

# An Improved Evolutionary Method with Test in Different Crossover Rates for PCR-RFLP SNP Genotyping Primer Design

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**Abstract**—An improved evolutionary method of PCR-RFLP (Polymerase Chain Reaction-Restriction Fragment Length Polymorphism) SNP (Single Nucleotide Polymorphism) genotyping primer design is presented in this paper. The core of SNP-RFLPing is combined with the method for available restriction enzymes mining. Based on the same criteria with the GA (Genetic Algorithm), we *in silico* simulated the method in SNPs of the SLC6A4 gene and compared these results with those of the GA. Furthermore, we test the method in different crossover rates settings for the PCR-RFLP SNP genotyping primer design. The experimental results showed that the method indeed improves the primer design for PCR-RFLP assay for SNP genotyping, and the crossover rate 1.0 is preferred. The observed results showed the increased crossover rate seems can improve the PCR-RFLP SNP genotyping primer design.

**Keywords**—GA, PCR-RFLP, SNP, genotyping, primer design.

## I. INTRODUCTION

PCR-RFLP (Polymerase Chain Reaction-Restriction Fragment Length Polymorphism) is a simple, inexpensive and accurate laboratory technique implemented to investigate the causes of genetic variations and mutations. It is extensively considered useful in small-scale research studies of complex genetic diseases [1, 2]. SNP (Single Nucleotide Polymorphism) is the most common genetic variations and always is used to be as the biological marker and applied to all kinds of studies including population genetics and evolutionary studies [3], pharmacogenetic analysis [4], malignancy studies [5, 6], preventive medicine [7, 8], personalized medicine [9] and forensics [10]. SNP genotyping is therefore important for the measurement of genetic variations. In order to achieve a PCR-RFLP assay for SNP genotyping, it is essential to find out available restriction enzymes and a feasible primer pair.

In the past literatures, there are several systems had provided the function for PCR-RFLP assay for SNP genotyping including V-MitoSNP [11], SNP Cutter [1], and Prim-SNPing [12]. However, those systems got some limits for primer design in PCR-RFLP assay for SNP genotyping; the limits had been

described in previous literature [13]. In 2013, an effective method that was implemented in an evolutionary computation-based genetic algorithm (GA) with the updated core of SNP-RFLPing was fundamentally proposed for the SNP PCR-RFLP SNP genotyping primer design [13]. Although many parameters of the GA were estimated to get better results for the primer design of PCR-RFLP assay for SNP genotyping, the GA in nature easily gets trapped into local optima. A local search mechanism is led into the GA can effectively avoid the problem. Therefore, we propose the improved evolutionary method with test in different crossover rates to make better for the PCR-RFLP SNP genotyping primer design.

In this study, we also applied the same core of SNP-RFLPing [14-16] to reliably mine available restriction enzymes. Based on the same criteria with the GA, we compare the results with the previous GA in SNPs of the SLC6A4 gene. In addition, the different parameter settings in crossover rates are *in silico* evaluated for the PCR-RFLP SNP genotyping primer design.

## II. METHODS

### A. PCR-RFLP SNP genotyping primer design problem

Assume a DNA template sequence  $T_D$  contains a target SNP for genotyping. The task of primer design for PCR-RFLP SNP genotyping is to give available restriction enzymes which can distinguish the target SNP and a feasible primer pair for PCR-RFLP assay. The  $T_D$  is formulated given as follows:

$$T_D = \{B_i \mid i \text{ is the index of DNA sequence,} \\ \exists! B_i \in \text{target SNP}\} \quad (1)$$

where  $B_i$  is a variable for the nucleotides of 'A', 'T', 'C', 'G' and SNP. The SNP is identified as SNP IUPAC code (M, R, W, S, Y, K, V, H, D, B or N) or the dNTPs format ([dNTP1/dNTP2]).

The symbol  $\exists!$  represents the existence and uniqueness. In order to achieve a widespread design, we only focus on true SNPs as described in dbSNP [17] of NCBI as the target SNP. For deletion/insertion polymorphisms (DIPs) and multi-nucleotide polymorphisms (MNP), we do not consider in the present design.

