Designing Multiple-Use Primer Set for Multiplex PCR by Using Compact GAs

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Abstract. Reducing the number of needed primers in multiplex polymerase chain reaction experiments is useful, or even essential, in large scale genomic research. In this paper, we transform this multiple-use primer design problem into a set-covering problem, and propose a modified compact genetic algorithm (MCGA) approach to disclose optimal solutions. Our experimental results demonstrate that MCGA effectively reduces the primer numbers of multiplex PCR experiments among whole-genome data sets of four test species within a feasible computation time, especially when applied on complex genomes. Moreover, the performance of MCGA further exhibits better global stability of optimal solutions than conventional heuristic methods that may fall into local optimal traps.

1 Introduction

Molecular analyses and extended diagnostic applications are often restricted by limited availability of biological materials. The Polymerase Chain Reaction (PCR) [1], which uses primers to amplify specific DNA segments, is thus with crucial essence to current genomic researches, such as constructing full-genome spotted microarrays [2] on the preparation of DNA spotting material. Multiplex PCR [3], while using multiple primers to concurrently amplify multiple target DNA segments in single reaction [4], is considered as a time and reagent saving technique for simultaneous amplification of different targets, respectively. In current multiplex PCR, the primer length is often designed between 17 and 25 nucleotides (nt) and the number of primers is exactly twice of target number (with forward and reverse primer pair). The primer length ranging 17~25 nt is due to that the specificity by all random permutations of 17 nt (4^{17} approximately equals to 1.7×10^{10}) has already exceeded the size of human genome (3×10^{9} bps) and therefore would cause least random priming in human genome. Many primer selection programs have been commercialized [5, 6], such as Primer 3 [7], and focused on designing unique left and right primers for each gene

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targets. Obviously, a substantial percentage of PCR experimental expense inevitably drained into the synthesis cost of needed unique primers in large scale research projects.

To greatly reduce the indicated synthesis cost of needed primers, a demand-driven multiple-use primer design is useful in concurrent amplification of several interested targets from a single sample. Welsh and McClelland [8] proposed the Arbitrarily Primed PCR (AP-PCR) method by using short (8 to 12 nt) arbitrary primers in order to match several targets and to produce more fragments while without prior sequence information. Nevertheless, the multiple-use primer design problem is a difficult and NP-complete [9] combinational optimization problem, which can not be solved by applying commercial primer selection programs mentioned above. Heuristic approaches were hence developed to find the minimal multiple-use primer set, such as linear time heuristic (LTH), densest subgraph heuristic (DSH) [9], and other greedy methods [10]. Albeit, these heuristic algorithms produce sub-optima solutions for multiple-use PCR primer design problem in which the probability of getting local optimal results tends to arise with the growing number of amplification targets in large scale projects.

This paper transforms the multiple-use primer design problem into a constrained set-covering problem and proposes a modified compact genetic algorithm (MCGA) in order to get global and optimal quality results and as well to save the number of required primers within a reasonable amount of time. The MCGA can save tasks from falling into local optima status and give tasks a quick estimate on computational difficulty [14]. Being considered as global search mechanisms, the genetic algorithms (GAs) are one of the main categories of evolutionary algorithms and have very well become increasingly popular for solving complex optimization problems, such as function optimization [11], traveling salesman problem [12], and set-covering problem [13]. The GAs are adaptable concepts for problem-solving and are especially well suited for solving difficult optimization problems based on the ideas borrowed from genetics and natural selection. The GAs have been applied to solve problems of large search spaces, where conventional optimization methods are less effective.

In this paper, our MCGA of primer design algorithm was applied on four test datasets with three different ranges of melting temperature (Tm). In order to match multiple positions in the DNA genome by one primer, the proposed method designs primers with a short length (8-12 nt). Our experimental results show that MCGA overcomes the trade-off between quality and time of feasible solution during minimizing needed primers. Specifically, the solution quality of MCGA is more stable and efficient when compared with most frequently used heuristic methods. Thus, this paper concludes that MCGA is a feasible solution for exploring complex genomes in minimizing multiple-use primers.

