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Original paper

Comparison of programs to design primers for Methylation-Specific PCR

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Abstract Three computer programs for designing alternative primers for MS-PCR were tested using both default and user-defined settings. The three primer design programs used in the study were MethPrimer, Primo MSP 3.4 and Methyl Primer Express Software v1.0. In each program, a DNA sequence is entered as the starting material. Then the program searches the sequence for potential CpG islands. Next, the primers are selected around the potential CpG islands designated by the user. The results of the primer selection are delivered through a web browser as text and a graphic preview. The programs use different algorithms, which results in the detection of different numbers and lengths of CpG islands. MethPrimer was the easiest to use and the most effective. Methyl Primer Express was also effective, but this is a program for at least moderately advanced users. An advantage of Methyl Primer Express and Primo MSP is their ability to analyse an entire genome without splitting it into fragments. MethPrimer has been used with its default settings in cancer research and in research on transgenic animals. Methyl Primer Express software has been widely used in basic research, as well as in research on the regulation of gene expression and in cancer research. MethPrimer and Methyl Primer Express can be recommended for research on honey bees.

Keywords Methylation-specific PCR (MSP), primer design, database, CpG islands, 5-methylocytosine, *in silico*

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Introduction

Recent years have seen a breakthrough in epigenetics, the study of mechanisms that modify gene expression without altering the DNA sequence. Methylation of DNA is one such epigenetic modification (ANDRASZEK & al. [1]; GRYZINSKA & al. [2]). Its main task is to activate and silence genes, and it operates as a gene promoter inhibitor (STRACHECKA & al. [3]; ANDRASZEK & al. [4]). Molecular biology methods enable analysis of an entire genome or of individual genes. There are currently quite a few techniques available for qualitative and quantitative determination of 5-methylcytosine (GRYZINSKA & al. [5]; STRACHECKA & al. [6]). When a gene is to be silenced, a CH3 methyl group attaches to cytosine at specific locations on DNA called CpG islands. This prevents the gene from being copied onto RNA and then translated (SUZUKI & al. [7]; FORET & al. [8]). The mechanism of DNA methylation seems to be very simple, but many factors are required for it to proceed correctly, such as specific methyltransferases, accessory proteins, or proper regulation of the process (KUCHARSKI & al. [9]; MALESZKA & al. [10]). According to VANYUSHIN [11], the DNA methylation pattern is usually preserved in the genome, except in the case of errors due to anomalies in the process.

DNA methylation takes place in specific sites in the genome, usually in CpG dinucleotides. In genetics, CpG islands are defined as genomic regions greater than 200 bp, with a high frequency of guanine/cytosine content above 0.5 and an observed or expected presence of above 0.6 (GARDINER-GARDEN & FROMMER [12]). Gene expression is directly proportional to the amount of methylated DNA within a promoter, which plays the most important role in the transcription process. Reduced expression of a gene results in greater DNA methylation. According to KUCHARSKI & al. [9] and GRYZINSKA & al. [13], this is important because it means that selected genes can be silenced or activated by removing or adding methyl groups.

Current methods allow for analysis of genome methylation. Additionally, quite a few methods enable qualitative and quantitative determination of 5-methylcytosine. Currently, one of the most common methods of qualitative analysis of the epigenome is methylation-specific PCR (MSP), based on traditional PCR (Figures 1-3). Methylated and unmethylated cytosine are determined by chemically modifying DNA with sodium bisulfite, during which unmethylated cytosine is converted to uracil, while methylated cytosine remains cytosine. Then specific fragments of DNA are subjected to allele-specific PCR (MS-PCR). The advantages of this method are its simplicity and low cost. Specific primers differentiating methylated and unmethylated cytosine can be designed using available computer programs, including internet software, which differ from one another.

