between 0%-33%.

Phenotypic discordance in MZ twins has traditionally indicated the roles of environmental factors. MZ twins show lower degrees of discordance, in comparison to discordance in DZ twins (Kaminsky & al. [16]). Epigenetic factors may explain phenotypic differences between MZ twins (Kaminsky & al. [17]). Cytosine methylation is the most common modification of DNA in eukaryotes. At gene promoters, methylation often leads to transcriptional silencing. For this reason, different methylation profiles cause differences between cells, tissues or organisms with identical DNA sequence. While MZ twins are genetically identical, their different methylation profiles acquired during development may lead to discordance. Syndromes known to be genetic in nature may have discordant expression. Kruyer & al. [18] observed that in MZ twins discordance is present in Fragile-X. They reported that one of the sisters has mental retardation while her twin has not. It was claimed that MZ twin concordance for a range of psychiatric conditions is rarely 100% (Mill & al. [19]). Therefore, most MZ twins are rarely absolutely identical (Machin [20]). We

aimed to determine whether MZ twins are epigenetically identical or not. The preliminary experiment to detect methylation variations was the digestion of genomic DNA samples with *Hpa*II and *Msp*I enzymes, simultaneously. As we expected, nearly all samples exhibited a wide smear after *Hpa*II digestion, indicating that most cytosines in genomic DNA are heavily methylated. Most of the human genome remains high molecular weight following digestion with *Hpa*II, which recognizes the motif (5`-CCGG-3`) (Singer & al. [21]). Nearly 55%-70% of *Hpa*II sites in animal genomes are methylated at the central cytosine (Bird [22]; Bestor & al. [23]). The part of genomic DNA cut into a size of hundreds of base pairs (Bird [24]) tend to localize with transcription start sites of genes active either constituvely (Larsen & al. [25]) or during embryogenesis (Ponger & al. [26]). We also analyzed methylation with CRED-RA technique which has been used by researchers to assess cytosine methylation status in various plant species (Prakash & Kumar, [13]; Tsaftaris & al., [27]; Labra & al. [28]; Leljak-Levanic & al. [12]; Temel & al. [15]). The cause of phenotypic discordance in MZ twins has traditionally been attributed to postnatal environmental factors unique to the individual siblings of the pair (*i.e.* non-shared environment). Nevertheless, there is increasing evidence that MZ co-twins may differ due to postzygotic genetic, epigenetic, and prenatal environmental factors, hence challenging the assumption of genetic or epigenetic similarity that underlies the classical twin model (Kaminsky & al. [17]). Environmental conditions are not always capable to explain discordance between MZ twins. Individuals of twin pair DZ-07 have different backgrounds *i.e.* one of the sibling is smoking while the other is not and one of them was exposed to radioactive or chemical agents and the other one was not. However, they showed highly monomorphic RAPD and CRED-RA profiles (Figure 1a). DZ-01 twin pair is younger (11 years old) than DZ-07 (46 years old) and has common backgrounds. DZ-01 individuals are genetically monomorphic (Figure 1b) however each sister has her own characteristic methylation profile. Interestingly, their *Msp*I digestion profiles were more similar than *Hpa*II profiles (Figure 3c). However, discordance in methylation levels between DZ twins were claimed to result from DNA sequence differences (Kaminsky & al. [16]). MZ-06 pair is one of the oldest (40 years old) twin pairs among our subjects, they showed highly monomorphic RAPD and CRED-RA profiles (Figure 3a). Although MZ-02 pair is the youngest set, siblings showed different methylation profiles (Figure 3b). In an another study, MZ discordance in methylation level in *COMT* gene was investigated. Some twin pairs showed a high degree of methylation condordance while the others did not. Subjects were consisted of 12 MZ twin pairs discordant for birth weight but clinically unaffected (Mill & al. [19]). In present study, we performed the fingerprinting of the subjects by means of RAPD and CRED-RA and found out that methylation profiles of MZ twins might be quite different. We did not try to establish any relationship between epigenetic profile and environmental conditions *e.g.* smoking, mutagenic agents. However, some internal factors *e.g.* cytosine methylation might be sometimes more effective in human development than external factors.

Conclusions

This paper is expected to be helpful for researchers to enlighten the relationship between epigenetics and twin discordance and to identify MZ twins according to their methylation profiles.

