Title: Effect of regeneration procedures on the genetic integrity of *Brassica oleracea* accessions

Authors: Soengas P, Cartea ME, Lema M, Velasco P

Department of Plant Genetics, Misión Biológica de Galicia (CSIC), Apartado 28, E-36080 Pontevedra, Spain.

Research supported by the project PIE20064-01-089 and the Excma. Diputación Provincial de Pontevedra, Spain.

Received:

To whom reprints should be addressed. e -mail address:

psoengas@mbg.cesga.es

TLF: 0034 986854800

ABSTRACT

A *Brassica oleracea* collection of landraces collected in the northwest of Spain is kept at the Gene Bank placed at 'Misión Biológica de Galicia'. Landraces of the collection are regenerated from time to time to restore the viability of the seed and to carry on field trials. The objective of this work is to study the effect of regeneration on the genetic integrity of three accessions of this collection, and to investigate the possible causes of the genetic changes observed. After characterizing the original populations and their following regenerated populations by 25 SSRs we concluded that there were significant changes in the population structure and the allelic frequency of individual loci due to the action of genetic drift, directional selection and probably assortative mating. Protocols to store and regenerate the accessions should be improved in order to avoid the effect of these forces in the genetic integrity of the collection.

KEYWORDS: *Brassica oleracea*, genetic drift, germplasm collection, population structure, regeneration protocols, SSRs

INTRODUCTION

The Gene Bank placed at Misión Biológica de Galicia (CSIC, Spain) keeps a *Brassica oleracea* collection of local landraces adapted to Atlantic conditions, which have been collected from the northwestern Spain since the 80s until the present. Currently the collection comprises 211 accessions of kales (*B. oleracea acephala* group), 39 accessions of cabbages (*B. oleracea capitata* group) and two accessions of tronchuda cabbage (*B. oleracea tronchuda* group).

Some aspects related to the potential usefulness of this collection and its conservation has been studied. The *B. oleracea* collection has been characterized based on morphological, agronomical, nutritional and sensory traits (Cartea et al 2003; Padilla et al 2007a, b; Vilar et al 2007), concluding that some of the landraces could be employed to broad the genetic base of cultivated *B. oleracea* varieties.

The characterization of the collection allowed the identification of redundant landraces, leading to a better management of the collection by reducing the number of accessions to be used in further critical trials (Padilla et al 2007a). Other important concerns respecting the collection maintenance are the storage conditions and regeneration procedures. At present, *B. oleracea* accessions are kept at 4°C and less than 50% of humidity. Regeneration of the accessions is needed in order to obtain enough seed to carry out field trials and to restore their ability to germinate, which decreases after several years of maintenance. Regeneration procedures should preserve the genetic integrity of the collection (Hintum et al 2007). Genetic variability present in the germplasm collections represent a valuable resource to look for new sources of resistance to biotic and abiotic stress, or to obtain new commercial varieties with desirable characteristics to release in the market.

B. oleracea is an outbreeding species with a well described self incompatibility system (Sato et al 2003). The multiplication of landraces is carried out in isolation cages or isolated plots. To minimize the effects of the genetic drift in the population structure, a minimum of 100 plants per landrace are used to regenerate the population. In addition, some changes in the collection can take place if selection (natural or artificial) or contamination occurs.

Effects of regeneration on the genetic composition of gene bank accessions have been studied previously on different *Brassica* species using molecular markers. Hintum et al (2007) observed some changes at specific loci in *B. oleracea* accessions after regeneration. Diaz et al (1997) showed that there is considerable genetic shift as a result of regeneration of *Brassica napus* and *Brassica rapa* accessions at different European institutions.

Microsatellite markers have been useful method to characterize, and study the diversity present in *Brassica* collections (Hasan et al 2005; Soengas et al 2006) and to study the genetic integrity of other outcrossing species (Chebotar et al 2003). The objective of this work is to study the effect of regeneration on the genetic integrity of *B. oleracea* accessions kept at Misión Biológica de Galicia Gene Bank and to investigate the possible causes of genetic changes observed after regeneration.

