Barley Genetics Newsletter (2005) 35:27-35

Barley Genetics Newsletter Volume 35

Adaptive methylation pattern of ribosomal DNA in wild barley from Israel

Shailendra Sharma[1], Harindra Singh Balyan[2] and Pushpendra Kumar Gupta[2*]

[1]Dept. of Fingerprinting, College of Biotechnology, SVBP University of Agriculture and Technology, Meerut-250 110, India; [2]Dept. of Genetics and Plant Breeding, Charan Singh University, Meerut-250 004, India; *corresponding author, e-mail: pkgupta36@ vsnl.com, Telefax: +91-121-2768195

Abstract

Forty four accessions of wild barley, *H. spontaneum* were assayed to study the methylation status of ribosomal DNA repeat units. For this purpose, *Bam*HI and *Hpa*II, which are, methylation sensitive restriction enzymes and *Msp*I, which is methyl insensitive enzyme, were used for restriction digestion. Southern blots were hybridized with pTa71 probe, which represented a complete ribosomal DNA repeat unit from bread wheat. Wild barley material studied belonged to two ecologically diverse climatic and edaphic microsites (the "Evolution Canyon" at Lower Nahal Oren, Mount Carmel, and Tabigha, Eastern Upper Galilee Mountains), from Israel, each having two different ecogeographcally contrasting microniches. *Hpa*II did not cleave ribosomal DNA, while *Msp*I gave a pattern typical of ribosomal DNA. In contrast, a total of 23 *Bam*HI phenotypes were observed due to methylation. Same rDNA repeat length units exhibited different methylation status at different microsites and miocroniches, as inferred from RFLPs observed. Differential methylation status of same rDNA repeat unit seems to be associated with ecological conditions dominating at a particular microsite or microniche suggesting a role of natural selection in determining methylation patterns.

Keywords: *Hordeum spontaneum* - Intergenic Spacer - Methylation - Natural selection - rDNA

Introduction

Eukaryotic ribosomal RNA genes (known as ribosomal DNA or rDNA), that encode 18S, 5.8S and 26S ribosomal RNAs (rRNAs), are found as parts of repeat units that are arranged as tandem arrays, located at the chromosomal sites known as nucleolar organizing regions (NORs) (Long and Dawid, 1980; Jorgensen and Cluster, 1988). In barley, there are two pairs of satellited chromosomes (6 or 6H and 7 or 5H), each carrying one rDNA locus (*Rrn*1 on chromosome 6H and *Rrn*2 on chromosome 5H) that is associated with the corresponding NOR (Saghai-Maroof *et al*., 1984; Brown *et al*., 1999). Each rDNA repeat unit consists of a highly conserved coding region (for 18S, 5.8S and 26S rRNAs) and a variable non-coding intergenic spacer (IGS) region. IGS itself consists of a non-transcribed spacer (NTS) region, which contains motifs referred to as subrepeats, and is itself flanked by external transcribed spacers (ETS) at its two ends. In the coding region also, on either side of 5.8S rRNA gene, are found internal transcribed spacers (ITS), described as ITS1 and ITS2.

Several studies suggest that natural selection is the major force that directs differentiation at the level of rDNA repeat unit (Flavell *et al*., 1986; Saghai Maroof *et al*., 1984, 1990; Gupta *et al*., 2002; Sharma *et al*., 2004). This differentiation may provide genotypes with variable ecological adaptations. Wild barley, *Hordeum spontaneum*, from fertile crescent region has been shown to be rich in genetic diversity and this genetic diversity is adaptive in nature (Balyan *et al*., 1996; Nevo *et al*., 1998; Gupta *et al*., 2002; Owuor *et al*., 1997; Sharma *et al*., 2004). In *Hordeum* species, besides restriction enzymes like *Sac*I, *Bam*HI has been used to study polymorphism in rDNA repeat length unit (Molnar *et al*., 1989; Gupta, 1996; Gupta *et al*., 2002). Molnar *et a*l., (1989) reported five *Bam*HI restriction site maps for 25 different *Hordeum* species and suggested grouping of different species on the basis of these maps.

The present study was undertaken to analyze the methylation patterns of rDNA in barley, and to study the role of natural selection in determining the current methylation status of rDNA, which could be partly adaptive.