In the PCR-RFLP SNP genotyping primer design, one restriction enzyme which can distinguish the genotype of the target SNP at least and a pair of sub-sequences of corresponding constraints retrieved from  $T_D$  must be found out. The available restriction enzyme can be searched from REBASE [18]. The

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pair of sub-sequences is respectively the forward primer ( $P_f$ ) and the reverse primer ( $P_r$ ). They have been formulated and respectively shown in formula (2) and (3) below.

$$P_f = \{B_i \mid \forall B_i \in \{'A', 'T', 'C', 'G'\}, F_s \leq i \leq F_e, i \text{ is the index of } T_D\} \quad (2)$$

$$P_r = \{\overline{B_i} \mid \forall B_i \in \{'A', 'T', 'C', 'G'\}, R_s \leq i \leq R_e, i \text{ is the index of } T_D\} \quad (3)$$

where  $F_s$  and  $F_e$  indicate respectively the start index and the end index of  $P_f$  in  $T_D$ ;  $R_s$  and  $R_e$  indicate respectively the start index and the end index of  $P_r$  in  $T_D$ .  $\{\overline{B_i}\}$  is the anti-sense sequence of  $B_i$ . For instance, a set [3]=“ATCTGCGCATCGATCTTATGCC”, its complementary sequence is “TAGACGCGTAGCTAGAATACGG”. The reason is that nucleotide ‘A’ is complemented to nucleotide ‘T’; nucleotide ‘C’ is complemented to nucleotide ‘G’, and vice versa. Finally, its anti-sense sequence  $\{\overline{B_i}\}$  is the reverse of the complementary sequence, i.e.,  $\{\overline{B_i}\} = \text{“GGCATAAGATCGATGCGCAGAT”}$ .

#### B. The improved evolutionary method for PCR-RFLP SNP genotyping primer design

In the evolutionary computation method, we use an encoding vector  $P_v$  (called “individual”) consisting of four elements  $F_s$ ,  $F_l$ ,  $P_l$  and  $R_l$  to determine the designed primers in a PCR-RFLP assay. The encoding vector is shown as:

$$P_v = (F_s, F_l, P_l, R_l) \quad (4)$$

Use the  $P_v$ , the start position  $R_s$  of the reverse primer can be calculated by:

$$R_s = F_s + P_l - R_l \quad (5)$$

From the above five variables of  $F_s$ ,  $F_l$ ,  $P_l$ ,  $R_s$  and  $R_l$ , we can immediately obtain forward primer and the reverse primer from  $T_D$ . Therefore, in the PCR-RFLP SNP genotyping primer design, we use  $P_v$  to perform the evolutionary computations.

Seven separate steps are mainly involved in the proposed method. The steps are respectively 1) available restriction enzymes mined for the target SNP, 2) availability evaluated for PCR-RFLP SNP genotyping primer design, 3) population generated for initialization, 4) fitness evaluated for feasibility, 5) local search applied for regional optimal solution, 6) termination criteria judged for finish, and 7) selection, crossover, mutation and replacement operated for diversity. These steps are described in detail below. Fig. 1 shows the work flowchart.

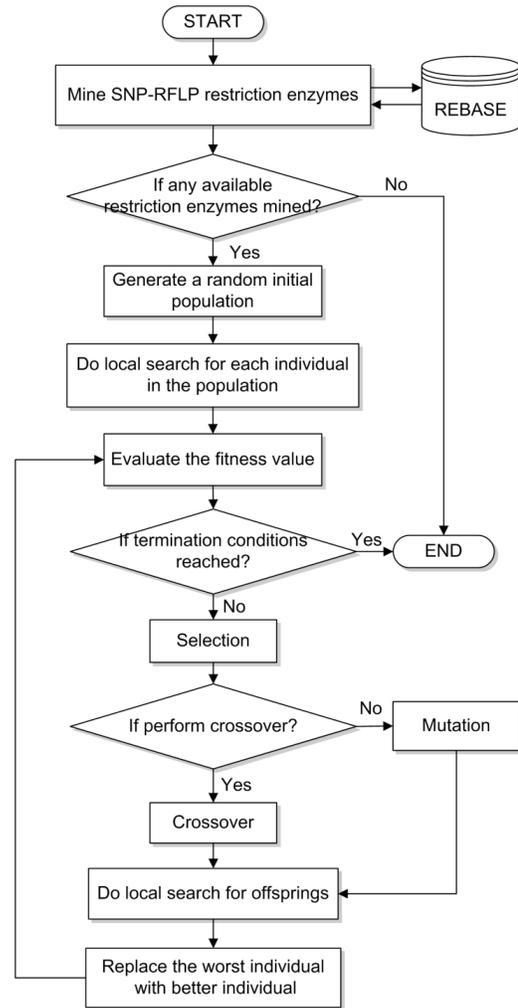


Fig. 1 The work flowchart of the improved evolutionary method for PCR-RFLP SNP genotyping primer design.

#### 1) Available restriction enzymes mined for the target SNP

The existed restriction enzymes have been completely gathered in the famous REBASE [18] and updated periodically. The proposed method like the previous GA uses the updated core of SNP-RFLPing [14-16] which provides an effectively way combined to REBASE to mine available restriction enzymes.

