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Genetic analysis and tagging of gene controlling fruit tubercles and fruit ridgeness pattern in bitter gourd using SSR markers

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Abstract

Thepresence of tubercles and pattern of ridges (continuous vs discontinuous) are important traits of bitter gourd which determine market price in India.The tuberculate fruits with broken or discontinuous ridges have more unit price than the non-tuberculate and continuous ridged fruits. In the present study, two different botanical varieties of *Momordica charantia* var. *charantia* (DBGS-2 and DBGS-54) and *M. charantia* var. *muricata* (DBGS-34) were used for inheritance study and molecular tagging of fruit tubercles and fruit ridgeness pattern. The findings revealed the monogenic control for both the traits. The bi-parental mapping populations (F_2) were developed from the crosses DBGS-54×DBGS-34 and DBGS-2×DBGS-34 for tagging of tuberculate (*Tb*) and continues ridgeness (*cr*) locus, using SSR markers. The single plant analysis of F_2 population (derived from DBGS-54×DBGS-34) based on SSR markers found McSSR-20 was linked to fruit tubercles gene (*Tb*) at 12.50 cM and another SSR marker JY004 in the cross DBGS-2×DBGS-34 was found linked with fruit ridgeness pattern (*cr*) gene at a distance of 19.6cM. It is the first report of SSR markers linked to fruit tubercles and ridgeness which will be helpful further in fine mapping of these traits with more PCR based markers. These findings will help in differenciating the genotypes with tubercles and pattern of ridgeness at the early stage of the crop and can be used in accelerating trait specific breeding programme in bitter gourd.

Key words: Bitter gourd, fruit tubercle, fruit ridgeness, molecular marker, gene tagging

Introduction

Bitter gourd *Momordica charantia* is an important vegetable crop with immense medicinal properties. It is predominantly cultivated in Asia and Africa and also available in South-American countries (Basch et al. 2003; Grover et al. 2004). It belongs to family Cucurbitaceae and its chromosome number is 2n=22. Bitter gourd is also called as vegetable insulin because of its incomparable hypoglycemic activities and nutraceutical values (Tan et al. 2008; Yang et al. 2015; Tan et al. 2016). The extrinsic fruit quality is a major focus in breeding programme which includes fruit tubercles, fruit colour, shape, size etc. Presence of fruit tubercles was dominant over non-tubercle fruits and controlled by single nuclear gene, whereas continuous ridges on fruit surface were recessive to discontinuous ridges and controlled by single gene (Srivastava and Premnath 1972; Dalamu et al. 2012). Breeding cultivars with tubercles is one of important objectives in bitter gourd improvement programme. Conventional approach for improvement of these traits is time and labour consuming and less effective. The traditional breeding approach supplemented with marker assisted selection (MAS) is more effective in selecting of desirable traits which quickens breeding process.

Very limited polymorphic molecular markers for mapping of different traits were developed in bitter gourd. Using FIASCO method Wang et al. (2010) and Guo et al. (2012) developed 26 SSR markers. Eleven SSR markers were generated through genomic library enrichment by Xu et al. (2011) while 43 were developed from other cucurbits (Xu et al. 2011; Chiba et al. 2003; Watcharawongpaiboon and Chunwongse 2008). In addition, a total of 160 SSR markers have been synthesized in *Momordica* species by Saxena et al. (2015) through enriched genomic library and 21 through genome wide analysis (Cui et al. 2018). Although bitter gourd has been considered as vegetable insulin, till date but very little research work has been published

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on QTL mapping of different economic traits. The linkage map in bitter gourd was reported by Kole et al. (2012) who used AFLP markers followed by Wang and Xiang (2013) using RAD-seq analysis for 13 horticultural traits and Matsumura et al. (2014) identified one SNP marker, GTFL-1 linked to the gynoecious locus. At present one draft genome sequence information is available in bitter gourd (Urasaki et al. 2017). Cui et al. (2018) identified QTLs for gynoecy, first flower node, female flower number, fruit epidermal structure and fruit color using RAD-based genetic map for anchoring scaffold sequences. Gangadhara Rao et al. (2018) identified a total of 22 QTLs for four traits *viz*., (gynoecy, sex ratio, node and days at first female flower appearance) were mapped on 20 LGs.