The rest sections of this paper are organized as of the followings. Section 2 describes the multiple-use primer design problems, and transforms it into the set-covering problem with more constraints. Section 3 introduces the evolutionary nature of the proposed MCGA approach. Section 4 demonstrates and discusses the experimental results on four datasets with three Tm ranges. Lastly, concluding comments are drawn in Section 5.

2 Multiple-Use PCR Primer Design Problems

2.1 Problem Definition

In a PCR experiment, two primers (forward and reverse primer) are required to amplify the target sequence (as shown in Fig. 1). A primer is an oligo-nucleotide with a constant length that is much shorter than the target sequence. This paper uses the following notations. Assuming there are n DNA sequences, a primer Pi is denoted by (j, p, f/r) to represent that primer Pi hybridizes to sequence j in which the start position is p, where f and r represent forward and reverse primer, respectively. |Pi| denotes the length of primer Pi.

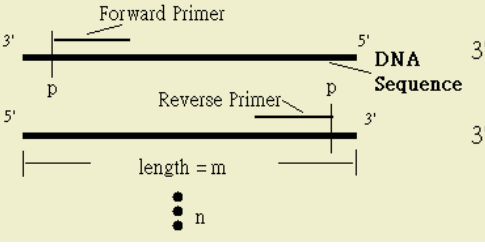

Fig. 1. The PCR primer design.

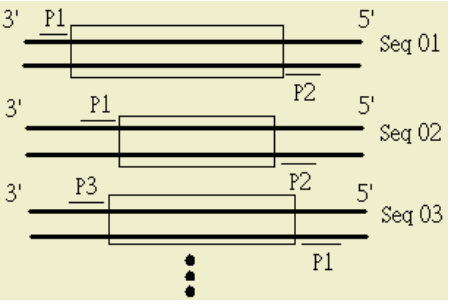

Fig. 2. The multiplex PCR with multiple-use primers. P1, P2, and P3 are three different primers. The rectangular boxes are the amplified DNA regions.

Figure 2 shows an example of the multiplex PCR problem with three target sequences simultaneously amplified by multiple-use primers. Specifically, primer P1 can be used as the forward primer for Seq01 and Seq02 and the reverse primer for Seq03 simultaneously, while P2 is used as the reverse primer for Seq01 and Seq02. Primers P1 and P2 are re-used for the PCR experiment and thus reduce the total primer number of the PCR experiment. Briefly, the defined multiple-use primer problem can be as follows. Given n DNA sequences, the multiple-use primer problem is to find a primer set with minimal number of primers that can amplify these n sequences simultaneously. Each primer in the set might be forward or reverse, and they satisfy certain desired constraints (described in the next paragraph). For each target sequence, the corresponding forward and reverse primers are contained in the set. Most importantly, primers can be used for amplifying different target sequences. In this paper, certain criteria and biological constraints are considered.

Primer Length: The length of a primer is 12 nt so that each primer can match multiple positions in the genome.

Primer Composition: Primers that contain a skewed AT/GC ratio can fail to give high specificity, or yield primers that are not well performed in other ways.

Melting Temperature (Tm): Tm is the key to deciding whether a primer candidate is appropriate or not. To enhance the discriminative ability of the oligo-nucleotides, the conditions of hybridization and washing need to be optimal. Therefore, it is crucial that all oligo-nucleotides should perform well under similar hybridization conditions. In this recent study, the Tm is set to 40 degrees centigrade and different ranges are used to accommodate more primer candidates.

GC Content: The GC content is 40-60% as in the common cases.

Length of Amplified Regions: The amplified regions are between 300-1200 base pairs long.