MATERIAL AND METHODS

To carry out this study three landraces of *B. oleracea*: two kales and one cabbage (Table 1), were chosen from the Gene Bank at the Misión Biológica de Galicia (CSIC, Spain). A seed sample of each population was collected in 1985 directly from the growers and preserved at the Misión Biológica de Galicia. A first regeneration of the original populations was done in the 90s. A second regeneration was done for each population, using the seed obtained in the 90s (Table 1). To regenerate the populations, they were first sown in seedbeds and transplanted into the field at the 5-6 leaf stage. One hundred plants were planted in rows spaced 0.8m apart, and the distance between adjacent plants was 0.6m. Crosses among plants were carried out by natural pollinators of the species. After silique formation, seeds of at least 50 different plants were sampled and mixed to form the regenerated population.

The original populations and their regenerations were sown in seedbeds and, forty days later, the fourth or fifth leaf was used to perform DNA extractions. DNA of each individual was extracted following the method of Liu and Whittier (1994) with minor modifications. DNA from 100-200 individuals of each original population was screened with a set of 25 SSRs. Individuals in the regenerated populations (between 100-200) were amplified only with primers that rendered clear polymorphic bands in the corresponding original population. The sequence of the primers was taken from Lowe et al (2004), Suwabe et al (2006) and from the webpage <http://osbornlab.agronomy.wisc.edu>.

Amplifications were performed by using a PTC-100™ Thermal Cycler (MJ Research, Watertown, MA). The amplification consisted of a denaturing step at 95 °C during 5 min followed by 35 denaturing cycles at 95 °C for 30 sec, annealing at 56 °C

for 30 sec, and elongation at 72 °C for 30 sec. The program ended with an extra elongation period of 10 min followed by a continuous cycle at 4 °C. PCR reactions were carried out in a volume of 25 µl containing 50 ng of each primer, 0.6 unit of Taq polymerase (ECOGEN), 200 µM each dNTP, 1 × reaction buffer, 2.0 mM MgCl₂, 50 ng DNA template, and distilled and autoclaved water. After amplification, SSR products were separated by electrophoresis on 6% non-denaturing acrylamide gels run in 1 × TBE buffer. Gels were stained with Ethidium bromide, run approximately for 2 h at 250V, and then were visualized under UV light. Fragments for each SSR locus were ordered and numbered according to increasing size.

Allelic frequencies were estimated for each original and regenerated population. The software Arlequin version 3.1 (available online at <http://cmpg.unibe.ch/software/arlequin3>) for population genetic analysis of molecular marker data was used to obtain the number of alleles per locus, the observed and expected heterozygosity (Nei 1978) and F_{ST} indexes between populations. For each SSR locus, the expected and observed number of heterozygotes was compared using Fisher's exact test (Guo and Thompson 1992). F_{ST} indexes were computed between the original populations and their following regenerations as a measure of short-term distances between populations. The significance of F_{ST} values was tested by permuting haplotypes between populations.

The probability of changes of individual allelic frequencies was estimated using the chi-square analysis of a 2 × 2 contingency table. The allelic frequencies of the second regenerations were compared to those of the original populations. The null hypothesis was rejected when $P \leq 0.05$. Standard Bonferroni (1936) corrections for multiple comparisons were applied.

To test whether the observed changes in allelic frequencies were due to genetic drift acting alone or to directional selection the test proposed by Schaffer was carry out (Schaffer et al 1977). This test relies on the fact that if directional selection is acting on the locus studied, then there should be an approximately linear directional trend in the gene frequency data that can be tested for significant deviations from the null hypothesis, i.e., random genetic drift acting alone. The model uses the transformed allelic data ($2 \sin^{-1} \text{ allelic frequency}^{1/2}$) expressed in radians and the variance-covariance matrix defined in Schaffer et al (1977). The sum of squares of the deviation calculated to test the null hypothesis has a central chi-square distribution with one degree of freedom less than the number of generations evaluated. In addition the sum of squares due to all departures from the model based solely on random genetic drift is partitioned in two components, one which has a central chi-square distribution with one degree of freedom that accounts for any linear trend in the gene frequency. Therefore, the significance of this linear component determines whether the allelic frequency changes due to directional selection were important. The null hypothesis was rejected when $P \leq 0.01$. Computations were carried out with SAS software (SAS 2000).