Quality requirements for the bitter gourd have received more and more attention, especially external quality, which is a direct factor in stimulating the purchase desire of consumers. Usually, in cucumber, smooth and non-warty (non tuberculate) fruit trait is more important to the fresh eaten cucumber types and advantageous in that the maintenance of smooth fruit produces less pollution, they are easy to clean and pack, and have higher resistance during transportation and storage, etc. (Wang et al. 2007). Similarly, bitter gourd fruit has either a smooth or a distinct warty exterior, and has broken or continuous ridges. The warty exterior is monogenically dominant over the smooth (Kole et al. 2012) however, research devoted to identifying genetic loci responsible for these important traits is limited (Cui et al. 2018). In bitter gourd both smooth and tubercles fruited varieties as well as the continuous and discontinuous (broken) ridge types were grown in India, in contrast to cucumber tuberculate with discontinuous ridge pattern varieties are more preferred as compared to fruit without tubercles (smooth type). Therefore, genetic analysis and tagging of gene controlling tuberculate and ridgness pattern will promote quality breeding of the bitter gourd. The present experiment was carried out to study inheritance and to identify closely linked molecular markers to fruit tubercles and ridgeness in bitter gourd.

Materials and methods

Plant materials and mapping population

The F_2 mapping populations were generated by using inter-botanical varieties *M. charantia* var. *charantia* (DBGS-2 and DBGS-54) and *M. charantia* var. *muricata* (DBGS-34). The bitter gourd line DBGS-54 (fruits without tubercles or smooth) was crossed with DBGS-34 (fruits with strong tubercles). Similarly, the inbred line DBGS-2 (fruits with continuous ridges) was crossed with DBGS-34 (fruits with dis-continuous ridges). These three parental lines were planted in insect proof net house condition during June-October-2015 in experimental block of the Division of Vegetable Science ICAR-Indian Institute of Agricultural Research at New Delhi. The F_1's DBGS-54×DBGS-34 and DBGS-2×DBGS-34 were grown in June-October 2016 and F_2, B_1 and B_2 were obtained by selfing and crossing F_1s with their respective parents. A total of 110 F_2 population derived from cross of DBGS-54 × DBGS-34 and 111 F_2 population from cross DBGS-2 × DBGS-34 were used for single plant analysis through both phenotyping and genotyping. For inheritance studies, the phenotypic observations were recorded during May-September, 2017 under insect proof net house condition and during March-July, 2018 under open field conditions.

DNA isolation and molecular analysis

Bitter gourd genomic DNA was isolated from fresh young leaves using modified CTAB method (Saghai-Maroof et al. 1984) as reported by Dey et al. (2006). PCR were performed in 10µl volume containing 1.0µl 10 X buffer; 1.0µl dNTP; 0.50µM each of forward and reverse primers; 0.3 unit of *Taq* DNA polymerase (Bangalore Genei Pvt. Ltd, Bangalore, India); ~50ng DNA and were performed using thermal cycler (Eppendorf Master Cycler Pro S and Applied Bio-System, California, USA). Amplification of SSR primers was performed with 4 min initial denaturation at 95°C followed by 35 cycles of 40 sec denaturation at 94°C, 40 sec annealing at temperature appropriate for SSR primers pairs, 1.0 min extension at 72°C and final extension for 10 min. The amplified DNA fragments were resolved on 4% agarose gels and visualized by ethidium bromide staining. The band size was obtained in comparison to a 100bp DNA ladder (BIOCHEM, New Delhi).

Statistical analysis and mapping

The Chi-square (χ^2) test suggested by Panse and Sukhatme (1985) was subjected to test the goodness of fit of the observed segregation ratio for the segregation of fruit tubercles and fruit ridgeness. These traits are expected as controlled by single gene. The χ^2 value was calculated as per standard procedure.

A total of 534 SSR primers (Wang et al. 2010; Guo et al. 2012; Xu et al. 2011; Chiba et al. 2003; Watcharawongpaiboon and Chunwongse 2008; Saxena et al. 2015 and Cui et al. 2018) were screened for

parental polymorphism and of which 10 and 11 SSR primers showed polymorphism for DBGS-54×DBGS-34 and DBGS-2×DBGS-34, respectively. The linkage association between the economical fruit trait and molecular marker was estimated using software MAPMAKER-3.0 (Lander et al. 1987) with LOD value of >3.00. Three non-tuberculate (*tb*) lines (DBGS-32, Gy-21 and DBGS-59) and four tuberculate (*Tb*) lines (DBG-34, G-23, DBGS-38 and Achhuba) were used for marker validation.