#### 2) Availability evaluated for PCR-RFLP SNP genotyping primer design

All restriction enzymes in REBASE [18] are evaluated for the target SNP. If no any available restriction enzymes are found to distinguish the target SNP, the PCR-RFLP SNP genotyping primer design is insignificant. In order to avoid this situation, the method will be terminated straightaway; else it continues to perform the following steps.

#### 3) Population generated for initialization

Initial population is the earliest solutions used in evolutionary computation methods. The method randomly generates a fixed number of unduplicated individuals ( $P_v$ ) as an initial population.

#### 4) Fitness evaluated for feasibility

The fitness value is minimized to evaluate the designed result.

The fitness value of each individual in the population is computed by an experienced fitness function. The experienced fitness function is designed according to the common primer constraints. The fitness function in the method is referred to [13].

#### 5) Local search applied for regional optimal solution

Local search looks for superior individuals in a preset limited area around the original individual. When a local search is performed, the experiences of individuals in the population will be improved to obtain local optimums. In the proposed method, all individuals are first subjected to the local search so that local optimal is acquired in an initial iteration. After crossover or mutation operation, the new offsprings are also subjected to the local search to obtain their local optimum. By iteration to iteration, the local search eventually brings a global optimum.

#### 6) Termination criteria judged for finish

The method implements two termination criteria: i) One of the fitness values of individuals in the population reaches to zero, and ii) a preset iterations is reached. The fitness value of an individual reaches to zero represents the designed primer pair completely conforms to the preset primer constraints. The value of the preset iterations is given based on DeJong and Spears' measure [19].

#### 7) Selection, crossover, mutation and replacement operated for diversity

The improved evolutionary method is like the GA that has the evolutionary computation including the operations for selection, crossover, mutation and replacement. The selection operation randomly selects two individuals from the population. The cross operation and the mutation operation is alternative in the method. When a given probability reaches the preset crossover rate, the two individuals perform the uniform crossover operation to generate two new offsprings. When a given probability reaches the preset mutation, one of the two individuals is randomly selected perform one point mutation to generate a new offspring. The detailed crossover and mutation operations can be found in [13].

### III. RESULTS AND DISCUSSION

PCR-RFLP assay for SNP genotyping is a wide and useful biotechnology for many laboratories. However, currently only very few methods and systems are provided for PCR-RFLP SNP genotyping primer design. In this study, an improved evolutionary method for PCR-RFLP SNP genotyping primer design is proposed. In order to measure the method, we fairly compare it with the previous GA method and perform it in different crossover rates.

#### A. The data set and computational environment

In this study, we use the same data set as the previous GA method which is 288 SNPs in the SLC6A4 gene. The 288 SNPs have excluded the deletion/insertion polymorphisms (DIPs) and multi-nucleotide polymorphisms (MNPs). A total length of 500 bp flanking sequence for every SNP had been retrieved from SNP-Flankplus (<http://bio.kuas.edu.tw/snp-flankplus/>) [20] for

as the DNA template for evaluating the primer design. These templates are referred to [13]. Furthermore, the method is also run on the same computational environment an Intel(R) Core(TM) 2 CPU of 1.86 GHz and 1GB RAM under the Microsoft Windows XP SP3 and JAVA 6.0 platforms for fairly compare to the previous GA method.

#### B. Parameter Settings

The common primer constraints include primer length between 16 and 28 nt, GC% between 40 and 60%, primer  $T_m$  between 45 and 62°C, primer  $T_m$  difference within 5°C, PCR product length larger than 100 bp and PCR product length ratio 1 : 2 : 3 were set in the method. Like the GA, four parameters, i.e., the number of iterations, the population size, the crossover rate and the mutation rate are also needed to be set. We first used the parameter settings based on the DeJong and Spears' measure [19]. The values were respectively given 1000, 50, 0.6 and 0.001. Furthermore, we also used different crossover rates to evaluate the PCR-RFLP SNP genotyping primer design.

#### C. In silico simulation results

##### 1) Results for DeJong and Spears parameter settings

Table 1 shows the *in silico* simulation results based on the DeJong and Spears parameter settings. In primer length difference, the proposed method is 1.59% better than the GA method. In GC%, the proposed method is 4.58% better than the GA method. In GC clamp, the proposed method is 31.07% better than the GA method. In  $T_m$ , the proposed method and the GA method is equivalent. In  $T_m$  difference, the proposed method is 2.39% better than the GA method. In PCR product length, the proposed method reaches to perfect 100% and 0.13% better than the GA method. In Dimer, both the proposed method and the GA method achieve perfect 100%. In Hairpin, the proposed method is 0.4% worse than the GA method. Finally, in specificity, both the proposed method and the GA method achieve perfect 100%. From the integral evaluation, the proposed method is superior to the GA method. The proposed method has the average fitness 1.17 and the GA has the average fitness 4.74 for 251 SNPs. Therefore, the proposed method for PCR-RFLP SNP genotyping primer design is more suitable for designing conformed primer constraints than the GA method based on DeJong and Spears parameter settings.