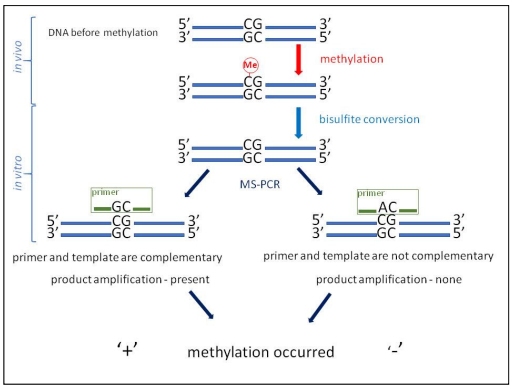

Figure 1. Reaction scheme for MS-PCR (DNA methylation has occurred).

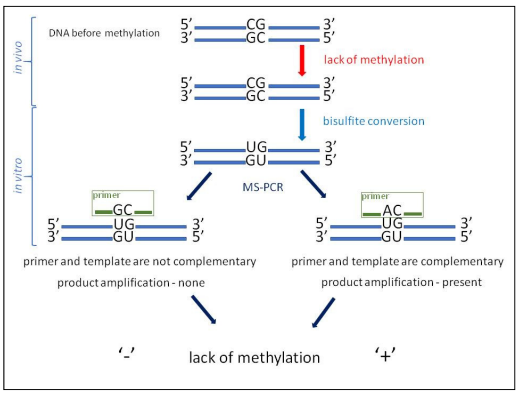

Figure 2. Reaction scheme for MS-PCR (no methylation).

Until now, programs designing primers for determining DNA methylation sites have been limited to the study of a single gene (more specifically its promoter gene site). At the same time, little attention has been paid to the mitochondrial epigenome, because preliminary studies have shown no DNA modification. However, research by DEVALL & al. [14] has shown that there are epigenetic changes in mtDNA, potentially playing an important role in mitochondrial gene dysfunction. In addition, studies on vertebrates (humans and mice) have shown modifications of the epigenetic D-loop (the non-coding region) within both CpGs and non-CpGs (previously described in fungi and plants) (BELLIZZI & al. [15]).

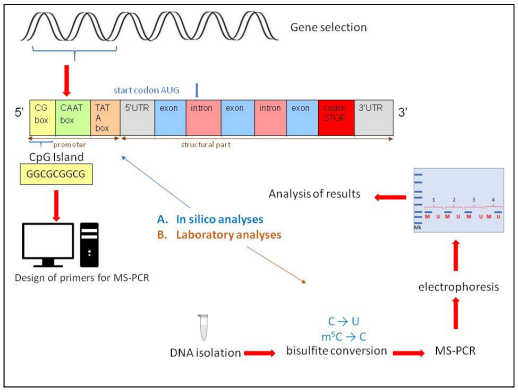

Figure 3. Stages of analysis of DNA methylation assessment.

The honey bee is used as a model organism in studies of epigenetic processes. Fertilized queens or sterile workers hatch from fertilized eggs. Despite similar genotypes, the future status of bees is determined by epigenetic mechanisms, such as DNA methylation and chromatin remodelling, which are affected by the diet during the larval period (KUCHARSKI & al. [9]). Substances present in the royal jelly have been shown to reduce methyltransferase enzyme activity and methyltransferase gene expression, which consequently decreased methylation within the dynactin p62 gene. This, in turn, resulted in a significant increase in the number of queens and a reduction in the numerical strength of workers or intercastes (SHI & al. [16]). Larvae reared as queens or workers have been found to differ in their gene expression patterns. Additionally, epigenetic modifications, mainly in the brains of honey bees, have been demonstrated in cases of behavioural reversal (HERB & al. [17], STRACHECKA & al. [18]).

The aim of this study was to design and compare MSP primers for the same DNA fragment using available software: MethPrimer, Primo MSP 3.4 and Methyl Primer Express Software v1.0. These programs were used to design primers to analyse methylation of honey bee (Apis mellifera) mtDNA in MSP.