Empirical estimates for the effective population size were obtained as well as their 95% confidence intervals using Waples' temporal method (Waples 1989), with the original populations as generation 1 and the second regenerations as the generation 3. The standardized variance in allele frequency change was calculated following the method of Nei and Tajima (1981).

RESULTS AND DISCUSSION

Seventeen SSRs were polymorphic in MBG-BRS0031, thirteen in MBG-BRS0055, and sixteen in MBG-BRS0072. The number of alleles amplified by each polymorphic locus varied between a minimum of 2 and a maximum of 5. Disregarding the alleles with a frequency lower than 0.05, the number of alleles per locus was similar for the three original population examined, being 2.18 for MBG-BRS0031, 2.31 for MBG-BRS0055 and 2.38 for MBG-BRS0072 (Table 2). The observed heterozygosity was higher for population MBG-BRS0072 compared to the rest of populations but not in a significant way.

A high percentage of the loci in populations MBG-BRS0072 and MBG-BRS0055 were not in Hardy-Weinberg equilibrium (62.5% and 50%, respectively), this percentage was considerably lower for population MBG-BRS0031 (5.6%) (Table 2). Deviations from Hardy-Weinberg proportions are mostly due to deficit of heterozygotes in the loci examined (data not shown). The way original populations were multiplied by growers could explain this deficiency. After harvesting leaves from kales, and heads from cabbages, growers normally leave few plants in the field to multiply the population. The number of plants employed to obtain seed can be as low as 5 or 6. Therefore, inbreeding, which occurs as a consequence of the small effective population size, could increase the level of homozygosity of the loci examined.

Also, in some landraces of the collection, there is a mixture of morphologically different types of plants for example in some cabbage landraces there is a mixture of head forming and non-forming plants (Padilla et al 2007b). The mixture of landraces with different allelic frequencies can cause subpopulation structure, leading to a reduction in the overall heterozygosity due to the Wahlund effect ([Wahlund 1928](#)). This

effect has been described before in natural populations of *Brassica insularis* (Sylvain et al 2005). Natural selection and selection carried out by the growers favoring more vigorous and resistant plants could also contribute to the Hardy-Weinberg disequilibrium.

Populations showed changes in the diversity after regeneration. The average number of alleles per locus and the observed heterozygosity decreased after two regenerations, although not significantly (Table 2). However, the loss of diversity had an effect in the differentiation of regenerated populations from the original populations MBG-BRS0055 and MBG-BRS0031 since F_{ST} values for both populations were significant (Table 2).

We would expect that, after two generations of random mating, the percentage of loci in Hardy-Weinberg disequilibrium decreased considerably, however this was not the case because the percentage of loci in disequilibrium increased across the regenerations for MBG-BRS0031, it did not change for MBG-BRS0055 and slightly decreased for MBG-BRS0072 (Table 2). Thus several factors affected the allele frequencies during regeneration provoking deviation from the equilibrium in the three populations examined. The changes over regenerations were examined by comparing the allele frequencies of individual loci. The frequencies of 15% of the alleles present in the second regeneration, showed significant differences compared to the original one in population MBG-BRS0031. This value was higher for MBG-BRS0055 and MBG-BRS0072 (25% and 34% of the alleles, respectively) (data not shown).

These results are in agreement with Diaz et al (1997) who showed that there was considerable genetic shift in allelic frequencies in several isozyme markers, as a result of regeneration of *B. napus* and *B. rapa* accessions, and disagrees with the results found by Hintum et al (2007), who observed some changes at few loci in *B. oleracea*

accessions after regeneration. Studies made in other species showed that changes in allelic frequencies after regeneration were quite substantial in rye (Chebotar et al 2003), and barley (Parzies et al 2000), but smaller in maize and wheat (Reedy et al 1995; Börner et al 2000).