Materials and methods

Plant materials

The material for the present study comprised 44 accessions of wild barley (*H. spontaneum*). Seed material was originally supplied by E. Nevo of University of Haifa, Israel and was multiplied at the Research Farm of Ch. Charan Singh University, Meerut, India. The 44 accessions of wild barley were collected from two different microsites in Israel. Twenty two (22) accessions were collected from "Evolution Canyon", out of which 9 belonged to NFS (North Facing Slope) and another 13 samples belonged to SFS (South Facing Slope). The remaining 22 accessions were collected from 'Tabigha' out of which 12 were collected from 'terra rossa' microniche and 10 were collected from 'basalt'. microniche. The two microsites are separated by 53 km. The details of two ecological microsites and those of microniches at each microsite are available elsewhere (Gupta *et al*., 2002).

DNA extraction and purification

Total cellular DNA, from one to two plants per accession, was isolated from one month old, field-grown individual plants using the modified CTAB method of Saghai-Maroof *et al*., (1984). The isolated DNAs were further purified by RNaseA treatment and phenol: chloroform: isoamyl alcohol following Sambrook *et al*., (1989).

Restriction enzyme digestion, Southern blotting, hybridization and autoradiography

Appropriate amounts (~10µg) of purified DNA samples were digested separately, with restriction enzymes *Bam*HI, *Hpa*II and *Msp*I according to manufacturer's instructions (Amersham, UK) (Figs. 1 and 2). Southern blotting, hybridization and autoradiography were performed as described earlier (Gupta *et al*., 2002).

Results and Discussion

Methylation pattern in the rDNA of wild barley

For the study of methylation patterns in rDNA, the genomic DNA from 44 accessions of wild barley, *H. spontaneum*, was restricted separately with each of the three restriction enzymes including *Hpa*II (sensitive to CG methylation) and *Msp*I (insensitive to CG methylation), a

isochizomeric pair, and *Bam*HI, a methylation sensitive enzyme. Both *Hpa*II and *Msp*I cleave the DNA sequence CCGG. However, *Hpa*II fails to cleave the rDNA, if the internal cytosine residue (CG) at the restriction site (CCGG) is methylated. In each of the above accessions, *Msp*I digestion and Southern hybridization with rDNA probe pTA71, showed many bands of varying sizes whereas similar experiments using *Hpa*II showed only uncut DNA in the autoradiogram (Fig. 1). This means that in wild barley the CCGG restriction site of *Hpa*II/*Msp*I in rDNA and consequently the rDNA repeat units are heavily methylated.

The restriction enzyme *Bam*HI that cleaves the rDNA at restriction site GGATC is also sensitive to cytosine methylation. In an individual rDNA repeat unit of barley, the *Bam*HI restriction enzyme has three (Molnar *et al*., 1989, Gupta *et al*., 1996) to four (Appels *et al*., 1980) cleavage sites. Two of the four cleavage sites are located in the IGS region and one of these two sites show considerable heterogeneity, which could be due to sequence alteration or to methylation. Of the remaining two sites, one each lies in 18S and 26S coding regions (Appels *et al.*, 1980, Gupta *et al*., 1996). However, the cleavage site in 26S coding region is presumed to be methylated (Appels *et al*., 1980, Gupta *et al*., 1996). In the present study, results obtained due to *Bam*HI digestion only were utilized for the study of methylation pattern of rDNA in above 44 accessions of wild barley. The digestion of DNA with *Bam*HI followed by Southern hybridization with rDNA probe pTA71 gave 23 different banding patterns each of which included 5 to 9 bands of variable sizes. These 23 banding patterns were classified as 23 (I to XXIII) different *Bam*HI phenotypes (Figure 2; Table 1).

Methylation status of rDNA repeat units

It may be recalled that in the same set of 44 wild barley accessions that were used for the study of methylation, *Sac*I restriction enzyme provided evidence of one to three rDNA repeat unit length variants at two rDNA loci in an individual accessions (Table 1; Gupta *et al*., 2002). Assuming four *Bam*HI cleavage sites in a single rDNA repeat unit (Appels *et al*., 1980), a maximum of four, seven and 10 fragments are expected following digestion of rDNA with *Bam*HI in wild barley accessions having one, two and three rDNA repeat unit length variants (having one common fragment in each case). However, on digestion with *Bam*HI during the present study, the individual wild barley accessions with one, two and three rDNA repeat units respectively gave 5 to 7, 6 to 9 and 8 to 9 *Bam*HI fragments of varying sizes. The availability of the varying number of *Bam*HI fragments suggested heterogeneity for *Bam*HI sites in rDNA repeat unit(s) of the individual wild barley accessions examined during the present study. As earlier reported, the observed heterogeneity for *Bam*HI sites may be attributed to the alteration in sequences/methylation of *Bam*HI sites located in IGS region, partial methylation of *Bam*HI sites and the methylation of the *Bam*HI site located in 26S rDNA (Appels *et al*., 1980, Molnar *et al*., 1989, Gupta *et al*., 1996).