Materials and Methods

1. Programs for the design of MSP primers

In the study we used three popular primer design programs. The first was MethPrimer, which uses the Java platform and is available on-line. Based on Primer3, it is a program for designing MSP primers used in the process of methylation mapping. It is also able to prepare primers for the COBRA and MS-SnuPE methods after slight modifications. The second program, Primo MSP 3.4, also uses the Java platform and is available on-line. The last program is Methyl Primer Express Software v1.0. Using each program, a DNA sequence is entered as the starting material. Then each of the programs searches the sequence for potential CpG islands. Next, the primers are selected around the potential CpG islands designated by the user. The results of the primer selection are delivered through a web browser as text and a graphic preview.

MethPrimer has an input limit on the number of nucleotides (5000 bp). To analyse the entire mtDNA, successive parts of the genome are entered: 1-5000 bp, 4001-9000 bp, 8001-13,000 bp and 12,001-16,343 bp. This enables accurate determination of the sites of CpG islands, owing to the 'overlap' of 1000 bp before each tested fragment of the mtDNA. The results are summed up and the localization of the CpG islands is calculated.

Primo MSP 3.4 allows the user to enter the entire DNA fragment to be tested, which is then scanned and processed according to the recommended settings.

Methyl Primer Express Software v1.0 allows the user to enter the entire DNA fragment to be tested, which is then scanned and processed according to the recommended settings.

2. Selection criteria for primers

One of the primer pairs for the reaction contains one or more fragments complementary to CpG sites. One of the primer pairs is designed so that the cytosine in the CpG site is treated as cytosine, while for the other it is assumed that cytosine has been changed to thymine. As a result of a parallel reaction we obtain one product. If the product is obtained using the first pair of primers, this indicates methylation of cytosine, and if it is obtained in a reaction with the second primer pair, cytosine is not methylated.

3. Transformation of fragments

To design primers for bisulfite-modified DNA, the user should enter the original sequence. No processing or editing is necessary. The program creates two versions of the modified fragments. The first is a bisulfite-modified and methylated fragment in which all cytosine, except 5mC, is converted to thymine, while the other is a bisulfite-modified and unmethylated DNA fragment in which all cytosine is converted to thymine.

The mtDNA of A. mellifera was entered into each of the programs in order to locate CpG islands. Three series of measurements were performed, which included different parameters of the search for CpG islands (the settings recommended by the manufacturer and two selected by the user). The CpG islands found were then used as a template for the design of MSP primers. The data obtained (the number of CpG islands and primers) were presented in Table 1 and the efficiency of the programs was compared. The level of difficulty for the user and the capabilities of each program were assessed, including their utility in epigenetic research. The mtDNA of the honey bee A. mellifera L., published online in the NCBI database (GenBank: L06178.1), was used to design the primers (CROZIER & CROZIER [19]).

Results and Discussions

1. Design of primers

The three simulations performed in each program on the entire mtDNA of A. mellifera L. resulted in different numbers and lengths of CpG islands. For Methyl Primer Express Software v1.0, four tests were conducted, due to the wider range of possibilities offered by this application. Table 1 presents a summary of the experimental results of design of MPS primers by each of the programs.

Many methods of analysis of DNA methylation are used in epigenetic studies. The most commonly used method is MS-PCR (GRYZINSKA & al. [20]). The MSP (methylation-specific PCR) reaction was first described in 1996 by HERMAN & al. [21]. This reaction, like many other methods of analysing levels of DNA methylation, is based on the chemical modification of nucleic acid by sodium bisulfite (NaHSO3). This leads to deamination of cytosine. An intermediate product is formed, which is converted to uracil when the pH changes from acidic to alkaline. The DNA matrix is subjected to PCR amplification. DNA amplification is performed using two pairs of primers that are designed to distinguish between methylated and unmethylated bases of DNA which have been modified by NaHSO3 from unmodified bases. The first pair of primers recognizes 5-methylcytosine as cytosine, is complementary, and attaches to the unchanged fragment of DNA. The second pair of primers is complementary to the sequence in which 5-methylcytosine has been converted to uracil. In the PCR reaction, uracil is treated as thymine and forms a complementary pair with adenine (HERMAN & al. [21]). The advantages of this method are the short time of analysis, the ability to obtain satisfactory results from small amounts of DNA (5 μg), specificity, and sensitivity (methylation can be detected even when only 0.1% of alleles are methylated). As with all PCR methods, a disadvantage of this method is the possibility of sample contamination. Moreover, poor primer design is a significant factor leading to a false positive result (SULEWSKA & al. [22]).