Several factors in the regeneration procedure used could cause significant shifts in allelic frequencies, as it was explained by Diaz et al (1997) and Hintum et al (2007), including the effects of genetic drift and directional selection. To detect if changes in allelic frequencies were due to one or the other cause, the Schaffer's test was carried out. Generally speaking, changes in the frequency of most part of the alleles in the three populations studied followed a model in which the genetic drift is acting alone on neutral loci. However, Schaffer's test detected that changes in allelic frequency of seven alleles in population MBG-BRS0031, 13 alleles in MBG-BRS0055 and nine alleles in MBG-BRS0072 (Table 3) were greater than expected from a model in which random drift is acting alone. Part of these deviations are only due to the residual component of the variation in allelic frequency that accounts for the action of any erratic factor, such as non-directional selection combined with random genetic drift and/or sampling error. Although regeneration of populations is done in isolated plots or isolated cages, some cases of contaminations could also take place (for example for allele OL11H06-4) in MBG-BRS0072). But, for three alleles in population MBG-BRS0031, five alleles in MBG-BRS0055 and eight alleles in MBG-BRS0072 (Table 3) the linear component of the deviation was also significant, proving that their frequency changed as a result of directional selection. Several alleles of SSR OL11-H06 showed a significant linear component in populations MBG-BRS0055 and MBG-BRS0072 (Table 3). SSR OL11-H06 has been associated before to a QTL controlling a component of seed yield in *B. napus* (Chen et al. 2007). Allele Ni2C12-4 in population MBG-BRS0072 was also

under the action of directional selection. A QTL for earliness has been found linked to this SSR in *B. napus* (Delourne et al., 2006). These results show that during the regeneration procedures we are favoring those plants which produce more seed and/or those plants that are early flowering. After silique formation, the amount of seed taken from each plant is not taken into account when harvesting, so more seed can be taken from those more productive plants. The seed is collected from all the plants in a plot and because there is phenotypic variation for flowering, the collection takes place at different dates. However seed from plants that are early flowering can be harvested more times than seed from late flowering plants.

To avoid the effect of the genetic drift when varieties were regenerated, seeds from at least 50 different plants were collected and mixed to form the regenerated populations, thus the effective population size should be 100 individuals. However, the estimated population effective size was close to 70 individuals for the three populations studied (Table 2). If directional selection is favoring those plants which produce more seed and/or those that are early flowering, then the probability that offspring have the same parents would be higher, increasing the variance in reproductive success and therefore the genetic hitchhiking. Other possible cause of the reduced effective size is the assortative mating due to differences in days to flowering that would produce population substructure and deviation from the Hardy-Weinberg equilibrium (Weis and Kossler 2004).

After studying three populations of *B. oleracea* that are kept currently at Misión Biológica de Galicia Gene Bank and the changes in their structure through the process of regeneration we can conclude that there were significant changes in the population structure and in the allelic frequency of individual loci due to the action of genetic drift, directional selection and probably assortative mating.

Since regeneration has negative effects on the genetic variability of accessions and is laborious and seed consuming, it should be reduced to a minimum by placing much more emphasis on the efficiency of seed preservation systems. We have recently adopted the method designed by Gómez-Campo (2002) to preserve wild accessions of the *Brassica* genus kept at Universidad Politécnica de Madrid (Spain). Following this method, seeds are dried with silica gel to equilibrium and placed afterwards in sealed glass containers besides silica gel and a cobalt indicator to prevent moisture intake sufficiently to be safely considered for use in long-term seed preservation (Gómez-Campo 2002). Glass containers are then stored at 4°C. This method is successfully used today by increasing number of Gene Banks (Gómez-Campo 2002).