Results

Inheritance of fruit tubercles and fruit ridges pattern

The F_1 plants of DBGS-54 (non tuberculate; *tb*) × DBGS-34 (tuberculate; *Tb*) had fruits with tubercle surfaceindicated that fruits with tubercle surface is dominant over non-tubercle fruit surface (Figs. 1 and 2). A total of 266 F_2 segregants were phenotyped under insect proof net house condition (110 plants) and open

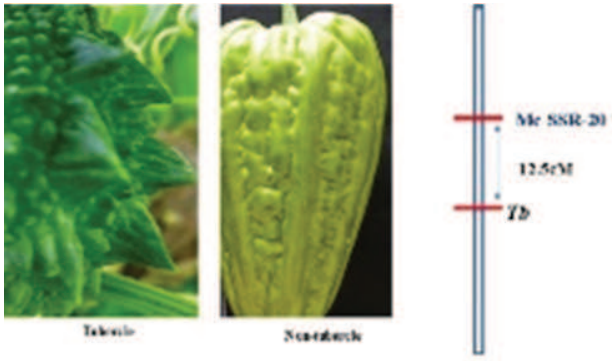

Fig. 1. inheritance of fruit tubercle

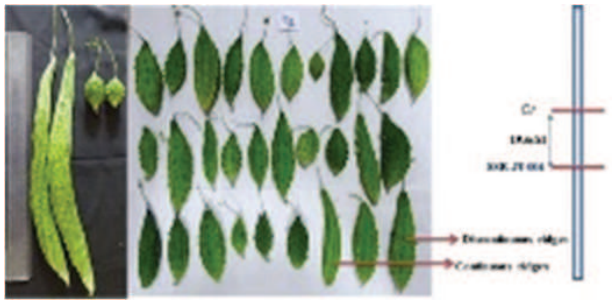

Fig. 2. Inheritance of fruit ridgeness

field (156 plants) condition, respectively. Out of 110 F_2 segregants evaluated under insect proof net house condition, 83 plants produced fruit with tubercle surface and 27 were with non-tubercle surface (χ^2 values of 0.01; P = 0.91). Under open field conditions, 156 F_2 plants were evaluated and it was observed that 118 segregants produced tubercle surfaced fruits and 38 bore non-tubercle fruits (χ^2 values of 0.03; P=0.85). The observed frequency of F_2 population fit well in the Mendelian segregation ratio of 3 (tubercles): 1 (non-tubercles) as evident from non-significant χ^2 values. The back cross (B_1) progenies segregated and fit well into ratio of 1 (tubercles) : 1 (non-tubercles) with non-significant χ^2 values (Table 1). These results suggested that the fruit tuberculate surface is controlled by a single dominant gene (*Tb*).

Table 1. Segregation pattern for tubercle and non-tubercle trait in F_2 and back cross generations of bitter gourd

Population	Total	Tubercle	Non-tubercle	Expected ratio	x^2-value	P-value
DBGS-54xBBGS-34 (Open field)						
P_1	10	0	10	-	-	-
P_2	10	10	0	-	-	-
F_1	20	20	0	-	-	-
F_2	156	118	38	3:1	0.03	0.85
B_1	30	16	14	1:1	0.13	0.72
B_2	30	30	0			
BBGS-54xDBGS-34 (Insect proof net house coalition)						
P_1	10	0	10			
P_2	10	10	0			
F_1	20	20	0			
F_2	110	79	31	3:1	0.59	0.44
B_1	20	12	8	1:1	0.50	0.48
B_2	20	20	0			
Pooled						
P_1	20	0	20			
P_2	20	20	0			
F_1	40	40	0			
F_2	266	197	69		0.13	0.72
B_1	50	28	22		0.72	0.40
B_2	50	50	0	-	-	-

The inheritance pattern of fruit surface (ridgeness pattern) was also investigated in the cross DBGS-2 (continuous ridge; *crcr*) × DBGS-34 (discontinuous ridge; *CrCr*), the F_1 generation had all fruits with discontinuous ridges (*Crcr*), indicating dominant in nature of discontinuous ridges. The 281 F_2 population were evaluated under two growing conditions (111 plants in insect proof net house condition during May-September, 2017 and 170 plants in open field condition during March-July, 2018) (Table 2). The F_2 generation fit well in the expected ratio of 3 discontinuous ridge:

Table 2. Inheritance pattern of trait ridgeness in F_2, B_1 and B_2 generations in bitter gourd