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References

1. http://www.neurodevnet.ca/ContactUs/Team.aspx, 08.07.2010.
2. J.G. HALL, E. LOPEZ-RANGEL, Twins and Twinning, A.E.H EMERY, D.L. RIMOIN, eds., Principles and Practice of Medical Genetics: Vol 1, Churchill Livingstone, inc., New York, 1996, 374-388.
3. A.G. CARDNO, F.V. RIJSDIJK, P.C. SHAM, R.M. MURRAY, P. McGUFFIN, A Twin Study of Genetic Relationships Between Psychotic Symptoms, *Am. J. Psychiat*, **159**, 539-545 (2002).
4. A. PETRONIS, I.I. GOTTESMAN, P. KAN, J.L. KENNEDY, V.S. BASILE, A.D. PATERSON, V. POPENDIKYTE, Monozygotic Twins Exhibit Numerous Epigenetic Differences: Clues to Twin Discordance? *Schizophrenia Bull*., **29**(1), 169-178 (2003).
5. M.F. FRAGA., E. BALLESTAR, M.F. PAZ, S. ROPERO, F. SETIEN, M.L. BALLESTAR, et al., Epigenetic differences arise during the lifetime of monozygotic twins, *P. Natl. Acad. Sci. USA*, **102**, 10604-10609 (2005).
6. K.D. ROBERTSON, A.P. WOLFFE., DNA methylation in health and disease, *Nat. Rev. Genet*., **1**, 11-19 (2000).
7. B.T. HEIJMANS, D. KREMER, E.W. TOBI, D.I. BOOMSMA, P.E. SLAGBOOM, Heritable rather than age-related environmental and stochastic factors dominate variation in DNA methylation of the human *IGF2/H19* locus, *Hum. Mol. Genet*., **16**(5), 547-554 (2008).
8. E. LI, Chromatin modification and epigenetic reprogramming in mammalian development, *Nat. Rev. Genet*.,**3**,662-673 (2002).
9. D. TAKAI, P.A. JONES, Comprehensive analysis of CpG islands in human chromosomes 21 and 22, *P. Natl. Acad. Sci*., USA, **99**, 3740-3745 (2002).
10. Holliday R., Ho T., Paulin R., Gene silencing in mammalian cells, In: Russo V.E.A., Martienssen R.A., Riggs A.D. (Eds.), Epigenetic mechanisms of gene regulation, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1996.
11. A. NEMETH, G. LANGST, Chromatin higher order structure: Opening up chromatin for transcription, *Brief. Funct. Genomic. Proteomic*., **2**, 334-34, (2004).
12. J.G.K. WILLIAMS, A.R. KUBELIK, K.J. LIVAK, J.A. RAFALSKI, S.V. TINGEY, DNA polymorphisms amplified by arbitrary primers are useful as genetic markers, *Nucleic Acids Res*., **18**(22), 6531-6535 (1990).
13. D. LELJAK-LEVANIC, N. BAUER, S. MIHALJEVIC, S. JELASKA, Changes in DNA methylation during somatic embryogenesis in *Cucurbita pepo* L., *Plant Cell Rep*., **23**(3) 120-127, (2004).
14. A.P. PRAKASH, P.P. KUMAR, Inhibition of shoot induction by 5-azacytidine and. 5-aza-2'-deoxycytidine, *Plant Cell Rep*., **16**(10), 719-724 (1997).
15. L. FERRUCCI, R. MEZZANOTTE, R. VANNI, R. STUPPIA, P. GUANCIALI-FRANCHI, G. CALABRESE, et al., Effect of *Hpa*II and *Msp*I restriction endonucleases on chronic myelogenous leukemia chromosomes: Detection of CpG dinucleotide demethylation in situ, *Cancer Genet. Cytogen*., **34**(2), 251-256 (1988).
16. A. TEMEL, G. KARTAL, N. GOZUKIRMIZI, Genetic and epigenetic variations in barley calli cultures, *Biotechnol. Biotech. Eq*., **22**(4), 911-914 (2008).
17. Z.A. KAMINSKY, T. TANG, S.C. WANG, C. PTAK, G.H.T. OH, A.H.C. WONG, et al., DNA methylation profiles in monozygotic and dizygotic twins, *Nat. Genet*., **41**(2), 240-245 (2009).
18. Z. KAMINSKY, A. PETRONIS, S.C. WANG, B. LEVINE, O. GHAFFAR, D. FLODEN, et al., Epigenetics of Personality Traits: An Illustrative Study of Identical Twins Discordant for Risk-Taking Behavior, *Twin Res. Hum. Genet*., **11**(1), 1-11 (2008).
19. H. KRUYER, M. MILA, G. GLOVER, P. CARBONELL, F. BALLESTA, X. ESTIVILL, Fragile X syndrome and the (CGG)n mutation: two families with discordant MZ twins, *Am. J. Hum. Genet*., **54**(3), 437-442 (1994).
20. [19] J. MILL, E. DEMPSTER, A. CASPI, B. WILLIAMS, T. MOFFITT, I. CRAIG, Evidence for monozygotic twin (MZ) discordance in methylation level at two CpG sites in the promoter region of the catechol-O-methyltransferase (*COMT*) gene, *Am. J. Med. Genet. B*, **141B**(4), 421-425 (2006).
21. G. MACHIN, Non-identical monozygotic twins, intermediate twin types, zygosity testing, and the non-random nature of monozygotic twinning: a review, *Am. J. Med. Genet. C*, **151C**(2), 110-127 (2009).
22. J. SINGER, J. ROBERTS-EMS, A.D. RIGGS, Methylation of mouse liver DNA studied by means of the restriction enzymes msp I and hpa II, *Science*, **203**, 1019-1021 (1979).
23. T.H. BESTOR, S.B. HELLEWELL, V.M. INGRAM, Differentiation of two mouse cell lines is associated with hypomethylation of their genomes, Mol. Cell. Biol., **4**, 1800-1806 (1984).
24. A.P. BIRD, DNA methylation and the frequency of CpG in animal DNA, *Nucleic Acids Res*., **8**, 1499-1504 (1980).
25. A.P. BIRD, CpG-rich islands and the function of DNA methylation, *Nature*, **321**, 209-213 (1986).
26. F. LARSEN, G. GUNDERSEN, R. LOPEZ, H. PRYDZ, CpG islands as gene markers in the human genome, *Genomics*, **13**, 1095-1107 (1992).
27. L. PONGER, L. DURET, D. MOUCHIROUD, Determinants of CpG islands: Expression in early embryo and isochore structure, *Genome Research*, **11**, 1854-1860 (2001).
28. A.S. TSAFTARIS, A.N. POLIDOROS, E. TANI, Gene regulation and its role in hybrid vigor and stability of performance, *Genetika*, **32**(3), 189-201 (2000).
29. M. LABRA, C. TANNINI, M. BRACALE, F. SALA, Methylation changes in specific sequences in response to water deficit, *Plant Biosyst*., **136**(3), 269-275 (2002).