The number of CpG islands is calculated using a simple algorithm through data flow controls. Along the DNA strand, the algorithm checks the content of GC dinucleotides in relation to expected GC dinucleotides (GARDINER-GARDEN & FROMMER [12]; GRYZINSKA & al. [23]). If the user selects primers with predicted CpG islands as a destination region, they should follow a set of rules reflecting the number and size of CpG islands found (GRYZINSKA & al. [20]; LI & DAHIYA [24]).

For standard PCR, an important parameter taken into account during the selection of primers is its ability to form a stable duplex with a specific fragment of the target DNA without hybridizing any other fragment of the test DNA. This also applies to the PCR test based on bisulfite conversion. To access these parameters, users of MethPrimer use Primer3 algorithms to calculate the hybridization, complementary pairs, GC content and melting temperature. There are also additional restrictions, such as differences in the melting point between the first and second primer and the permitted length in relation to the CpG islands (LI & DAHIYA [24]).

The results of our study indicate that the various programs use different searching algorithms, which results in the detection of different numbers and lengths of CpG islands. These results guide the search of primers, which differ significantly from one another.

Primo MSP 3.4 lacks some important features, such as an adjustable number of non-CpG Cs and CpG island searches. Most likely the application is not sufficiently optimized (DAVIDOVIĆ & al. [25]). MethPrimer and Methyl Primer Express were found to be the most frequently used programs in the available research of other authors. MethPrimer has been used with its default settings in cancer research (SUN & al. [26]) and in research on transgenic animals (SU & al. [27]), while Methyl Primer Express has found wide application in basic research, as well as in research on regulation of gene expression and cancer research (OKAZAKI & al. [28]; QUENTMEIER & al. [29]). These programs can also be used in research on honey bees.

Table 1. Summary of experimental results of design of MPS primers by different programs.

Settings	Parameters	MethPrimer®	Primo MSP 3.4®	Methyl Primer Express® Software v1.0
		Number found		
Recommended settings	CpG islands	0	0	0
	Primers (pair)	5	0	0
1st run	CpG islands	1	1	0
	Primers (pair)	5	0	0
2nd run	CpG islands	0	0	0
	Primers (pair)	5	0	0
3rd run	CpG islands	-	-	3
	Primers (pair)	-	-	0
Difficulty of use	-	Easy	Easy	Intermediate

Conclusions

Of the programs tested, the easiest to use and most effective was MethPrimer, which found one CpG island and designed five pairs of primers. Methyl Primer Express Software v1.0 is also effective, but this is a program for at least moderately advanced users who are able to specify the search criteria. Another advantage of both Methyl Primer Express Software v1.0 and Primo MSP 3.4 is the ability to analyse an entire genome without splitting it into parts. The utility of the primers will be confirmed when an amplification product is obtained in an MSP reaction. Primo MSP 3.4 proved to be the least flexible of those analysed.

Conflict of interest disclosure

There are no known conflicts of interest in the publication of this article. The manuscript was read and approved by all authors.

Compliance with ethical standards

Any aspect of the work covered in this manuscript has been conducted with the ethical approval of all relevant bodies and that such approvals are acknowledged within the manuscript.