However, even if the seed can be preserved without any loss of viability, regenerations are sometimes necessary to produce enough seed to carry on field trials or to exchange with other research centers. To avoid the effect of genetic drift and directional selection as much as possible, protocols to regenerate the accessions should be improved. The effect of genetic inbreeding can be reduced by keeping the effective population size to a minimum of 100 individuals (50 males and 50 females), as it is done in other outcrossing species as maize. To reach this objective the same number of seeds per plant should be taken at harvest during regeneration. The effect of the assortative mating could be reduced if populations flowered uniformly, but most *B. oleracea* populations kept at Misión Biológica de Galicia are highly heterogeneous for their flowering date. A possible solution is to control and direct the pollinations. However we could only do that if the number of accessions to be regenerated is reduced. This can be achieved by improving the storage conditions of the collection.

Acknowledgements

Authors want to thank Rosaura Abilleira, Elisa Santiago, Eva Fernández, and Elena Zubiaurre all the laboratory work. Pilar Soengas acknowledges an I3P postdoctoral contract from the Ministry of Sciences and Education of Spain. This research was supported by the project PIE20064-01-089 and the Excma. Diputación Provincial de Pontevedra.

REFERENCES

- Bonferroni CE (1936) Teoria statistica delle classi e calcolo delle probabilita.
Pubblicazioni del R Istituto Superiore di Scienze Econmiche e Commerciali di
Firenze 8:3-62
- Börner A, Chebotar S, Korzun V (2000) Molecular characterization of the genetic
integrity of wheat (*Triticum aestivum* L.) germplasm after long-term
maintenance. Theor Appl Genet 100:494-497
- Cartea ME, Picoaga A, Soengas P, Ordás A (2003) Morphological characterization of
kale populations from northwestern Spain. Euphytica 129:25-32
- Chebotar S, Röder MS, Korzun V, Saal B, Weber WE, Börner A (2003) Molecular
studies on genetic integrity of open-pollinating species rye (*Secale cereale* L.)
after long-term genebank maintenance. Theor Appl Genet 107:1469-1476
- Chen W, Zhang Y, Liu X, Chen B, Tu J, Tingdong FT (2007) Detection of QTL for six
yield-related traits in oilseed rape (*Brassica napus*) using DH and immortalized
F2 populations. Theor Appl Genet 115:849-858
- Delourne R, Falentin C, Huteau V, Clonet V, Horvais R, Gandon B, Specel S, Hanneton
L, Dheu JE, Deschamps M, Margale E, Vincourt P, Renard M (2006) Genetic
control of oil content in oilseed rape (*Brassica napus* L.). Theor Appl Genet
113:1331-1345
- Diaz O, Gustafsson M, Astley D (1997) Effect of regeneration procedures on genetic
diversity in *Brassica napus* and *Brassica rapa* as estimated by isozyme analysis.
Genet Res Crop Evol 44:523-532
- Gómez-Campo C (2002) Long term seed preservation: the risk of selecting inadequate
containers is very high. Monographs ETSIA, Universidad Politécnica de Madrid 163:1-

- Guo S, Thompson E (1992) Performing the exact test of Hardy-Weinberg proportion for multiple alleles. *Biometrics* 48:361-372
- Hasan M, Seyis F, Badani AG, Pons-Kühnneman J, Friedt W, Lühs W, Snowdon RH (2005) Analysis of genetic diversity in the *Brassica napus* L. gene pool using SSR markers. *Genet Resour Crop Evol* 53:793-802
- Hintum TJL van, Wiel CCM van, Visser DS, Treuren R van, Vosman B (2007) The distribution of genetic diversity in a *Brassica oleracea* gene bank collection related to the effects on diversity of regeneration as measured with AFLPs. *Theor Appl Genet* 114:777-786
- Liu YG, Whittier RF (1994) Rapid preparation of megabase plant DNA from nuclei in agarose plugs and microbeads. *Nucleic Acids Res* 22:2168-2169
- Lowe AJ, Moule C, Trick M, Edwards KJ (2004) Efficient large-scale development of microsatellites for marker and mapping applications in *Brassica* crop species. *Theor Appl Genet* 108:1103-1112
- Nei M (1978) Estimation of average heterozygosity and genetic distance from a small number of individuals. *Genetics* 89:583-590
- Nei M, Tajima F (1981) Genetic drift and estimation of effective population size. *Genetics* 98:625-640
- Padilla G, Cartea ME, Ordás A (2007a) Comparison of several clustering methods in grouping kale landraces. *J Amer Soc Hort Sci* 132:387-395
- Padilla G, Cartea ME, Soengas P, Ordás A (2007b) Characterization of fall and spring plantings of Galician cabbage germplasm for agronomic nutritional and sensory traits. *Euphytica* 154:63-74