Possible role of ecogeographic factors in governing methylation of rDNA

Interestingly, out of the above 23 *Bam*HI phenotypes, 21 (91.31%) phenotypes showed microsite specific distribution and only 2 (8.69%) *Bam*HI phenotypes were shared by the two microsites i.e. 'Evolution Canyon' and Tabigha, separated by 53 km. Similarly, at the 'Evolution Canyon' microsite, out of nine exclusive phenotypes, 5 phenotypes (55.55%) exclusively belonged to NFS (cooler more humid representing south European and

Mediterranean dense Macquis live oak forests) and three phenotypes (33.33%) belonged to SFS (drier and much warmer representing African Savannah) and only one (11.11%) phenotype was common between the two microniches. At Tabigha edaphic microsite, out of 12 exclusive phenotypes, six phenotypes (50%) belonged to terra rossa (drier and shallow soil layer) and five phenotypes (41.66%) belonged to basalt (humid and flat soil type) microniches with one (8.33%) phenotype common between the two microniches. This suggests that the distribution of *Bam*HI phenotypes is not random, and has a definite relationship with the climatic/edaphic factors at the two microsites/microniches. Since a particular *Bam*HI phenotype is dependent on the alteration of sequences/methylation of *Bam*HI cleavage sites, the ecogeographical factors might have played an important role in determining microsite/microniche specific patterns of rDNA methylation. This is in agreement with the previous studies on several plant species (*Nicotiana tabacum, Arabidopsis thaliana,* etc.) showing alteration of cytosine methylation patterns due to environmental conditions/stress (Burn *et al*., 1993; Schmitt *et al*., 1997; Riddle and Richards, 2002).

Methylation at Nor loci and rDNA gene expression

In most eukaryotes, rRNA genes are found in multiple copies and only a subset of these genes is expressed in most cells (Conconi *et al.*, 1989; Dammann *et al*., 1993). These studies showed that very active loci have a higher proportion of rRNA genes with demethylated cytosine residues compared to less active loci (Doerfler, 1983; Cedar, 1988, Flavell *et al*., 1988; Riddle and Richards, 2002). The length of IGS region has also been correlated with methylation status of cytosine residues in wheat (Sardana *et al*., 1993). For instance, longer intergenic spacers have more unmethylated CCGG sites than shorter intergenic spacers. Since most of the polymorphic bands contributing to microsite specific *Bam*HI phenotypes involved major part of IGS region, the possible role of selection at the IGS regions is speculated. It has been noticed in studies involving both plant and animal species that IGS region contains some regulatory sequences that play an important role in the expression of neighboring rRNA genes (Doerfler, 1983; Cedar, 1988; Sardana *et al*., 1993). In the present study also, microsite/microniche specific *Bam*HI phenotypes suggests that natural selection plays an important role in the methylation of nucleotides of the IGS and, therefore, in the switching on or off of rDNA genes expression. Moreover, the results indicate that the two rDNA loci differ in their response towards methylation, which can be attributed to selection forces regulating the sequence activity at spacer region. This differential behavior of two loci towards methylation could be related with one or more important traits. In the past also, polymorphism at *Rrn2* locus was shown to be associated with water sensitivity (Powell et al. 1991). Our earlier studies also suggested a role of ecogeographical factors in the differentiation of rDNA in wild barley (Gupta *et al*., 2002; Sharma *et al*., 2004). However critical experiments, like sequence analysis of different IGS regions, are still needed to verify this.

Acknowledgements

The work was done under the tenure of a Emeritus Scientist project of Council of Scientific and Industrial Research (CSIR), New Delhi, India. PKG is currently working as a Senior Scientist of Indian National Science Academy (INSA), New Delhi. Thanks are also due to Prof. E. Nevo of

University of Haifa, Israel for supply of wild barley seed material.

References

Appels, R., Gerlach, W.L., Dennis, E.S., Swift, H. and Peacock, W.J. (1980) Molecular and chrosomal organization of DNA sequences coding for the ribosomal RNAs in cereals. Chromosoma 78:293-311.