TABLE I  
IN SILICO SIMULATION RESULT BASED ON THE DEJONG AND SPEARS PARAMETER SETTINGS IN 251 SNPs OF THE SLC6A4 GENE

Constraints	GA	the improved evolutionary method
Primer length difference	97.61%	<b>99.20%</b>
GC%	78.69%	<b>83.27%</b>
GC clamp	65.94%	<b>97.01%</b>
$T_m$	97.61%	97.61%
$T_m$ difference	94.82%	<b>97.21%</b>
PCR product length	99.87%	<b>100.00%</b>
Dimer	100.00%	100.00%
Hairpin	<b>99.80%</b>	99.40%
Specificity	100.00%	100.00%
Average fitness	4.74	<b>1.17</b>

The bold font shows the better result between the GA and the improved evolutionary method.

## 2) Results for different crossover rates

The crossover operation can assist the individual to exchange their information with other individual to obtain feasible solution. In order to observe the influences of the crossover rates, we set the crossover rate respectively to 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, and 1.0 to perform the method for PCR-RFLP SNP genotyping primer design. The results are shown in Table 2. Among results of using different crossover rates, we found the crossover rate set to 1.0 obtain better average fitness 0.81. In these different crossover rates, all primer constraints in the mean and SD achieve to the satisfied results except the GC% (all the means are more than 97%, and all the SDs are less than 0.8% excluding the GC clamp and  $T_m$  which are 2.00% and 1.24%, respectively). To observe the results in the different crossover rates, the average fitness seems to be improved gradually by the increased crossover rate. When the crossover rate reaches the highest value 1.0, the average fitness is also got the best. Compare to the previous GA method, the crossover rate is also set to 1.0 obtain the better average fitness 3.49 but it is obviously worse than that of the proposed method.

## IV. CONCLUSIONS

To provide an effective and reliable PCR-RFLP SNP genotyping primer design method is helpful for small-scale basic research studies of complex genetic diseases associated with SNPs. In the past, we had provided an evolutionary computation method which uses the GA for PCR-RFLP SNP genotyping primer design. However, the nature of the GA method usually gets trapped into local optima. That prompts us to propose a better method for designing more feasible primers for PCR-RFLP assay for SNP genotyping. Comparing to the previous GA method, the method is indeed meaningfully improved for PCR-RFLP SNP genotyping primer design. The test in different crossover rates for the proposed method has been cautiously evaluated and we get the result which the crossover rate is set to 1.0 is preferred.

TABLE II  
IN SILICO SIMULATION RESULT USING DIFFERENT CROSSOVER RATES IN 251 SNPs OF THE SLC6A4 GENE

Crossover rate	Constraints									
	Primer length difference	GC%	GC clamp	$T_m$	$T_m$ difference	PCR Product length	Dimer	Hairpin	Specificity	Average fitness
0.1	98.41%	80.28%	94.62%	97.21%	98.01%	100.00%	100.00%	99.80%	99.80%	1.64
0.2	99.60%	80.48%	96.22%	97.01%	99.60%	100.00%	100.00%	99.80%	100.00%	1.6
0.3	98.41%	79.88%	97.41%	98.41%	98.80%	99.73%	100.00%	100.00%	99.80%	1.14
0.4	98.01%	81.67%	96.81%	97.61%	99.20%	100.00%	100.00%	100.00%	100.00%	1.45
0.5	99.20%	83.67%	96.22%	98.21%	98.41%	99.73%	100.00%	100.00%	100.00%	1.16
0.6	99.20%	83.27%	97.01%	97.61%	97.21%	100.00%	100.00%	99.40%	100.00%	1.17
0.7	99.60%	84.46%	97.21%	98.80%	98.80%	99.87%	100.00%	100.00%	100.00%	0.95
0.8	98.41%	82.07%	98.61%	98.41%	99.20%	100.00%	100.00%	99.80%	100.00%	0.93
0.9	99.60%	82.07%	98.21%	98.80%	99.20%	99.73%	100.00%	100.00%	100.00%	0.9
1.0	99.60%	83.07%	99.00%	98.61%	99.60%	100.00%	100.00%	100.00%	100.00%	0.81
mean± SD	99.00%± 0.63%	82.09%± 2.98%	97.13%± 2.00%	98.07%± 1.24%	98.8%± 0.74%	99.91%± 0.39%	100.00%± 0.00%	99.88%± 0.40%	99.96%± 0.13%	1.18± 0.26

※The highlighted rows show the better average fitness among the used population sizes.

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