Population	Total	Dis-continuous	Con-tinuous	Expected ratio	x^2-value	P-value
DBGS-2xDBGS-34						
P_1	10	0	10			
P_2	10	10	0			
F_1	20	20	0			
F_2	111	84	27	3:1	0.03	0.87
B_1	20	11	9	1:1	0.2	0.66
B_2	20	20	0			
DBGS-2 xDBGS-34						
P_1	10	0	10			
P_2	10	10	0			
F_1	20	20	0			
F_2	170	128	42	3:1	0.13	0.72
B_1	30	16	14	1:1	0.13	0.72
B_2	30	30	0			
Pooled						
P_1	20	0	10			
P_2	20	10	0			
F_1	40	40	0			
F_2	281	212	69	3:1	0.03	0.86
B_1	50	27	23	1:1	0.32	0.57
B_2	50	50	0			

1 continuous ridge (χ^2 = 0.87 and χ^2 = 0.72), respectively under both insect proof net house condition and open field condition). The results indicated that the trait is governed by a single dominant gene.

Genotyping of the F_2 population

Two F_2 populations developed from the crosses, DBGS-54 × DBGS-34 and DBGS-2 × DBGS-34 with 110 and 111 individuals, respectively were evaluated under insect proof net house condition and used for genotyping using SSR markers. Out of 534 SSR markers used for polymorphic study, 10 SSRs (1.87 %) showed polymorphism between the parents DBGS-54 and DBGS-34 and 11 SSRs (2.06%) %) showed polymorphism between the parents DBGS-2 and DBGS-34 (Table 3). The SSR markers segregated and fit well into Mendelian ratio of 1:2:1 with non-significant χ^2 values and these markers were used for linkage map construction (Table 3). Because of limited number of polymorphic markers we followed single marker analysis to determine linkage between these markers with fruit surface traits.

Linkage analysis for fruit tubercle and fruit ridgeness pattern

The genetic linkage analysis was carried out using MapMaker (EXP 3.0) softwere (Lander et al. 1987) and genetic distance between trait of fruit tubercles and position of the marker was analyzed. A minimum LOD score of 3.0 was first used to associate loci into initial linkage groups. The conversion from recombination frequency to map distance was done by Kosambi function (Kosambi 1943). The mapping of fruit tubercles gene (*Tb*) was carried out by using linkage analysis of 110 F_2 population phenotypic data and genotypic data generated by amplifying with McSSR-20 marker from the cross DBGS-54× DBGS-34. The SSR marker, McSSR-20 was mapped at a distance of 12.50 cM (LOD=14.87). The gene action was dominant and allele originated from male parent (DBGS-34) could promote the more tubercle traits expression (Table 4). The linkage map showing the linkage of fruit tubercle gene *Tb* with McSSR-20 marker is depicted in the Fig. 1.

The linkage relationship among the molecular marker and fruit ridges (*crg)* gene was analyzed using scored phenotypic data and genotypic data generated by running SSR marker JY004 on 111 F_2 population derived from cross DBGS-2 × DBGS-34. The SSR

Table 3. Segregation analysis of the genotypic data of the SSR markers in the F_2 populations

Marker	Total plants genotyped	Allele			Genetic ratio	χ^2 value	P-value
		A	H	B			
DBGS-54 × DBGS-34 (Tubercles)							
McSSR-20	110	24	55	31	1:2:1	0.89	0.65
DBGS-2 × DBGS-34 (Fruit ridgeness)							
Jy-004	111	27	53	31	1:2:1	0.54	0.77

A = ABGS-54 allele; B = DBGS-34 allele; H = Heterogygote

Table 4. Mapping of fruit tubercles and fruit ridgeness in bitter gourd

Trait name	Marker name	LOD	PVE (%)	Add	Dom
DBGSS-54×DBGS-34					
Fruit Tubercles	McSSR-20	14.87	46.34	-0.68	0.67
DBGSS-2×DBGS-34					
Fruit ridges	Jy-004	10.65	35.71	0.27	-0.29

marker JY004 was flanked to fruit ridges at a distance of 19.60 cM (LOD = 10.65) and accounting genotypic variance of 35.71%. The dominance gene action was observed for dis-continuous fruit ridges and the value of positive additive effect indicating the continuous ridges are contributed by the female parent (DBGS-2). The linkage map showing the linkage of dis-continuous ridge gene *Cr w*ith JY004 marker is depicted in the Fig. 2.