- Parzies HK, Spoor W, Ennos RA (2000) Genetic diversity of barley landrace accessions (*Hordeum vulgare* ssp. *vulgare*) conserved for different lengths of time in ex situ gene banks. *Heredity* 84:476-486
- Reedy ME, Knapp AD, Lamkey KR (1995) Isozyme allelic frequency changes following maize (*Zea mays* L.) germplasm regeneration. *Maydica* 40:269-273
- SAS Institute (2000) SAS OnlineDOc, version 9.1. SAS Institute. Cary, N.C.
- Sato Y, Fujimoto R, Toriyama K, Nishio T (2003) Commonality of self-recognition specificity of S haplotypes between *Brassica oleracea* and *Brassica rapa*. *Plant Mol Biol* 52:617-626
- Schaffer HE, Yardley D, Anderson WW (1977) Drift or selection: a statistical test of gene frequency variation over generations. *Genetics* 87:371-379
- Soengas P, Velasco P, Padilla G, Ordás A, Cartea ME (2006) Genetic relationships among *Brassica napus* crops based on SSR markers. *Hortscience* 41:1195-1199
- Suwabe K, Tsukazaki H, Iketani H, Hatakeyama K, Kondo M, Fujimora M, Nunome T, Fukuoka H, Hirai M, Matsumoto S (2006) Simple sequence repeat-based comparative genomics between *Brassica rapa* and *Arabidopsis*. *Genetics* 173:309-319
- Sylvain G, Gaude T, Guillemin ML, Lourmas M, Olivieri I, Mignot A (2005) Balancing selection in the wild: testing population genetics theory of self-incompatibility in the rare species *Brassica insularis*. *Genetics* 171:279-289
- Vilar M, Cartea ME, Padilla G, Soengas P, Velasco P (2007) The potential of kales as a promising vegetable crop. *Euphytica* 159:153-165
- Wahlund S (1928) Zusammensetzung von populationen und korelationserscheinungen vom standpunkt der vererbungslehre aus betrachtet. *Hereditas* 11:65-66

- Waples RS (1989) A generalized approach for estimating effective population size from temporal changes in allele frequency. *Genetics* 121:379-391
- Weis A, Kossler T (2004) Genetic variation in flowering time induces phenological assortative mating: quantitative genetic methods applied to *Brassica rapa*. *Amer J Bot* 91:825-836

Table 1. Name, origin, year of collection and year of regeneration of the three landraces studied.

Population name	Group	Origin	Year of collection	Years of regeneration
MBG-BRS0031	<i>Brassica oleracea acephala</i> group	Pontevedra (A Ramallosa)	1985	1995, 1999
MBG-BRS0055	<i>Brassica oleracea acephala</i> group	Pontevedra (Bueu)	1985	1997, 2001
MBG-BRS0072	<i>Brassica oleracea capitata</i> group	Lourido (Poio)	1985	1999, 2002

Table 2. Genetic diversity, percentage of loci in Hardy-Weinberg disequilibrium, F_{ST} values and effective population size for the original populations MBG-BRS0031, MBG-BRS0055 and MBG-BRS0072 and their following regenerations.