2.2 Problem Transformation

In this paper, we transform the multiple-use primer design problem into a constrained set-covering problem. Given n sequences and m primer pairs, there are l primers where $l \leq 2m$ since some primers might be used for more than one target sequence. An incidence matrix A (a_{ij}, $1 \leq i \leq m$, $1 \leq j \leq l$) is used to represent the relationship between primers and primer pairs. $a_{ij} = 1$ if primer j is a forward (or reverse, or both) primer in primer pair i, and zero otherwise. For sequence k, there might be t primer pairs which can amplify it. Denote the set of these t primer pairs as $pair_k$. Define $X_j = 1$ if primer j is in the solution and $X_j = 0$ otherwise.

In the transformed set-covering problems, we intend to find a subset of primers with minimal number of primers which can amplify all target sequences. A sequence k is covered if at least one of $pair_k$ is included in the solution set. Formally, the problem can be stated as a set-covering problem in the following way:

$$\text{Minimize} \sum_{j=1}^{l} X_j \tag{1}$$

subject to

$$\forall k, 1 \leq k \leq n \,,\ \exists i \in pair_k \ \text{ s.t.} \sum_{j}^{l} a_{ij} X_j = \sum_{j=1}^{l} a_{ij} \,. \tag{2}$$

Constraint (2) guarantees that each sequence k is covered by at least one primer pair, which is different from the traditional set-covering problem. It is well known that set-covering problem has been proven to be NP-complete [15] and is a model for several important applications [16, 17]. Description of set-covering problem could be found in [18, 19].

3 Modified Compact Genetic Algorithm (MCGA)

A genetic algorithm, combining the spirits of compact genetic algorithm (CGA) [14] and a local search based on edge replacement, is proposed to minimize the multiple-use primer number of PCR. The main mechanism of CGA is to represent the popula-

tion as a probability distribution over the set of solutions, applying the principle of survival of the fittest [20] to produce successively better approximations to a solution. The local search, inspired by the study [9], is considered as an efficient heuristic. Based on the CGA with local search modification, our proposed MCGA approach substantiates both global and local strategies by probability-based mutations.

Figure 3 shows the main steps of MCGA. First, a probability vector V is generated where each entry is equal to 0.5. Then two individuals are generated according to V. A uniform crossover is applied on these two individuals to generate two new intermediates. The local search (Section 3.2) is applied on two intermediates to produce two children. The competition mechanism (Section 3.3) then updates V according to these two children. The above process is repeated until the probability V converges. In the following subsections, the chromosome representation, the local search, and the competition mechanism are described in detail.

3.1 Chromosome Representation

Assume there are l primers. A chromosome is represented as $(u_1, ..., u_l)$, where, $u_j = 1$ implies that primer j should be selected and $u_j = 0$ otherwise ($1 \le j \le l$). A probability vector $V=(v_1, ..., v_l)$, is used to represent the population. v_j represents the possibility of selecting the primer j. In the beginning, all v_j is set to 0.5 for random sampling. All chromosomes are generated according to the probability vector V. That is, the probability of setting u_j to 1 is v_j.

3.2 The Local Search

After applying a uniform crossover, two intermediates are generated. A new local search is applied to improve them. The local search mechanism works as follows. Since an intermediate is not always feasible, we first select proper primer pairs into the primer set to make sure that all targets are fully covered. Then for each primer pair, we replace it with other primer pair for the same target sequence to check if the number of primers decreases or not. If the number of primers is less than the previous one, the primer pair is replaced. After all pairs are checked, all primer pairs in the primer set without any shared common primer for other sets are removed. The above process is repeated until there is no more reduction in primer number.

3.3 The Competition Mechanism

After the local search, two children are produced. The one with higher fitness is *winner*, while the other one is *loser*. The competition mechanism applies the following steps to update probability vector V. For a primer i, there are three cases to update v_i: 1) If *winner* selects primer i but *loser* does not, then $v_i=v_i+1/n$; 2) If *loser* selects primer i but *winner* does not, then $v_i=v_i-1/n$; 3) If both *winner* and *loser* selects primer i, then v_i stays the same, where n is the population size. The above updating steps are repeated for l times, where l is the number of primers.