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References

1. K. ANDRASZEK, M. GRYZINSKA, S. KNAGA, E. WÓJCIK, E. SMALEC. Number and size of nucleoli in the spermatocytes of chicken and Japanese Quail. Folia Biol. (Krakow), 60 (3-4): 121-127 (2012).
2. M. GRYZINSKA, J. BATKOWSKA, K. ANDRASZEK, B. HORECKA, G. JEŻEWSKA-WITKOWSKA. Changes in plumage color and patterns in Polbar breed chicks (Polish conservative breed) during their first weeks after hatching. Europ. Poult. Sci, 78: 1-10 (2014).
3. A. STRACHECKA, G. BORSUK, K. OLSZEWSKI, J. PALEOLOG, M. GAGOŚ, J. CHOBOTOW, A. NAWROCKA, M. GRYZINSKA, M. BAJDA. The effect of amphotericin b on mortality, body-surface protein concentrations and DNA methylation level in the honey bee (Apis mellifera). J. Apicl. Sci, 56 (2): 5-11 (2012).
4. K. ANDRASZEK, M. GRYZIŃSKA, E. WÓJCIK, S. KNAGA, E. SMALEC. Age-dependent change in the morphology of nucleoli and methylation of genes of the nucleolar organizer region in the Japanese quail model Coturnix japonica (Temminck and Schlegel, 1849) (Galliformes: Aves). Folia Biol. (Krakow), 62 (4): 293-300 (2014).
5. M. GRYZINSKA, A. JAKUBCZAK, P. LISTOS, P. DUDKO, K. ABRAMOWICZ, G. JEŻEWSKA-WITKOWSKA. Association between body weight and age of dogs and global DNA methylation. Med. Weter, 72 (1): 64-67 (2016).
6. A. STRACHECKA, J. CHOBOTOW, J. PALEOLOG, A. ŁOŚ, M. SCHULZ, D. TEPER, H. KUCHARCZYK, M. GRZYBEK. Insights into the biochemical defence and methylation of the solitary bee Osmia rufa L: A foundation for examining eusociality development. PLoS ONE, 12(4): e0176539 (2017).
7. M.M. SUZUKI, A.R.W. KERR, D. DE SOUSA, A. BIRD. CpG methylation is targeted to transcription units in an invertebrate genome. Genome Res, 17 (5): 625-631 (2007).
8. S. FORET, R. KUCHARSKI, Y. PITTELKOW, G. LOCKETT, R. MALESZKA. Epigenetic regulation of the honey bee transcriptome: unravelling the nature of methylated genes. BMC Genom, 10: 472 (2009).
9. R. KUCHARSKI, J. MALESZKA, S. FORET, R. MALESZKA. Nutritional control of reproductive status in honeybees via DNA methylation. Science, 319 (5871): 1827-1830 (2008).
10. R. MALESZKA. Epigenetic integration of environmental and genomic signals in honey bees. Epigenetics, 3 (4): 188-192 (2008).
11. B.F. VANYUSHIN. Enzymatic DNA methylation is an epigenetic control for genetic functions of the cell biochemistry. Biochem-Moscow, 70 (4): 488-499 (2005).
12. M. GARDINER-GARDEN, M. FROMMER. CpG islands in vertebrate genomes. J. Mol. Biol, 196 (2): 261-282 (1987).
13. M. GRYZINSKA, K. ANDRASZEK, G. JEŻEWSKA-WITKOWSKA. Estimation of global content of 5-methylcytosine in DNA during alantoic and pulmonary respiration in the chicken embryo. Folia Biol. (Krakow), 62 (2): 97-101 (2014).
14. M. DEVALL, J. MILL, K. LUNNON. The mitochondrial epigenome: a role 1 in Alzheimer's disease? Epigenomics, 6 (6): 665-75 (2014).
15. D. BELLIZZI, P. D'AQUILA, T. SCAFONE, M. GIORDANO, V. RISO, A. RICCIO, G. PASSARINO. The control region of mitochondrial DNA shows an

