GENETIC PURITY OF THE COMMON BEAN (*Phaseolus vulgaris* L. cv. 'INTA ROJO') DURING SEED PRODUCTION IN NICARAGUA

Oswalt Jiménez
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University of Helsinki
Department of Applied Biology
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Tiedekunta/Osasto — Fakultet/Sektion — Faculty	Laitos — Institution — Department
Faculty of Agriculture and Forestry	Department of Applied Biology

Tekijä — Författare — Author
Oswalt Rafael Jiménez Caldera

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Common bean (*Phaseolus vulgaris* L.) is one of the most important crops in Nicaraguan agriculture. Bean production is carried out on small scale farms, where farmers generally lack key inputs. Seeds have been identified as the most critical input in bean production. For this reason, national seed program will be a priority during next ten years. Among the four main seed quality components, genetic component has been the least studied. The occurrence of offtype seeds and plants in the cultivar 'INTA ROJO' during seed production has hindered the seed certification process and questioned the genetic quality of the cultivars produced in Nicaragua. The present study aimed to compare the genetic composition of different seed categories in the cultivar 'INTA ROJO', and to confirm the genetic identity of offtype seeds and plants found in this cultivar. The genotype frequencies of fourty individuals were analyzed using ten microsatellite markers in the following seed categories: Breeder's seed, foundation seed, registered seed and certified seed. The genotype frequencies were analyzed using Fisher's exact test, where breeder's seed was assigned as a reference population. Additionally, twenty offtype seeds and plants, among them the offtype seeds known as "frijol viterra" and "frijol rojo oscuro", were contrasted with breeder's seed through pairwise comparisons of genetic distances. The results suggest that changes in genotype frequencies take place during seed production and they are ascribed to the selection pressures caused by environmental differences among production regions and inadequate varietal depuration procedures during seed production. In addition, the offtypes denominated "frijol rojo oscuro" were identified as an unknown cultivar probably derived from natural segregations, mutations and out-crossing among bean seed lots, and in less degree from accidental seed mixtures. In contrast, "frijol viterra" was confirmed to be the same cultivar 'INTA ROJO'. Its differences in seed weights were associated rather to environmental effects than to genotypic ones. The seed technology implications of these findings and further perspectives are discussed.

Avainsanat — Nyckelord — Keywords
Phaseolus vulgaris, genetic purity, bred cultivars, seed production, microsatellites

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Muita tietoja — Övriga uppgifter — Further information
helena.korpelainen@helsinki.fi

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1 INTRODUCTION

The common bean (*Phaseolus vulgaris* L.) has two possible centers of origin, the Andes and Mesoamerica. Studies of chloroplast and mitochondrial DNA polymorphisms have supported this hypothesis and allowed classifying beans from the centers of origin into diverse racial groups and even different domestication moments (Khairallah *et al*., 1992; Bofana *et al*., 1999; Chacón *et al*., 2005).

The common bean is a diploid crop (2n=22) belonging to the family Leguminoceae. The genus *Phaseolus* has 55 species, of which four are cultivated. The cultivated species are *Phaseolus vulgaris* L**.** (Common bean); *P. coccineus* L. (Scarlet runner bean), *P. lunatus* (Lima bean) and *P. acutifolius* A. Gray (frijol tepari). There are no inter-species genetic barriers that would prevent crosses between wild relatives and cultivated species (Mora, 1997).

Since its domestication, common bean has been one of the most important crops around the world. In Africa and Latin America, beans represent the cheapest source of protein, fiber, complex carbohydrates, folic acid, vitamin B, potassium, and zinc (Santalla *et al.*, 1999). In Central America, common bean production was about two million hectares in 2007 (FAO, 2007).

Beans represent one of the most important crops for Nicaraguan families. In rural areas, where people live in extreme poverty, beans are the sole protein source. This fact positions bean as a key crop in the current Nicaraguan Food Security Program. Unquestionably, common bean is and will be an important component to be improved for achieving sustainability in rural families.

In Nicaragua, the main bean production areas are located in the following regions: I (Estelí), IV (Rivas, Granada, and Carazo), VI (Matagalpa and Jinotega) and in RAAS (Región Autónoma del Atlántico Sur, Nueva Guinea). In these areas, bean varieties meet the best environmental conditions for production. According to Ministerio Agropecuario y Forestal (MAGFOR) (2007a), the national production was estimated to be 179,716.5 tm in 2006 with an average yield of 780 $kg.ha^{-1}$. The highest yields (1,287 $kg.ha^{-1}$) are obtained from bred varieties, among which red-colored seed cultivars are most important (‘INTA MASATEPE’, ‘INTA CANELA’, and ‘INTA ROJO’). However, black-colored seed cultivars, such as ‘INTA CARDENAS’ and ‘INTA NUEVA GUINEA’, have risen in importance due to the interest of other countries in importing them. These varieties have higher protein contents and a high acceptance in international markets.

In spite of governmental efforts to improve common bean production, Nicaraguan bean farmers harvest one of the lowest yields in the Central American region (760 $kg.ha^{-1}$). Every year, bean farmers have to face several problems during the production process, such as unpredictable raining seasons, presence of pests and diseases, low soil fertility, and low quality of seeds (INTA, 2004). All these factors are translated into low production. Recently, the Nicaraguan government has identified the high quality of seeds as one of the most important inputs in crop production. Therefore, many efforts have been focused on the Seed Production Program, which will provide a better seed quality to the farmers at low costs.

1.1 Bean seed production in Nicaragua

According to MAGFOR (2007b), bean production remained almost constant during the period from 2001 to 2006, showing a yearly average of 198,412.9 tm. Contrary to this tendency, seed production showed an increase from 897 tm to 2,260 tm during this period (figure 1). Nonetheless, the seed production system still covers only about 17 % of the national seed demand. The rest of the farmers use their "own seeds", that are produced on traditional farming systems. These seeds are produced below an artisanal system that hardly ever follows a seed certification process. The first observed consequence of this fact is the low plant densities on fields.