Population	Year	Average	Average	Average	% loci in H.-W. disequilibrium ^x	F_{ST} ^y	N_e
		No. Alleles	Observed Het.	Expected Het.			
MBG-BRS0031	1985	2.18 ± 0.64	0.31 ± 0.18	0.38 ± 0.19	5.6	0.000	
	1995	2.18 ± 0.64	0.28 ± 0.18	0.37 ± 0.18	37.5	0.002	
	1999	1.94 ± 0.96	0.24 ± 0.19	0.37 ± 0.17	47.1	-0.001	66.81 (66.66;66.90) ^z
MBG-BRS0055	1985	2.31 ± 0.48	0.25 ± 0.15	0.41 ± 0.15	50.0	0.000	
	1997	2.31 ± 0.48	0.31 ± 0.20	0.42 ± 0.13	50.0	0.015*	
	2001	2.15 ± 0.38	0.25 ± 0.20	0.36 ± 0.14	50.0	0.014*	70.42 (68.21;71.52)
MBG-BRS0072	1985	2.38 ± 0.50	0.34 ± 0.19	0.45 ± 0.09	62.5	0.000	
	1999	2.31 ± 0.48	0.33 ± 0.14	0.42 ± 0.11	50.0	0.030*	

2002	2.31 ± 0.48	0.32 ± 0.17	0.42 ± 0.14	56.0	0.029*	71.91 (65.25;76.02)
------	-----------------	-----------------	-----------------	------	--------	---------------------

^x Percentage of loci that are in Hardy-Weinberg disequilibrium at $P = 0.05$.

^y F_{ST} values computed between regenerations and the original populations.

* Significantly different from 0.000 at $P = 0.05$.

^z 95% confidence interval.

Table 3. Frequencies of alleles which showed a significant χ^2 in the Schaffers' test trough the regeneration process at SSR markers in the three populations studied. Significant χ^2 values at $P = 0.01$ for deviations from random genetic drift (deviations χ^2) and for directional selection (linear χ^2) of allelic frequency changes.

			Allelic frequencies				
			Original	First	Second		
Population	SSR	Allele	population	regeneration	regeneration	Deviations χ^2	Linear χ^2
MBG-BRS0031	Na10-A08	3	0.04	0.39	0.00	91.78	
		4	0.17	0.17	0.44	17.07	10.04
		5	0.17	0.12	0.00	27.04	16.18
	Na12-E05	1	0.07	0.03	0.17	14.74	
	FITO 035	1	0.21	0.03	0.03	13.5	11.18
	FITO 130	1	0.06	0.06	0.00	9.88	
		2	0.94	0.94	1.00	9.88	
MBG-BRS0055	Na10-F06	1	0.80	0.57	0.94	62.65	
		2	0.21	0.43	0.06	62.65	

MBG-BRS0072	Na10-G08	1	0.16	0.16	0.01	17.45	6.51
		3	0.32	0.19	0.52	23.12	
	Na10-H03	1	0.71	0.58	0.16	49.94	32.05
		2	0.29	0.42	0.84	49.94	32.05
	OI10-D03	1	0.31	0.12	0.37	19.54	
	OI11-H06	1	0.55	0.44	0.26	11.05	8.73
		2	0.40	0.48	0.74	17.80	11.32
		3	0.06	0.09	0.00	16.79	
	FITO 035	3	0.61	0.53	0.77	12.04	
	FITO 063	1	0.33	0.73	0.23	58.48	
		2	0.67	0.27	0.75	54.20	
	Na10-D09	1	0.48	0.73	0.76	15.85	14.50
	Na12-E05	1	0.71	0.90	0.94	17.36	17.06
		2	0.06	0.00	0.00	13.92	11.81
	Ni2-C12	4	0.00	0.00	0.23	40.60	19.01

Ni4-D09	1	0.55	0.78	0.88	19.46	19.45
	2	0.45	0.22	0.12	19.46	19.45
OI11-H06	1	0.59	0.07	0.11	69.83	50.11
	3	0.07	0.34	0.44	29.65	28.84
	4	0.00	0.05	0.01	9.98	