Validation of SSR marker linked to fruit tubercle trait

The accuracy of SSR marker McSSR-20 was tested using three (*tb*) non-tubercles lines (DBGS-32, Gy-21 and DBGS-59) and four tubercles (*Tb*) bitter gourd line (DBG-34, G-23, DBGS-38 and Achhuba) (Fig. 3) with the same parental allele as DBGS-54 (without tubercles) and DBGS-34 (with tubercles). The result was identical to the phenotype investigation. Based on analysis of 7 bitter gourd lines of diverse origin McSSR-20 could correctly predict the fruit phenotype (Fig. 3).

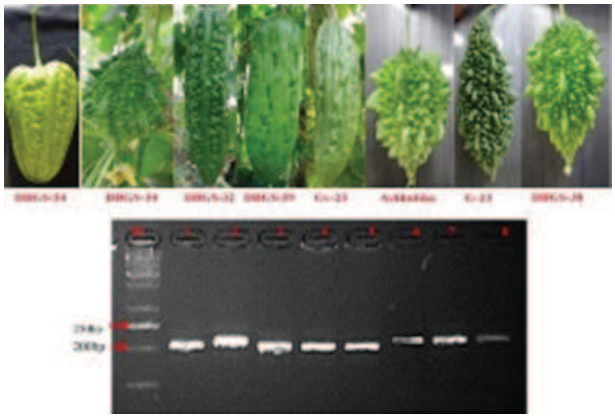

Fig. 3. McSSR_20 marker validation

Discussion

The inbred DBGS-54 with non-tubercle fruit was crossed with DBGS-34 with fruit tubercle surface, and the segregation ratio of tubercle fruit to non-tubercle fruit was found to fit 3:1 ratio. In another study, parent DBGS-2 with continuous ridges (*cr*) was crossed with DBGS-34 with discontinuous (broken) ridge (*Cr*) fruit surface, and the segregation ratio of discontinuous to continuous ridges fit well to Mendelian ratio of 3:1. These results suggested that the tubercle fruit surface and discontinuous ridges in DBGS-34 were controlled by the single dominant gene '*Tb*' and '*Cr*', respectively. The mode of inheritance provided a basis for the further study the genes *Tb* and *Cr* at molecular level. Genetic inheritance of fruit tubercle and ridges in bitter gourd has been reported earlier, which showed monogenic nature of these traits (Srivastava and Premnath 1972; Vahab 1989; Dalamu et al. 2012; Kumari et al. 2015). Cui et al. (2018) also reported similar inheritance pattern which showed monogenic segregation (3:1) for fruit surface wart (*fwa*) in two different cross combinations.

Gene tagging and QTL detection are very useful tools for MAS and trait improvement in bitter gourd. The use of sequenced markers that have a concrete biological meaning for linkage map construction allows obtaining a functional map (Mc Couch, 2001). The SSR markers were selected for this study because they present in the expressed regions of the genome and they could be transferred across species (Varshney et al. 2005; Gonzalo et al. 2015). In the present study, we reported co-dominant SSR marker McSSR-20 linked to fruit tubercles gene (*Tb*) in bitter gourd at a distance of 12.50cM with the phenotypic variance of 46.34%. Several studies have previously shown that the single dominant gene control the fruit tubercles (tubercles dominant over non-tubercles) in bitter gourd (Vahab 1989; Kumari et al. 2015). McSSR_20 microsatellite marker was synthesized from SSR-enriched genomic libraries and which had core repeat of 9 bp (TTTCTCCAT) with high PIC value of 0.775 (Saxena et al. 2015).

In another population derived from DBGS-2× DBGS-34, another SSR marker JY004 found to be linked to fruit ridge pattern (*crg*) gene at a distance of 19.60cM (LOD = 10.65) with phenotypic variance of 35.71% based on single plant analysis of 111 F_2 individuals. The fruit ridgeness pattern was controlled by single gene, discontinuous (broken) ridge was found dominant over continuous ridges (Srivastava and Premnath, 1972; Dalamu et al. 2012). Markers that are closely linked to desirable traits can be used for MAS programme (Tanksley 1983). The present study, reports that SSR markers McSSR-20 linked to Tb/tb

locus (12.50cM) and SSR-JY004 linked to Cr/cr locus (19.60cM) were not so closely associated to the fruit tubercle and fruit ridge pattern traits in bitter gourd. However, it is the first report of SSR markers linked to fruit tubercles and ridgeness which will facilitate the fine mapping in further studies and bitter gourd improvement through marker assisted selective for these traits with more PCR based markers. In the present study, the marker McSSR-20 was also validated on diverse group of genotypes and results indicated that the allelic variation of marker was observed in tubercle fruit and non-tubercle fruited germplasms of bitter gourd. Allelic variation in microsatellite has also been reported earlier for warty and smooth fruit in cucumber (Zhang et al. 2010).