Bean seed production starts with the materials that are provided by the Regional Bean Breeding Program. This program together with different authorities breeds new varieties and releases them to the Central American Region. After receiving elite material from the Regional Bean program, national breeders start to increase the seed amount from breeder's seed to certified seed (figure 2). However, it is possible to start the bean seed production program by selecting from a bean population (with a known origin) around 400-500 individuals that exhibit the features of the cultivar. Each individual is harvested as an independent sample (lines) and sown in a row for several cycles. While plants are growing on the row, breeders select the lines that display the attributes of the cultivar and whose variation among individuals within the row is not so high. This process of varietal deputation is carried out together with several authorities from MAGFOR and the Instituto Nicaraguense de Tecnología Agropecuaria (INTA).

After being harvested, the seeds of selected lines are evaluated according to the seed features of the cultivar. Color, shape, and size of seeds are evaluated with awareness by bean researchers. It is noticeable that the criteria of the involved people prevail during this process. The promissory lines are mixed as a whole seed lot and they become in the breeder's seed.

The breeder's seed is the first seed category in the chain of production and it is managed by INTA at the Centro Nicaraguense de Investigación Agropecuaria y Biotecnología (CNIAB). The volumes of seed managed during this stage are small, therefore the varietal maintenance can be carried out without any problems related to technical issues. From 140 to 270 kg of seeds can be produced for the category breeder's seed at any cropping season.

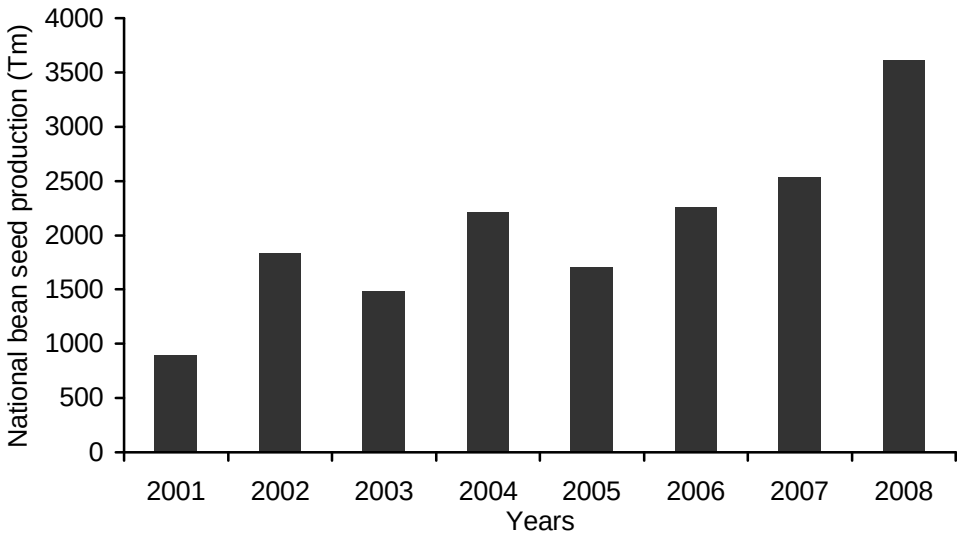

Figure 1. Bean seed production in Nicaragua during the period 2001-2008. (source: MAGFOR, 2009). The bean seed production in 2008 was based on prognostics, taking into accounts the current areas and historical production indexes.