Balyan, H.S., Jana, S. and Selvaraj, G. (1996) Polymorphism, ecogeographical differentiation and adaptation of ribosomal DNA (rDNA) alleles in wild and cultivated barley. In: Chopra VL, Sharma RP, Swaminathan MS (eds) Agricultural biotechnology, 2nd Asia Pacific conference. Oxford & IBH Publishing Co Pvt Ltd, New Delhi, India, pp 39-49.

Brown, S.E., Stephens, J.L., Lapitan, N.L.V. and Knudson, D.L. (1999) FISH landmarks for barley chromosomes (*Hordeum vulgare* L.). Genome 42:274-281.

Burn, J.E.D., Bagnali, J.D., Metzger, E.S., Dennis and Peacock W.J (1993) DNA meth-ylation, vernalization and the initiation of flowering. Proc. Natl. Acad. Sci. USA 90:287-291.

Cedar, H. (1988) DNA methylation and gene activity. Cell 53:3-4.

Conconi, A., Widmer, R..M., Koller, T. and Sogo, J.M. (1989). Two different chromatin structures coexist in ribosomal genes throughout the cell cycle. Cell 57:753-761.

Dammann, R., Lucchini, R., Koller, T. and Sogo, J.M. (1993). Chromatin structures and transcription of rDNA in yeast *Saccharomyces cerevisiae*. Nucleic Acids Res. 21:2331-2338.

Doerfler, W. (1983) DNA methylation and gene activity. *Annu Rev Biochem* 52:93-124.

Flavell, R.B., O'Dell, M., Sharp, P., Nevo, E. and Beiles, A. (1986) Variation in the intergenic spacer of ribosomal DNA of wild wheat, *Triticum dicoccoides*, in Israel. Mol. Biol. Evol. 3:547-558.

Flavell, R.B., O'Dell, M. and Thompson, W.F. (1988) Regulation of cytosine methylation in ribosomal DNA and nucleolus organizer expression in wheat. J. Mol. Biol. 204:523-534.

Gupta, P.K. (1996) Use of molecular probes for the study of DNA polymorphism in the genera *Hordeum* and *Avena*. In: Chopra VL, Sharma RP, Swaminathan MS (eds) Agricultural biotechnology, 2nd Asia Pacific conference. Oxford & IBH Publishing Co Pvt Ltd, New Delhi, India, pp 27-37.

Gupta, P.K., Sharma, P.K., Balyan, H.S., Roy, J.K., Sharma, S., Beharav, A. and Nevo, E. (2002) Polymorphism at rDNA loci in barley and its relation with climatic variables. Theor. Appl. Genet. 104:473-481.

Jahoor, A. and Fischbeck, G. (1987) Genetical studies of resistance of powdery mildew in barley lines derived from *Hordeum spontaneum* collected from Israel. Plant Breeding 99:265-273.

Jorgensen, R.A. and Cluster, P.D. (1988) Modes and tempos in the evolution of nuclear ribosomal DNA: new characters for evolutionary studies and new markers for genetic and population studies. Ann. Miss. Bot. Gard. 75:1238-1247.

Long, E.O. and Dawid, I.B. (1980) Repeated genes in eukaryotes. Ann. Rev. Biochem. 49:727-764.

Molnar, S.J., Gupta, P.K., Fedak, G. and Wheatcroft, R. (1989) Ribosomal DNA repeat unit polymorphism in 25 *Hordeum species*. Theor. Appl. Genet. 78:387-392.

Nevo, E., Baum, B., Beiles A. and Johnson, D.A. (1998) Ecological correlates of RAPD DNA

diversity of wild barley, *Hordeum spontaneum*, in the Fertile Crescent. Genet. Resour. Crop Evol. 45:151-159.

Owuor, E.D., Fahima, T., Beiles, A., Korol, A, and Nevo, E. (1997) Population genetic response to microsite ecological stress in wild barley, *Hordeum spontaneum*. Mol. Ecol. 6:1177-1187.

Powell, W., Thomas, W.T.B., Thompson, D.M. and Swanston, J.S. (1991) Associations between rDNA alleles and qunatitative traits in doubled haploid populations of barley. Genetics 130:187-194.

Riddle, N.C. and Richards, E.J. (2002) The Control of Natural Variation in Cytosine Methylation in Arabidopsis. Genetics 162:355-363.