4 Experimental Result

In the experiments, we first solved general set-covering problems and then applied our MCGA to the real-world multiple-use primer design problems. In the test of solving general set-covering problems, we used the test sets from Beasley's OR library [21] and compared our approach with present literature [13], including a direct genetic algorithm labeled BeCh [22], a Lagrangean-based heuristic labeled CFT [18], an indirect genetic algorithm labeled IGA [13], a linear time heuristic labeled LTH and a densest subgraph heuristic labeled DSH [9]. Moreover, we applied our CGA to primer design problems in genome-level PCR for four different species, *Schistosoma mansoni* (SMA), *Medicago truncatula* (MTR), *Hordeum vulgare* (HV), and *Ciona intestinalis* (CIN). The proposed MCGA method was tested on three Tm ranges, including 39-41, 38-42, and 37-43 degrees in centigrade, which are much narrower when compared with previous studies. Furthermore, the proposed MCGA approach was compared with the currently fast heuristic LTH and best heuristic DSH of multiple-use primer design to manifest MCGA's optimal performance and stability.

4.1 The Result of Set-Covering Problem by MCGA

The computational experiment was carried out on a 450 MHz Pentium II PC with 128 MB RAM. From the data of Beasley's OR library [21], 60 test sets were used, ranging in size from 200 rows × 1000 columns to 1000 rows × 10000 columns and in density (average proportion of rows covered by a column) from 2% to 20%. The deviation (Dev.) represents the ratio of variance between the problem's optimal solution and the best results out of 10 runs for each data instance. The corresponding deviation and time consumed in finding optimal solutions for these testing problems using different methods are summarized in Table I, where we can see the trade-off between solution quality and time consumption when using commonly available methods. Most excitingly, the MCGA seems to overcome the above drawbacks effectively. The MCGA offered better solution quality of no deviation in the experiments than most other methods did with exceptions of CFT and DSH. Moreover, MCGA spent fewer seconds to find the optimal solution than CFT and DSH did. Based on these comparisons, we are confident to claim that the proposed MCGA approach can find a better solution in a shorter amount of time than common used methods.

4.2 The Improvement in Reducing Needed Primers of LTH Within Acceptable Calculation Time

The multiple-use primer design problem is a set-covering problem with more constraints in the real world. We used the MCGA to design genome-level PCR primers for four datasets of different species, including *Schistosoma mansoni* (SMA), *Medicago truncatula* (MTR), *Hordeum vulgare* (HV), and *Ciona intestinalis* (CIN), downloaded from NCBI UniGene database. The GC content and the length of a primer were set to 40-60% and 12, respectively. We focused on amplifying segments of length between 300-1200 bps. In our implementation, the program automatically

translates the genome files in the FASTA database format into appropriate input for the Primer 3 [7].

The proposed method was tested on three Tm ranges of 39-41, 38-42, and 37-43 degrees in centigrade, which are much narrower when compared with previous studies. Obviously, a larger range of melting temperatures produces more primer candidates and gets more reduced primers even when the reduced rate does not improve. However, the range of melting temperature from 39 to 41 is the most desirable condition for real-world experiments since all the primer candidates can work at the most similar temperature in a multiplex PCR experiment.

The proposed approach was compared with the currently fast heuristic LTH and best heuristic DSH of multiple-use primer design to manifest its optimal performance and stability. The experiments were performed on a Pentium 2.6 GHz PC with 1 GB memory running on Linux operation system.

4.2.1 Reducing Needed Primers Within Feasible Calculation Time

Table II shows the simulation results on primer pair design in the test datasets with three Tm ranges by MCGA, DSH and LTH. Each experiment is repeated 30 times, and the results are summarized in Table II. From Table II, we can see that the rate and number of reduced primers grows as the number of primer pair candidates grows. This indicates that the importance of multiple-use primers is more explicit when we plan to explore more complex organisms.