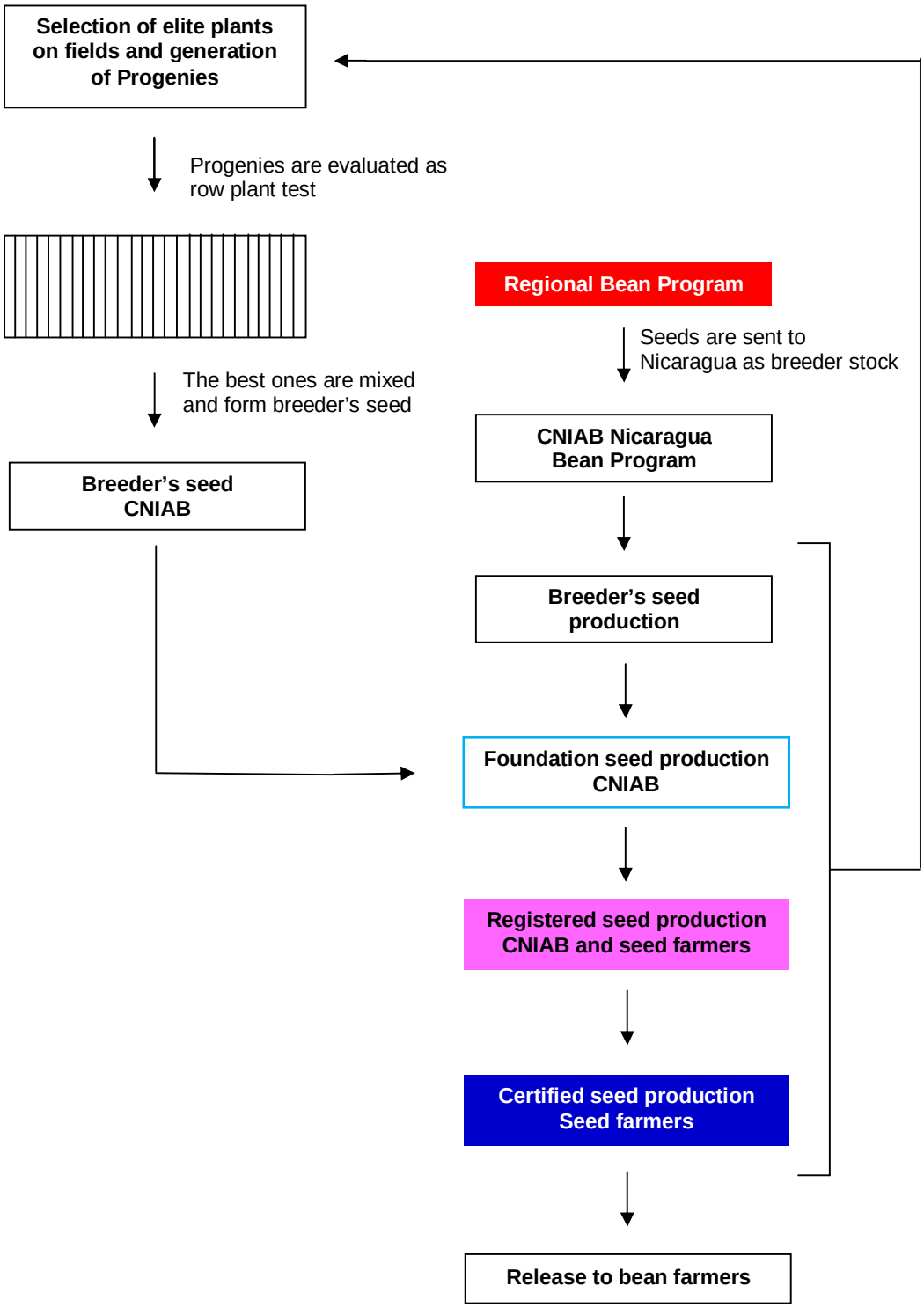

Figure 2. Diagram of the common bean seed production in Nicaragua. The red-colored box represents the start point in the system. The white, pink and blue colored boxes represent the label color depending on the seed category (MAGFOR, 2002). Notice that the seed production from breeder's seed to certified seed are inspected by MAGFOR.

The seed produced from the breeder's seed is known as foundation seed. During this increasing of seed, no selection must be carried out on the population, instead a varietal depuration is accomplished and offtype plants are discarded from the seed lot as well as weak plants and plants with symptoms of diseases.

An offtype plant is a plant that exhibits a different phenotype from the one reported by the plant breeder during the registration (MAGFOR, 1998b). A seed sample of one kilogram is sent to the Centro Nacional de Diagnostico Fitosanitario Y Semillas (CNDF y Semillas) for carrying out the external control. During this quality control, seed lots are screened by seed specialists looking for offtype seeds. An offtype seed is a seed that exhibits a phenotype different from the one reported by the plant breeder during the registration (MAGFOR, 1998b). Offtype plants and seeds have a limit of tolerance stated by the national regulations for seed production and certification.

Registered and certified seed are the product of subsequent generations where the varietal depuration is carried out constantly. During all these stages seed inspectors from MAGFOR examine the seed quality on fields. Additionally, in concordance to the Law 280 (Ley de la Produccion y Comercio de Semillas) (MAGFOR, 1998a & 1998b) it is possible to use the seed category "able for cultivation". This is an extraordinary seed category that can be used if there is no seed in the market or after facing a national urgency. During this process seed inspectors select a known commercial bean lot (preferable before flowering) and the offtype plants are roughed out, and the quality control is carried out as for certified seed.

1.1.1 Bean seed quality

Thompson (1980) defined seed quality as the sum of several components, where the genetic, physical, physiological and pathological components are the most important ones. During the quality control of seeds, all these quality components are tested. However, unfortunately, the genetic component has not received attention in the last years, although it determines the genetic quality of the materials. This quality can also be named genetic purity.

Genetic purity is a key component in seed production, because it involves the expression of the genetic potential into a cultivar, sometimes expressed in high yields and resistance to diseases. Also, it can be observed as uniform rates of growth and uniformity in flowering, maturing and harvesting. All these characteristics facilitate the maximization of production, and are important in national and international seed markets where uniform products are highly demanded.

Indeed, a new cultivar must meet several criteria of distinctness, uniformity and stability (DUS) in order to be considered a cultivar (UPOV, 2005). Throughout the process of seed certification, inspectors verify these criteria on fields and laboratories through the application of diverse national regulations. Abnormal plants found on fields are classified as offtype plants, and the seeds that exhibit patterns different from the parental lot are reported as pure seeds of other cultivar (ISTA, 2004).

Nicaraguan government approved a regulation for common bean seed production in 2002. It states that a seed lot should not have more than 0.2% offtype plants on field and less than six seeds of other varieties per kilogram in the storage (MAGFOR, 2002). Nevertheless, in recent years the authorities have found that some lots exceed these limits. For example, red-colored varieties show different seed patterns in color, shape and size. These variations have not been explained, and sometimes they are ascribed to natural variation in populations.

Figure 3. Offtype seeds found in a seed lot, cultivar ‘INTA ROJO’. Right: light red seeds. Left: frijol rojo oscuro seeds. Seed Laboratory, CNDF y Semillas (MAGFOR). Photo: Oswalt Jiménez.

The most common seed variation observed in the bean cultivar ‘INTA ROJO’ is known as “frijol viterra”. These seeds are bigger than normal seeds and sometimes have low intensity in color. Another variation found in ‘INTA CANELA’, ‘INTA ROJO’ and ‘INTA MASATEPE’ is named “frijol rojo oscuro”, commonly confused with the cultivar ‘DOR 364’, which has bright dark red color, kidney shape and about 0.21 g.seed^{-1} (Rosas *et al*., 2004) (Figures 3 and 4). This situation has bent confusion among producers, researchers, and certification authorities. This phenomenon has been ascribed to environmental effects without clear evidence.