unusual CpG and non-CpG methylation pattern. DNA Res, 20 (6): 537-547 (2013).
16. Y.Y. SHI, ZY HUANG, Z.J. ZENG, Z.L. WANG, X.B. WU, W.Y. YAN. Diet and cell size both affect queen–worker differentiation through DNA methylation in honey bees (Apis mellifera, Apidae). PLoS One, 6 (4): e18808 (2011).
17. B.R. HERB, F. WOLSCHIN, K.D. HANSEN, M.J. ARYEE, B. LANGMEAD, R. IRIZARRY, G.V. AMDAM, A.P. FEINBERG. Reversible switching between epigenetic states in honeybee behavioral subcastes. Nat. Neurosci, 15 (10): 1371-1373 (2012).
18. A. STRACHECKA, K. OLSZEWSKI, M. BAJDA, J. PALEOLOG. Natural larval diet differently influences the pattern of developmental changes in DNA 5-methylcytosine levels in Apis mellifera queens as compared with the workers and drones. Biochem-Moscow, 8 (8): 1019-1025 (2015).
19. R.H. CROZIER, Y.C. CROZIER. The Mitochondrial Genome of the Honeybee Apis Mellifera: Complete Sequence and Genome Organization. Genetics, 133 (1): 97-117 (1993).
20. M. GRYZINSKA, K. ANDRASZEK, G. JOCEK. DNA methylation analysis of the gene CDKN2B in Gallus gallus (chicken). Folia Biol. (Krakow), 61 (3-4): 165-171 (2013).
21. J.G. HERMAN, J.R. GRAFF, S. MYOHANEN, B.D. NELKIN, S.B. BYLIN. Methylation-specyfic PCR: a novel PCR assai for methylation status of CpG island. Proc. Natl. Acad. Sci. USA, 93 (18): 9821-9826 (1996).
22. A. SULEWSKA, W. NIKLINSKA, M. KOZŁOWSKI, L. MINAROWSKI, W. NAUMNIK, J. NIKLINSKI, K. DABROWSKA, L. CHYCZEWSKI. DNA methylation in states of cell physiology and pathology. Fol. Histochem. Cytobiol, 45 (3): 149-158 (2007).
23. M. GRYZINSKA, A. JAKUBCZAK, R. STRYJECKI, G. JEZEWSKA-WITKOWSKA. In silico analysis of methylation of the selected genes using computer programmes based on various analytical techniques. Biocybern. Biomed. Eng, 35 (3): 169-175 (2015).
24. L.C. LI, R. DAHIYA. MethPrimer: designing primers for methylation PCRs. Bioinformatics, 18 (11): 1427-1431 (2002).
25. R.S. DAVIDOVIĆ, A.M. BOŽOVIĆ, V.L. MANDUŠIĆ, M.M. KRAJNOVIĆ. Methylation-specific PCR: four steps in primer design. Cent. Eur. J. Biol, 9 (12): 1127-1139 (2014).
26. X.F. SUN, Z.Y. SUN, B. PAN, L. LI, W. SHEN. Alteration in methylation pattern of oncogene Akt1 promoter region in bladder cancer. Mol. Biol. Rep, 39 (5): 5631-5636 (2012).
27. J.M. SU, B. YANG, Y.S. WANG, Y.Y. LI, X.R. XIONG, L.J. WANG, Z.K. GUO, Y. ZHANG. Expression and methylation status of imprinted genes in placentas of deceased and live cloned transgenic calves. Theriogenology, 75 (7): 1346–1359 (2011).
28. R. OKAZAKI, A. OOTSUYAMA, Y. YOSHIDA, T. NORIMURA. Establishment of methylation-specific PCR for the mouse p53 gene. Mol. Biol. Internat, 938435 (2011).
29. H. QUENTMEIER, S. EBERTH, J. ROMANI, H.A. WEICH, M. ZABORSKI, H.G. DREXLER. DNA methylation regulates expression of VEGF-R2 (KDR) and VEGF-R3 (FLT4). Cancer, 12: 19 (2012).