As shown in Table II, MCGA reduces more primers than LTH in all the tested dataset, even small ones. Moreover, the number of reduced primers of MCGA is much greater than that of LTH when applied to larger genomes. Take *Ciona intestinalis* (CIN) with Tm range of 37-43 as an example. MCGA reduces 3239.5 primers more than LTH does. If a 12mer primer cost $7 at least, we could save about $22673 in this experiment. Figure 4 further demonstrates that MCGA can reduce more primers in average than the LTH at all conditions. The y-axis represents the difference in reduced primers between MCGA and LTH; the x-axis contains the four datasets. Each curve represents different Tm ranges. MCGA gets a significant reduction when the data set grow. While MCGA spends a little more time than LTH, it saves a noticeable experimental cost. Because the calculation time of MCGA is acceptable, such a trade-off is worthwhile when the size of simultaneously amplified genes becomes larger.

In addition, Table II also reveals that MCGA finds optimal solutions much faster than DSH in all the test datasets, even in large ones. In such a short calculation time, the number of reduced primers of MCGA is quite close to and even better than that of DSH when they are applied to larger genomes. Take *Ciona intestinalis* (CIN) with Tm range of 37-43 as an example. MCGA spends fewer 455334.85 secs (which is close to 5.27 days) than DSH while the average solution quality of MCGA is better than DSH. MCGA saves more time than DSH when the data set grows. The result shows that MCGA is a more applicable way to deal with larger data set such as the human genome data and get reliable solutions within an acceptable amount of time.

4.2.2 Stable Solution Quality

Figure 5 displays the comparison of reduced primer numbers among LTH, DSH, and MCGA applied on *Ciona intestinalis* (CIN) under Tm range of 37-43 degrees in centigrade for 30 repeated experiments. According to [9], LTH will make a random selection with probability of 1/2 when there is no improvement of the primer's reduction. Therefore, the LTH will get the sub-optima solution depending on its initial state. From figure 5, we can see that MCGA exhibits a stable performance in all the 30 repetitions, whereas the other two with highly variable solutions. Although the maximal reduced primer number of DSH is 17707, which is a little larger than the one of CGA (17254), the average reduced primer number and reduction rate of DSH is much less than those of MCGA. More specifically, the standard deviations of primer numbers reduced by LTH and DSH in the 30 repetitions are 417.16 and 1163.67, respectively. Meanwhile, MCGA is only 26.2. The experimental results indicate that our MCGA approach derives much more reliable solutions than the other two methods do.

Furthermore, Table II shows that the standard deviation of reduced primer number in parentheses seems to be increasing as the data set increases. There are some exceptions such as HV of LTH and MTR of DSH because of the instable solution quality may cause the result. Based on the observation of the standard deviation of solution quality, MCGA has the best solution stability and solution quality in large datasets

5 Conclusion and Future Work

In this paper, we formulized the multiple-use PCR primer design problem for multiplex PCR experiments, and proposed the MCGA approach to solve it with good solution quality and feasible calculation time. We demonstrated MCGA's superiority towards previous heuristic method in reducing the primers needed for PCR experiments of four species. In the experiments, MCGA shows its ability in achieving good and stable reduction of needed primers even in large datasets. The reasons why MCGA performs better may be uniquely due to its generation from the local optima that was often not considered in most heuristic cases.

As in the case of multiple-use PCR primer design, GAs incorporating powerful heuristic methods as the local search mechanisms may offer an adequate way for detecting pathogens causing similar syndrome in the same time. For instance, the multiple-use primers may be designed to simultaneously detect respective *SARS* virus, *Flu* virus, and even the common cold virus in a clinical task-oriented set of target sequences.

In the future, we proceed to combine better heuristic method and genetic algorithms to design multiple-use PCR primers with optimal solution quality and feasible computation time. Our recent work on establishing a better heuristic method may focus on more biological characteristics in order to directly preselect the qualified candidates for subsequent optimization. For the optimal solution quality, we pursue to develop more efficient selection and adaptation rules in GAs. Furthermore, our goal is to develop a user-friendly tool for biologists to design multiple-use PCR primers and develop applications on detecting task-oriented pathogen sets.