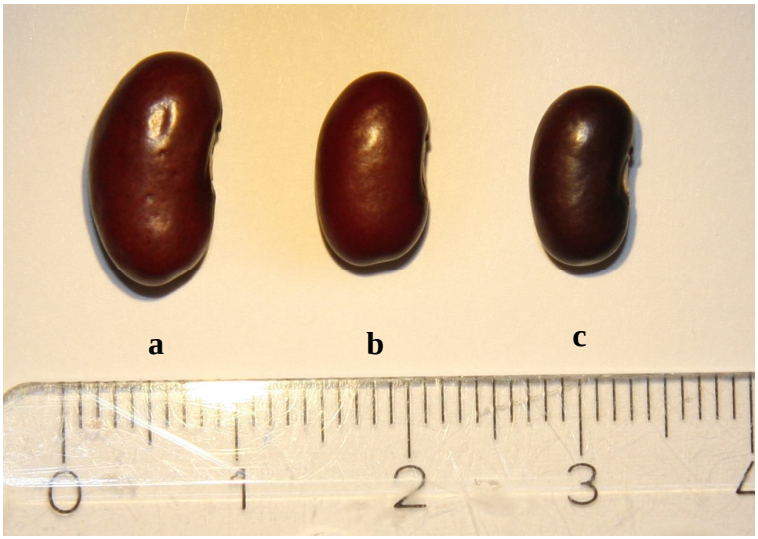

Figure 4. Frijol viterra (a), normal seed (b), and frijol rojo oscuro (c). Photo: Oswalt Jiménez.

It is known that the genetic purity of a cultivar deteriorates along the several cycles of production. Desai *et al* (1997) stated that developmental variation, mechanical mixtures, mutations, natural crossing, minor genetic variation, selected influence of diseases, and the technique of the plant breeder are the most important factors that deteriorate the genetic purity of a cultivar.

Nowadays molecular tools have been used in studies that aim at the molecular characterization of germplasm and marker assisted selection in plant breeding. Nonetheless, not many studies have aimed to study the genetic structure and cultivar identification of bred bean cultivars. However, some molecular markers, most importantly microsatellites or simple sequences repeats (SSRs), have been developed for common bean and some of them have been shown to be highly polymorphic (Yu *et al*., 2000; Gaitan *et al*., 2002; Guerra, 2004; Blair *et al*., 2006; Buso *et al*., 2006; Blair *et al*., 2008). The highly polymorphic characteristic of microsatellites can be exploited for detecting small changes in the genetic purity of a bean cultivar, and this may be extremely useful when evaluating the genetic purity of a bean cultivar during the seed production.

The genetic quality of bean varieties has never been studied by applying molecular tools in Nicaragua. Also, the seed production as a whole process has been evaluated following traditional methods that only give a phenotypic estimation. These phenotypic values have a high environmental component that easily leads us to a wrong estimation creating confusion and probably accelerating the deterioration of the quality of materials evaluated on field.

The study and understanding of the factors that affect the genetic purity of a bean cultivar are very important for bean seed production. Additionally, it is important to consider that seeds are the main input during bean production and that all improvements in bean production will be translated to benefits for the poorest families in rural areas where common bean is considered an important part of people's incomes and food security.

2 OBJECTIVES

The present study was focused on the following objectives:

- To compare the genetic composition of different seed categories in the bean cultivar 'INTA ROJO'.

- To confirm the genetic identity of offtype plants and seeds found in the cultivar 'INTA ROJO'.

We hypothesized that there are changes in genotype frequencies during the process of seed production and that at least one of the offtype groups is different from the breeder's seed at the loci analyzed.

3 MATERIALS AND METHODS

3.1 Seed lots used in this study

The cultivar used in this study was 'INTA ROJO'. The selection of this cultivar was based on the fact that it represented about 77% of the areas destined to bean seed production in 2008 (MAGFOR, 2008). Therefore, it can be considered as a representative cultivar of Nicaraguan bean seed production.

'INTA ROJO' is a small red dry bean that was created under the name 'EAP 9510-77'. This cultivar was developed at The Escuela Agricola Panamericana (EAP), Zamorano, Honduras, and released in Central America in a collaboration with the national programs of Honduras, El Salvador, Nicaragua and Costa Rica, and the University of Puerto Rico in 2003 (Rosas *et al.*, 2004). It was obtained through the cross between the lines 'TIO CANELA' and 'DICTA 105'. 'TIO CANELA' has small red seeds and resistance to Bean Golden Yellow Mosaic Virus (BGYMV, a Geminivirus) (Rosas et al, 1997). On the other hand, 'DICTA 105' is a small red-seeded cultivar with resistance to pod weevil (*Trichapion godmani* Wagner). This line was bred at the Centro Internacional de Agricultura Tropical (CIAT), Cali, Colombia and the Dirección de Ciencia y Tecnología Agropecuaria (DICTA), Tegucigalpa, Honduras.

'INTA ROJO' has an intermediate upright bush, Type II growth habit with short vine. It flowers in 36 to 38 days. Stem color is green with red pigmentation. Green pods turn yellow with red pigmentation at physiological maturity. Additionally, it has long pods containing seven to eight seeds per pod. Also, it has ovoid elongated seeds, averaging 25g.100seeds^{-1}. Seed coat color is shiny red (Rosas *et al*, 2004). This color quality placed 'INTA ROJO' in an advantageous position in the national market, where landraces are preferred due to their color and culinary properties.

The study was conducted for the following seed categories: breeder's seed, foundation seed, registered seed, and certified seed. One population or seed lot was taken from each seed category. For the three first seed categories only the existing lot was sampled. In contrast, for certified seed one representative lot was chosen following a random process from the lot list. Additionally, one population of offtype plants and seeds were included in the study.

3.2 Seed and plant sampling

3.2.1 Seed lots sampling

All the chosen populations were sampled following the international rules for seed sampling (ISTA, 2004) and the recommendations stated by the seed inspectors from MAGFOR. However, the intensity of sampling was greatly increased (between ten primary samples for breeder's seed and 30 for certified seed) in order to obtain a representative sample for each seed lot and thus to have a better estimation of the genetic changes among populations.