Saghai-Maroof, M.A., Soliman, K.M., Jorgensen, R.A. and Allard, R.W. (1984) Ribosomal DNA spacer length polymorphisms in barley: Mendelian inheritance, chromosomal location, and population dynamics. Proc. Natl. Acad. Sci. USA 81:8014-8018.

Saghai-Maroof, M.A., Allard, R.W. and Zhang, Q. (1990) Genetic diversity and ecogeographical differentiation among ribosomal DNA alleles in wild and cultivated barley. Proc. Natl. Acad. Sci. USA 87:8486-8490.

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning: a laboratory manual, 2nd edition. Cold Spring Harbor Laboratory Press, Plainview, New York, USA.

Sardana, R., Michael, O'Dell. and Flavell, R.B. (1993) Correlation between the size of the intergenic regulatory region, the status of cytosine methylation of rRNA genes and nucleolar expression in wheat. Mol. Gen. Genet. 236:155-162.

Schmitt, F.E., Oakeley, J. and Jost, J.P. (1997) Antibiotic induces genome- wide hypermethylation in cultured Nicotiana tabacum plants. J. Biol. Chem. 272:534-1540.

Sharma, S., Beharav, A., Balyan, H.S., Nevo, E. and Gupta, P.K. (2004) Ribosomal DNA polymorphism and its association with geographical and climatic variables in 27 wild barley populations from Jordan. Plant Sci. 166:467-477.

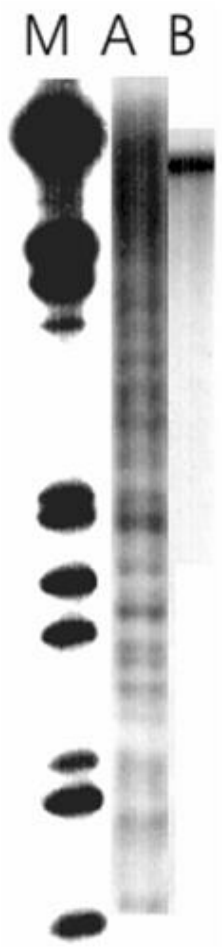

Fig. 1 Representative autoradiogram showing digestion pattern obtained using isochizomeric pair of *Msp*I and *Hpa*II restriction enzymes. Lane A: *Msp*I showed large scale digestion depicted by number of bands from top to bottom of lane. Lane B: *Hpa*II gave only single dense band at the top of lane depicting undigested DNA. M represents λ DNA/*Eco*RI+*Hin*dIII marker.