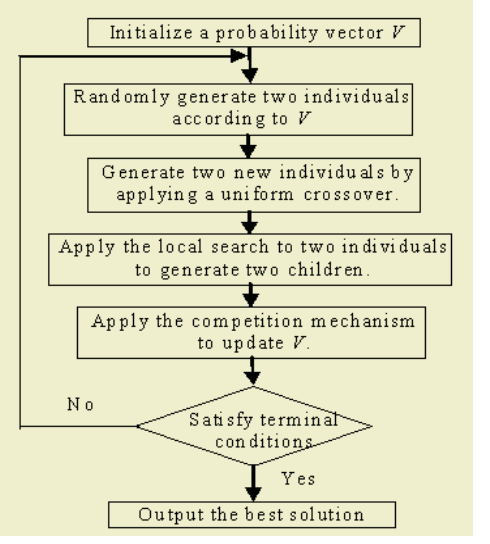

Fig. 3. Overview of the proposed genetic algorithm.

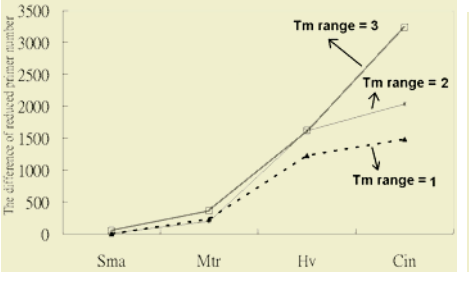

Fig. 4. The difference in the number of reduced primers between MCGA and LTH. The size increasing order of the 4 datasets are from SMA to CIN.

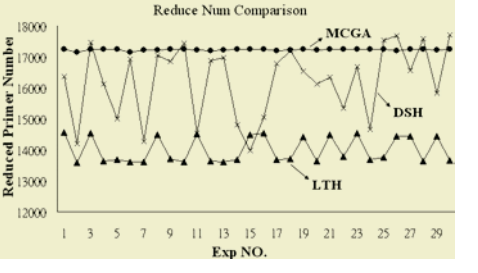

Fig 5. Comparison of reduced primer numbers between LTH, DSH and MCGA at Ciona intestinalis (CIN) in 30 repeated experiments.

Table 1. Summarized results averaged for data sets of same size and density.

	BeCh		CFT		IGA		LTH		DSH		MCGA	
Problem	Dev.	Time	Dev.	Time	Dev.	Time	Dev.	Time	Dev.	Time	Dev.	Time
4	0.00	163.00	0	6.50	0.00	93.30	0.00	3.11	0	28.10	0	44.59
5	0.09	540.20	0	3.20	0.00	61.20	0.00	1.11	0	13.10	0	28.98
6	0.00	57.20	0	9.40	0.00	7.60	0.00	2.25	0	36.33	0	3.52
A	0.00	149.40	0	106.60	0.00	81.00	5.56	27.36	0	466.44	0	38.37
B	0.00	155.40	0	7.40	0.00	30.40	5.53	2.34	0	33.77	0	15.07
C	0.00	199.20	0	66.00	0.00	82.80	3.56	12.00	0	279.38	0	37.13
D	0.00	230.40	0	17.20	0.32	69.00	4.47	5.49	0	76.67	0	30.27
E	0.00	8724.20	0	118.20	0.00	56.00	6.31	28.55	0	499.73	0	25.87
F	0.00	2764.80	0	109.00	0.00	142.80	10.01	10.49	0	452.84	0	67.47
G	0.13	12851.40	0	504.80	0.13	342.80	4.72	62.34	0	2299.68	0	152.04
Overall	0.02%	2583.52	0%	94.83	0.05%	96.69	4.02%	15.50	0%	418.60	0%	44.33

Table 2. Primer reduction in four species with different Tm range. (Primer length = 12)**.**