Breeder's seed, foundation seed and registered seed were produced at the research station "La Compañia" in Carazo and sampled at CNIAB INTA. The certified seed was produced in Matagalpa and sampled *in situ* in collaboration with the seed inspectors from MAGFOR (table1). After sampling each lot, the composed sample was reduced to one-kilogram sample (sending sample).

Table1. Seed lots sampled during this study.

Seed category	Lot size (tm)	Date of harvesting	Origin
Breeder's seed	1.74	September 9, 2008	CNIAB-INTA San Marcos , Carazo
Foundation seed	2.64	September 9, 2008	CNIAB-INTA San Marcos, Carazo
Registered seed	5.72	December, 2007	CNIAB-INTA San Marcos, Carazo
Certified seed	13.64	October, 22 2008	EMPROSECAGRO, Matagalpa

3.2.1.1 Seed analyses in laboratory

The samples were homogenized using a heavy duty boerner divider (Seedburo) and reduced (until 100 seeds) following recommendations stated by the Seed Laboratory of the CNDF y Semillas, and the spoons methodology cited by ISTA (2004). Nonetheless, in this case 8 centimeter petri plates were used. Standard germination rates, 1000 seed weight, and moisture content were determined for each sample following the rules stated by ISTA (2004) (table 2). This information provided valuable information for the next steps in this research and an estimation of the physiological quality of each lot.

Table 2. Physiological and physical quality of the seed lots used in this study.

Seed category	Germination rate (%)	Moisture content (%)	Weight of 1000 seeds (g)
Breeder's seed	90	12.2	254.6*
Foundation seed	92	12	248.3
Registered seed	91	11	246.8
Certified seed	92	12	241.0

*=value taken from MAGFOR (2002)

3.2.2 Screening for offtype seeds

In collaboration with the Seed Laboratory of the CNDF y Semillas, all samples evaluated from the national production between September and November 2008 were screened for offtype seeds. The evaluation was carried out by contrasting the offtype seeds found with a reference sample provided by a plant breeder. The offtype seeds were classified in "frijol viterra" and "frijol rojo oscuro". Before evaluating the seed analysts explained how they evaluate the presence of offtype seeds in the samples. Briefly, they screen about 700 g from the sample. All the seeds are compared with the reference sample (provided by the plant breeder). Only the offtype seeds that showed a clear difference in shape, size and color were selected. Ten offtype seeds (the most extreme ones) were compared with our reference population at molecular level.

3.2.2.1 Phenotypic analyses of offtype seeds

When all offtype seeds were classified, a phenotypic analysis was carried out in order to describe and contrast the frijol viterra and rojo oscuro seeds with 'INTA ROJO' varietal features. Three offtype seed features were recorded (color, shape and weight) in one hundred seeds. The shape and kind of seed was determined following the methodology described by Muñoz *et al*. (1993) (figure 5). The average weight of one seed in 'INTA ROJO' was calculated as follows: ten groups containing 100 seeds (following a randomized process) were weighted. As the variation coefficient was less than four percent, the weight of an individual seed was determined by averaging weights. For offtype seeds, all seeds were weighted and the average was calculated.

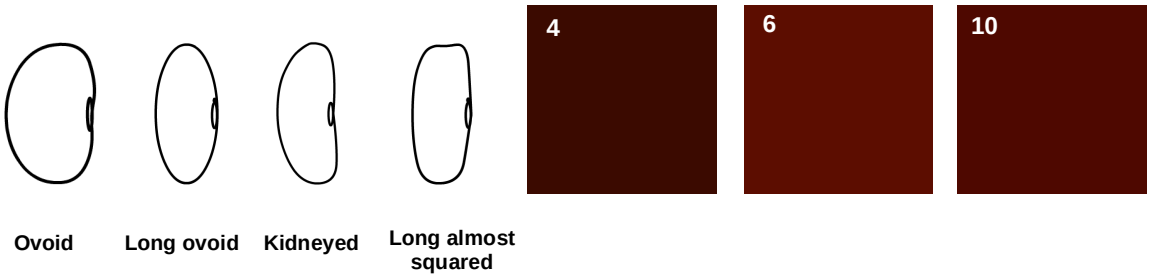

Figure 5. Left: Bean shapes evaluated in viterra, rojo oscuro, and breeder's seed. Right: Different brown red color intensities evaluated in the viterra, rojo oscuro and breeder's seed. (These colored squares and bean seed shapes are a representation of those showed in Muñoz, *et al*., 1993, but recreated using MACROMEDIA FLASH SORFTWARE 8.0v.)

3.2.3 Plant sampling

Over the last week of November 2008, varietal depuration was carried out in most of the bean seed lots in Nicaragua. The offtype plant sampling was carried out on certified seed lots at "La Compañia", Carazo. The field trip was carried out in cooperation with seed inspectors from MAGFOR and a plant breeder from INTA. They previously described how the varietal depuration is carried out and what criteria they take into account during this activity. The offtype plants were chosen according to these criteria and based on the experience of the field workers who collaborate in this process.

The sampled area was about five ha. Five kinds of offtype plants were found and sampled on that research station. They were: plants with a growth habit type III and green stems (undetermined prostrated non climbing or viny semi climbing), contrary to type II found in 'INTA ROJO', plants with a growth habit type III and red pigmentation on stems, plants with red pigmentation on pods, plants showing over growth, and a plant with four foliar lobules instead three (figures 6 and 7). In order to avoid confusing the evaluated traits with environmentally masked effects the sampled plants were first identified on the field by contrasting them with the phenotype described by the plant breeder during the registration. Also, when plants showing over-growth were found they had to have neighbor plants at 10-cm distance. Plants were removed from the field and examined carefully on a table. Disease-affected plants were not sampled, because of the complex symptoms that virus-affected plants can show.

Figure 6. Offtype plants found in an 'INTA ROJO' population during the plant sampling at the research station "La Compañia" (Carazo, Nicaragua). Left: INTA field worker showing an abnormal long red pigmented main stem previously marked for further observations. Right: unusual red pigmented pods at the beginning of the physiological maturity. Photo: Oswalt Jiménez.