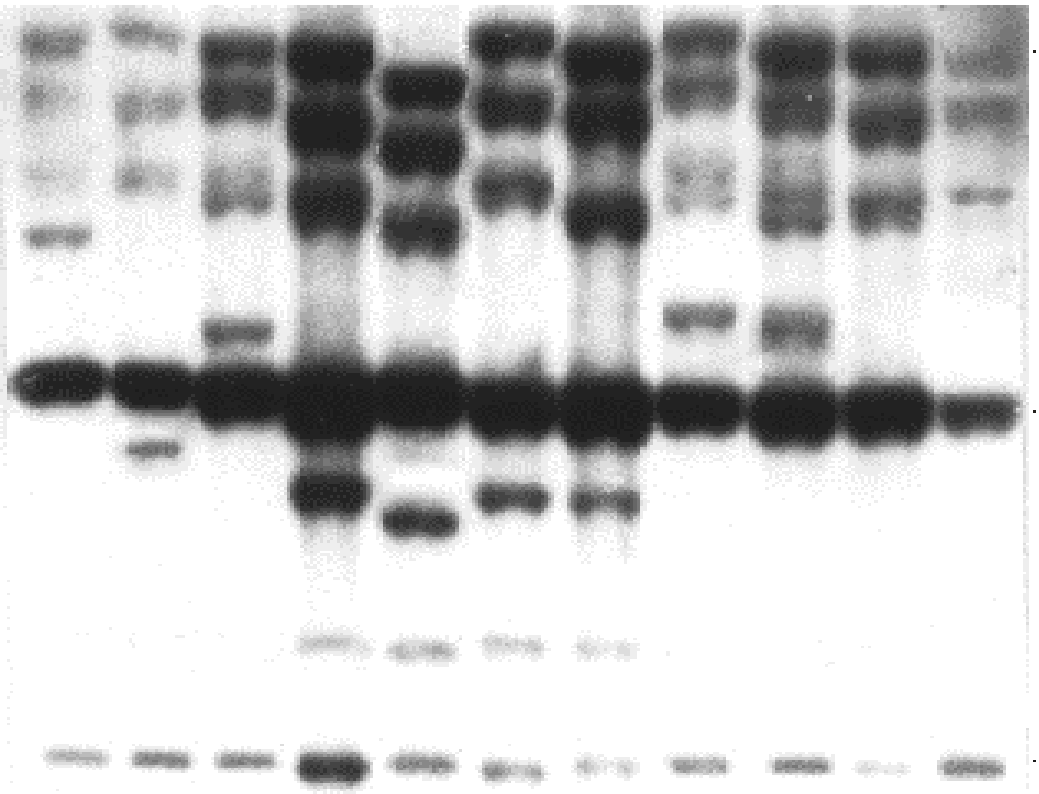

Fig. 2 Representative autoradiogram showing 11 different phenotypes obtained after *Bam*HI digestion.

Table 1. Details of phenotypes and number of bands with their sizes obtained after *Bam*HI digestion

*Bam*HI phenotype	TNB	Size of *Bam*HI bands (bp)	No. of accessions & relative frequency				Phenotypes common at:		
			Evolution Canyon		Tabigha		Microsites	Niches	
			NFS	SFS	TR	B		Slopes	Soils
I	5	9540, 7740, 5640, 3900, 1800	1 (0.1)	-	-	-	-	-	-
II	5	9425, 7625, 5525, 3900, 1800	3 (0.3)	1 (0.07)	-	-	-	+	-
III	7	9425, 7625, 5525, 3900, 3125, 2400, 1800	-	-	-	1 (0.09)	-	-	-
IV	7	9655, 7855, 5755, 3900, 3255, 2500, 1800	-	-	2 (0.15)	2 (0.18)	-	-	+
V	6	9310, 7510, 5410, 3990, 3610, 1800	-	3 (0.23)	-	-	-	-	-
VI	6	9195, 7395, 5295, 3900, 3495, 1800	-	3 (0.23)	-	1 (0.09)	+	-	-
VII	6	9310, 7510, 5180, 3900, 3380, 1800	-	2 (0.15)	-	-	-	-	-
VIII	6	9080, 7855, 5180, 3900, 3380, 1800	-	4 (0.30)	-	-	-	-	-
IX	6	9310, 7510, 5755, 3955, 3900, 1800	1 (0.1)	-	-	1 (0.09)	+	-	-
X	7	9310, 7625, 5525, 5410, 3955, 3900, 1800	-	-	2 (0.15)	-	-	-	-
XI	9	9383, 8085, 5985, 5483, 4185, 3900, 3083, 2400, 1800	-	-	-	2 (0.18)	-	-	-
XII	7	9383, 8085, 5483, 5985, 4185, 3900, 1800	1 (0.1)	-	-	-	-	-	-
XIII	8	9540, 8315, 7740, 5640, 6215, 4415, 3900,1800	-	-	-	1 (0.09)	-	-	-
XIV	7	9540, 8315, 6215, 5640, 4415, 3900, 1800	1 (0.1)	-	-	-	-	-	-
XV	7	9425, 7625, 6215, 5525, 4415, 3900, 1800	1 (0.1)	-	-	-	-	-	-
XVI	6	9383, 7583, 6100, 4300, 3900, 1800	1 (0.1)	-	-	-	-	-	-
XVII	7	9383, 7970, 5870, 5483, 4070, 3900, 1800	-	-	1 (0.07)	-	-	-	-
XVIII	7	9383, 7583, 5775, 3900, 3255, 2500, 1800	-	-	4 (0.30)	-	-	-	-
XIX	7	9195, 7395, 5525,3900, 3025, 2500, 1800	-	-	-	1 (0.09)	-	-	-
XX	8	9540,7740, 5410, 3900, 3610, 2810, 2600, 1800	-	-	1 (0.07)	-	-	-	-
XX1	8	9310, 7855, 6215, 3900, 5755, 3955, 5410, 1800	-	-	1 (0.07)	-	-	-	-
XXII	9	9540, 8965, 7740, 6215, 5640, 5065, 4415, 3900, 1800	-	-	-	1 (0.09)	-	-	-
XXIII	9	9310, 7740, 5640, 5410, 4950, 3900, 3130, 2510, 1800	-	-	1 (0.07)	-	-	-	-

TNB= Total number of bands obtained from *Bam*HI digestion; NFS= North facing slope; SFS= South facing slope; TR=Terra rossa; B=Basalt;
+ indicates common phenotypes at microsites and microniches