Input Properties				LTH			DSH			MCGA		
Species[a]	Tm	Amplified Genes[b]	Primer Pair Candidates[c]	Primer Reduction[d]	Reduced[e] Rate	Time (Sec.)	Primer Reduction	Reduced[e] Rate	Time (Sec.)	Primer Reduction	Reduced[e] Rate	Time (Sec.)
SMA[a]	39-41	706	83,915	193.5 (**0.94**)*	13.7 % (**0.066**)*	0.53	196.2 (**2.13**)*	13.9 % (**0.151**)*	637.74	196.1 (**1.70**)*	13.89 % (**0.120**)*	55.50
	38-42	785	403,092	308.5 (**3.16**)*	19.65 % (**0.201**)*	0.80	319.9 (**5.11**)*	20.38 % (**0.325**)*	1875.26	319.9 (**3.01**)*	20.38 % (**0.192**)*	135.50
	37-43	817	1,015,285	340.7 (**19.28**)*	20.85 % (**0.176**)*	8.03	407.2 (**19.23**)*	24.92 % (**1.180**)*	30151.11	398.1 (**3.26**)*	24.36 % (**0.200**)*	1824.70
MTR[a]	39-41	3937	313,744	2534.7 (**11.31**)*	32.19 % (**0.144**)*	2.00	2854.3 (**73.64**)*	36.25 % (**0.935**)*	6676.01	2771.9 (**5.32**)*	35.2 % (**0.068**)*	359.19
	38-42	4305	1,471,105	3229.7 (**22.16**)*	37.51 % (**0.257**)*	7.14	3495.9 (**58.01**)*	40.6 % (**0.674**)*	28743.73	3428.9 (**7.38**)*	39.83 % (**0.086**)*	1373.17
	37-43	4466	3,711,383	3448.6 (**25.62**)*	38.61 % (**0.287**)*	18.52	3771.4 (**100.18**)*	42.22 % (**1.122**)*	74549.02	3815.6 (**10.81**)*	42.72 % (**0.121**)*	3121.42
HV[a]	39-41	10796	3,370,774	11712.8 (**61.85**)*	54.25 % (**0.286**)*	38.18	13360.4 (**374.23**)*	61.88 % (**1.733**)*	45317.04	12942.5 (**13.09**)*	59.94 % (**0.061**)*	1711.25
	38-42	10982	15,171,875	12855.1 (**56.62**)*	58.53 % (**0.258**)*	153.41	14742.2 (**435.23**)*	67.12 % (**1.982**)*	108060.02	14470.5 (**15.79**)*	65.88 % (**0.072**)*	3922.50
	37-43	11180	36,571,728	14155.1 (**59.79**)*	63.31 % (**0.267**)*	1069.92	16055.37 (**477.83**)*	71.8 % (**2.137**)*	227001.40	15778.5 (**16.10**)*	70.57 % (**0.072**)*	7294.19
CIN[a]	39-41	11690	4,202,249	11973.4 (**48.36**)*	51.21 % (**0.207**)*	45.38	13360.3 (**432.36**)*	57.14 % (**1.849**)*	75286.80	13459.0 (**17.06**)*	57.57 % (**0.073**)*	2213.37
	38-42	12260	19,965,597	13034.0 (**120.57**)*	53.16 % (**0.492**)*	177.30	15881.4 (**698.13**)*	64.77 % (**2.847**)*	186067.80	15071.6 (**24.56**)*	61.47 % (**0.100**)*	5103.37
	37-43	12669	48,507,108	13990.0 (**417.16**)*	55.21 % (**1.646**)*	1765.03	16212.4 (**1163.67**)*	63.98 % (**4.593**)*	467867.35	17229.5 (**26.20**)*	68 .0 % (**0.103**)*	12532.50

Note:

a. The data set of four respective species: *Schistosoma mansoni*, SMA; *Medicago truncatula*, MTR; *Hordeum vulgare*, HV; and *Ciona intestinalis*, CIN.

b. the gene can be amplified with appropriate primer pair.

c. the number of primer pair candidates which were generated by Primer3.

d. the mean value of primer's reduced number in 30 repetitions.

e. the mean value of primer's reduced rate in 30 repetitions.

*. the standard deviation of 30 repetitions.

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