At the end of the field trip, sixty putative offtype plants were sampled. However, only the ten more extreme ones were chosen and analyzed each plant representing a group (table 3). After classifying the plants as offtypes, seven leaves were taken from each individual, one leave was used for DNA extraction and the other six leaves were dried at room temperature (about 26 ^{o}C) and conserved for further DNA extraction if needed.

Figure 7. ‘INTA ROJO’ offtype plant exhibing an over-growth when compared with other normal plants. Photo: Oswalt Jiménez

Table 3. Offtype plants found into a ‘INTA ROJO’ seed lot and sampled in the research station “la Compañia”

Individual	Characteristics that make them offtypes
1	Long green main stem
2	Long green main stem, leaves showing over-growth, and red* pigmentation in pods
3	Long red* main stem
4	Five-leaved plant, red* long main stem
5	Red* long main stem, over-growth
6	Long green main stem
7	Long red* main stem
8	Red* pigmentation in pods
9	Red* pigmentation in pods, over-growth
10	Long green main stem, over-growth

*Notice that even registers notified this possible red pigmentation in stems and pods (Rosas *et al*., 2004), certification authorities and even plant breeders point out them as offtypes. Observations show than in some cases red pigmentation in stems is almost total and in pods cover about 15% of the surface.

3.3 Laboratory analyses

3.3.1 DNA extraction

After sampling and reducing the samples, four populations and two groups of offtype seeds (frijol viterra and rojo oscuro) were obtained. From each population, 100 randomized individuals were taken. Those seeds were sown in plastic trays containing sterilized soil and kept in a room at 30^{o}C for six days (figure 8). Forty individuals from each population, ten individuals of offtype plants and ten individuals of offtype seeds were analyzed.

The DNA extraction was carried out at The Biotechnology Laboratory placed at CNIAB-INTA in Nicaragua. The Mini mini preparation protocol (Dellaporta *et al*, 1983) was modified for being used in common bean. Briefly, about 20 mg of leaf tissue was taken from each individual. The plant material was placed into an Eppendorf tube. After that, 200 µl of cold miniprep II extraction buffer (containing 100 mM Tris-HCl, 50 mM EDTA, 500 mM NaCl, and 20 mM 2-mercaptoethanol) was added, and the tissue was macerated and homogenized using a plastic pestle. After that, 24 µl of sodium dodecyl sulfate (SDS) (10%) were added and mixed. Subsequently, the samples were placed into a wet cabin at 65 ^{o}C for ten minutes. After that, 111.6 µl of 3M potassium acetate (KAc) was added and the samples were incubated on ice for 30 minutes. The samples were centrifuged at full speed (14,800xg) at 5^{o}C for 15 minutes. Then, the supernatant was removed carefully and placed into a clean Eppendorf tube avoiding the debris. Afterward, 0.6 volumes of isopropanol were added and the samples were kept at -22^{o}C for 30 minutes. Then, the samples were centrifuged at full speed (14,800xg) at 5^{o}C for 15 minutes, the isopropanol was removed, and the pellet was washed twice with ethanol (70%) and left to dry. The pellet was dissolved into 100 µl of TE (containing 10 mM Tris-HCl pH 8, and 1 mM EDTA). Then, 1 µl of RNAse (10 ng. μl^{-1}) was added and the DNA samples were placed into a warm wet cabin at 37^{o}C for one hour. Finally, the samples were placed into a freezer at -24^{o}C. The DNA amount was checked by running it on an agarose gel (1%).

Figure 8. Seedlings obtained from offtype seeds. Seedlings placed at right in red and blue trays are frijol viterra, seedlings at left and right in red and yellow trays respectively are frijol rojo oscuro, and the two seedlings placed at left in the yellow and blue trays are breeder's seed seedlings. CNIAB-INTA, Nicaragua. Photo: Oswalt Jiménez

3.3.2 Microsatellite genotyping

Microsatellite genotyping was carried out in the Laboratory of the Department of Applied Biology, University of Helsinki, Finland. Twelve microsatellites markers (at least one per linkage group) were selected for this study: BM-053, BM-143, BM-172, BM-199, BM-175, BM-137, BM-210, BM-189, BM-188, BM-212, BM-184, and GATS091 (table 1A). All these primers were developed by Gaitán *et al* (2002) and they were selected attending their high polymorphism values and good discrimination power shown in many studies on bean landraces (Blair & Díaz 2006; Blair *et al.*, 2007).

The PCR reactions were carried out in 10 µl volumes by mixing the following components: 1µl of 10x buffer, 0.2 µl of dNTPs (10 mM each 500 µl), 6 µl of MQ water, 1 µl of each primer (5 pmol). The forward primers were fluorescently labeled (Applied Biosystems). Finally, 0.3 µl of DNA polymerase (Dynazyme, $2U.\mu l^{-1}$) was added. All these components were mixed with 0.5 µl DNA template (about 40 ng). The

PRC reactions were carried out as follows: DNA denaturation at 94°C for 4 minutes followed by 30 cycles of denaturation at 94°C for 45 seconds, annealing at 50-62°C (depending on the primer) for 45 seconds, and elongation at 72°C for 1 minute, with a final elongation at 72 °C for 10 minutes. After amplification the PCR products were diluted with MQ water at 1:20 ratio. From this dissolution 0.5 µl of each template was mixed with 20 µl of HiDi-formamide and 0.15 µl of size standard (GeneScan 500 ROX). After mixing, the samples were denatured for five minutes at 95 °C. Finally, DNA fragments were analyzed in a capillary electrophoresis system 3730 DNA Analyzer (Applied Biosystems) in the Sequencing Laboratory of the Institute of Biotechnology, University of Helsinki, Finland. The different peaks showing the fragment sizes were read using PEAK SCANNER v1.0 software (Applied Biosystems).