Fruit warts and ridges are distinct in shape and epidermal distribution, both of which act as identifying features of bitter gourd. The width of the fruit ridge is correlated with the presence of fruit warts, which provides a novel measurement to dissect the genetic loci of fruit epidermal structure (Cui et al. 2018). Their results indicated that the two characters (fruit wart and ridge width) of fruit epidermal structure were possibly controlled by a same dominant gene (Fwa/Wr) and suggested pleiotropic effect. In cucumber, fruit warts consist of spines (non-glandular trichomes) and tubercules governed by csgl1/mict (Li et al. 2015; Zhao et al. 2015) and Tu (Yang et al. 2014), respectively. Consequently, cloning the genes underlying fruit tubercles and ridgness pattern of bitter gourd is essential in understanding the molecular mechanisms that control the formation of fruit tubercles and ridges.

Authors' contribution

Conceptualization of research (TKB, VR); Designing of the experiments (VR, TKB, ABG); Contribution of experimental materials (TKB, ZH); Execution of field/lab experiments and data collection (VR, ABG, ZH); Analysis of data and interpretation (VR, KTB, ABG); Preparation of manuscript (VR, TKB).

Declaration

The authors declare no conflict of interest.

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Supplementary Table S1. Segregation analysis of the genotypic data of the SSR markers in the F_2 population from the cross

Marker	Total plants genotyped	Allele			Genetic ratio	χ^2 value	P-value
		A	H	B			
DBGS-54 × DBGS-34 (Tubercles)							
McSSR-20	110	24	55	31	1:2:1	0.89	0.65
DBGS-2 × DBGS-34 (Fruit ridgeness)							
Jy-004	111	27	53	31	1:2:1	0.54	0.77

Supplementary Table S2. List of SSR-Primers used for mapping for economical important traits

Marker	Annealing temp. (^{0}C)	PCR product size (bp)	Sequence (Forward) 5'-3'	Sequence (Revers) 5'-3'
McSSR-20	56.50	200/230	GGAATTCAGGTGAACCTGACG	CCAGGAGGAAGAGGAACTGC
McSSR-35	53.00	300/230	TTAGCTGCTCGCTTGAGGAT	CAAGGATTCTCACATTTCCACA
MCSSR-62	54.00	143/160	GAGCTTCGAAACGACTTTCA	AAACCCAAGACCACCAACAC
JY-003	56.00	190/170	GTGGGTGCAATGGGTGTC	CTGCTGCTGTTGCTTCTTC
JY-004	55.00	100/94	GTCAACTGCCATCGGTAC	AGGGAAGAAGAAGAAGAAG
S-12	56.00	160/150	GACATCCTTCTTGCCTCTTACA	GAAACGGAACGAAACCTCA
S-32	56.00	110/150	CTAAATCACGCAAACCCATC	GAGCAAAAGACTGAGGAAAACT
Mc-01-4761	54.00	140/122	TAACGAAACGGAACGAAACC	CCTGGCAATTGGAGATCAGT
MC-06-73242	54.20	440/500	AGCGAAGCAGCTTTATCGAG	CGAAACGCACTATTCCCATT
MC-11-158010	53.50	130/150	GTCCAAAAATGGAGGCAAAA	TTGTAGTGGAGGGGATCGAG
AVRDC-BG-2	51.05	190/160	GAGCACACAGAAAATTGGGT	TGATCCACTCCCAATCTTAGC
AVRDC-BG-7	53.85	180/160	CAGAATCATTGAGAGTGCCG	GGCCGTAAGCTCTCACACCTC
AVRDC-BG-74	53.80	150/180	AACACCTTCTGACTCCACCC	CGTTCAATCCTCTCCTCCTC
AVRDC-BG-85	50.70	227/200	TGCAACCACTTGGGTTCTAA	CAGGCCAGTAGCTTCAACAT
AVRDC-BG-87	51.75	140/120	GAGGAACTCCCAGTTCGAGA	AATTCCCTGCGATTATGGAG
N-6	54.00	160/150	GGGAATTCTCAAAGAGCCAGA	TGGCACACTCTGCATGAAAT