3.4 Statistical analyses

3.4.1 Genetic assumptions

Before analyzing the data, we must state some assumptions about the nature of this study. Common bean is a self-pollinated species, expected to form very homogeneous population composed by very homozygous individuals for the loci involved (Acquaah, 2007). 'INTA ROJO' a bred cultivar obtained as a $F_{2:6}$ line derived from a cross between two lines (Rosas *et al.*, 2004). At least the breeder's seed should be a homogenous population. The successive generations can exhibit some changes in the allele frequencies as Rodrigues & Santos (2006) suggested in their study. These changes were ascribed to fitness differences and natural crossings, but in this study we have to take into account that in the successive seed generations following breeder's seed (foundation seed, registered seed, and certified seed) the plant breeder, the seed farmers and the seed inspectors from MAGFOR rough out all the offtype plants from the seed lots, and try to keep the population as original as possible in genetic terms. Before this constant depuration process, it makes sense to consider the breeder's seed as a reference population when comparing the next generations.

3.4.2 Genotype frequency analysis

All genotypes found at the different loci were recorded (as a binary combination of successive alleles as: A_1, A_2, A_3,…, A_n). The genotype frequencies of the reference population (breeder's seed) were compared with the successive seed categories (foundation, registered, and certified seeds) through Fisher's exact test using R software (Bioconductor).

3.4.3 Comparison between seed populations

The genetic structure of the reference population was contrasted with the foundation, registered and certified seed populations. The genetic diversity parameters were estimated (total number of alleles, and observed and expected heterozygosity; H_{obs} and H_{exp}). Genetic distances of the reference population and successive seed generations were tested for the level of significance with a pairwise t-test. Furthermore, the F_{IS} index was calculated for each population.

Additionally, the offtype individuals were subdividided into three groups: offtype plants, frijol viterra and frijol rojo oscuro. To estimate pairwise differences between these groups and the reference population, F_{ST} values were calculated using ARLEQUIN 3.1. The pairwise matrix was contrasted by UPGMA test and the phylogenetic tree was plotted using MEGA 4.1v software.

4 RESULTS

4.1 Phenotypic analysis of offtype seeds

The breeder's seed used as a reference population showed a very uniform pattern, contrary to the frijol viterra and rojo oscuro seeds with considerably ranging seed features. We can summarize the phenotypic results as follows: breeder's seed had a medium weight, was entirely long-ovoid in shape, and brown-red in color. Frijol viterra was predominantly of medium weight, long-ovoid in shape and brown-red in color. However, the color was the same, but the intensity was different frijol viterra being lighter. On the other hand, frijol rojo oscuro was predominantly small weight seed, long-ovoid shaped and brown-red colored (with a darker color, 10) (Table 4).

Table 4. Seed features of the materials used in this study

Material	Shape (%)				Red color intensity (%)			Weight (Muñoz *et al*., 1993)		
	2	5	7	8	4	6	10	S	M	B
Breeder's seed	-	100	-	-	-	-	100	-	X	-
Frijol viterra	19	59	1	21	87	3	10	-	X	-
Frijol rojo oscuro	31	61	7	1	1	49	50	X	-	-

Shape: 2= ovoid, 5= long ovoid, 7= long almost squared, and 8= kidneyed. Size: S= small, M= Medium, and B= big.

4.2 Genetic structure of 'INTA ROJO'

Among the set of twelve microsatellites, ten worked adequately. The microsatellites BM-053 and BM-212 did not produce any PCR products, even after testing several PCR programs. The other primers were successful in amplifying DNA fragments. GATS091 and BM-199 were the most informative or polymorphic loci, while BM-175 and BM-188 were the least variable loci. Some examples of the genotyping peaks are showed in figure 9.

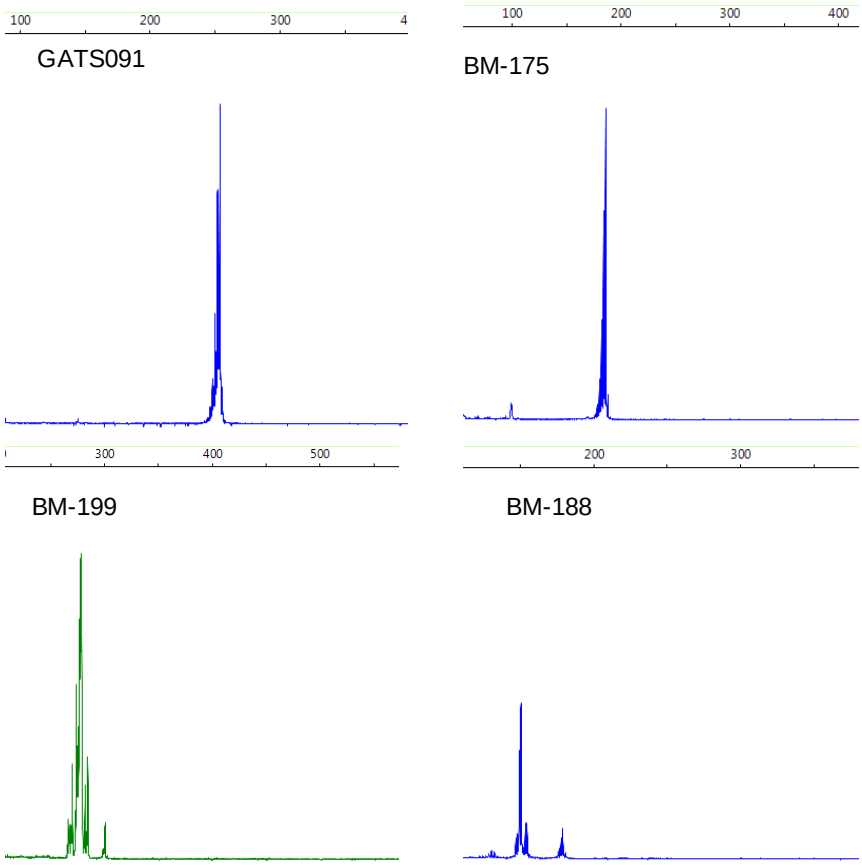

Figure 9. Examples of peaks obtained in this study. When tested on seed categories, loci GATS091 and BM-199 showed the highest allele numbers (5 and 4 alleles respectively). In contrast, BM-188 and BM-175 were monomorphic. However, when tested on offtypes, they discriminated them from breeder's seed. The upper line shows the peak sizes in basepairs (Images generated using PEAK SCANNER SOFTWARE v1.0, Applied biosystems)

A total of 27 different alleles were identified in the four seed categories at the ten microsatellite loci, with an average of 2.7 alleles per locus. In each seed group, from two to five loci were monomorphic. Registered seed group showed the least number of monomorphic loci (2) and foundation seed group the highest (5). When the H_{exp} and H_{obs} were contrasted it was evident that the observed heterozygocity values of these groups (breeder's seed, 0.0075; foundation seed, 0.0000; registered seed, 0.0075; and certified seed, 0.0100) were very low. The average F_{IS} showed that the four groups were highly homozygous, with the exception of the locus BM-189 with an average value of 0.6556 (table 5).

Table 5. Number of alleles and F_{IS} index found in the four seed categories at ten microsatellite loci.

Locus	Seed category				Mean	Total number of alleles	F_{IS}
	Breeder's seed	Foundation	Registered	Certified			
	Number of alleles						
BM-143	2	1	1	1	1.25	2	1.000
BM-172	2	2	2	2	2.00	2	1.000
BM-199	3	3	3	3	3.00	4	1.000
BM-175	1	1	2	1	1.50	2	1.000
BM-137	2	2	2	2	2.00	2	1.000
BM-210	1	1	3	2	1.75	4	0.883
BM-189	2	2	2	2	2.00	2	0.656
BM-188	1	1	1	1	1.00	1	1.000
BM-184	1	1	2	1	1.25	2	1.000
GATS091	4	2	4	1	2.75	6	0.937
Average	1.9	1.6	2.2	1.6	1.83	2.7	0.948

The F_{ST} values between the four groups of seed categories ranged from 0.0635 to 0.2894 (table 6). In all comparisons, the difference was statistically significant (table 6). The average F_{ST} value was 0.1390.

Table 6. F_{ST} values between different seed categories at ten microsatellite loci.

Seed category	Breeder's seed	Foundation seed	Registered seed	Certified seed
Breeder's seed	0			
Foundation seed	0.0747*	0		
Registered seed	0.0641*	0.0635*	0	
Certified seed	0.2288*	0.2894*	0.1136*	0

*Significant at $P < 0.05$

A phylogenetic tree of the four seed categories showed a strong differentiation of the certified seed from the other seed categories. Breeder's seed was in the same main branch as registered and foundation seed. Nonetheless, registered and foundation seed were considered as one group (figure 10)

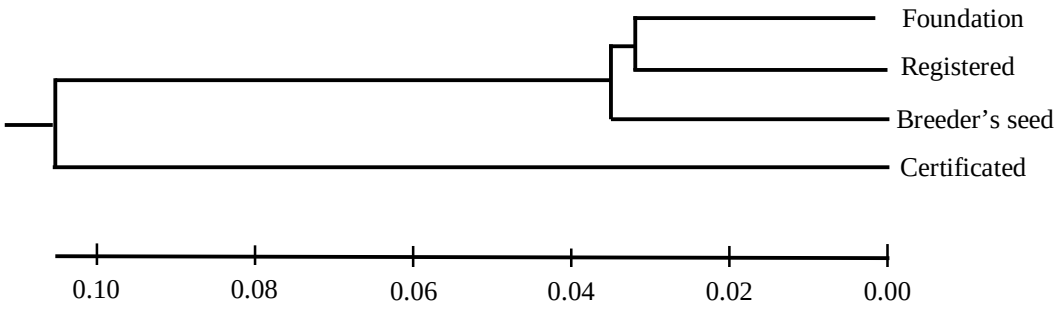

Figure 10. Rectangle cladogram showing the relatedness among the four seed categories analyzed in the cultivar 'INTA ROJO' during seed production in Nicaragua.

4.3 Genotype frequency analysis

4.3.1 Genetic structure of breeder's seed as reference population

The ten microsatellites revealed in total 19 different alleles and five different genotypes (table 2A). Four loci were monomorphic (BM-210, BM-175, BM-184, and BM-188). The loci BM-137, BM-143, BM-189 and BM-172 had two alleles, and the loci BM-199 and GATS091 possessed three and four alleles, respectively. The average number of alleles per locus was 1.9 (table 5)

4.3.2 Comparison between breeder's seed and foundation seed

Foundation seed population showed 16 alleles and six different genotypes at ten loci analyzed. The loci BM-143, BM-175, BM-210, BM-188 and BM-184 exhibited only one allele. In contrast, BM-172, BM-189, BM-137 and GATS091 showed two alleles. Only the locus BM-199 had three alleles.

When this population was contrasted with breeder's seed, the Fisher's exact test revealed changes at genotype frequencies at the locus GATS091 (table 2A). This locus showed a new genotype not detected before. The other loci exhibited no new genotypes.

4.3.3 Comparison between breeder's seed and registered seed

Registered seed population exhibited 22 alleles and seven genotypes at ten loci. Only the loci BM-143 and BM-188 were monomorphic. Loci BM-172, BM-175, BM-137, BM-189 and BM-184 showed two alleles. Loci BM-199 and BM-210 exhibited three alleles. Finally, locus GATS091 exhibited four alleles. When compared with the reference population through Fisher's exact test, registered seed showed changes at genotype frequencies at the locus GATS091 (table 3A). As well, two new genotypes appeared in registered seed at the locus GATS091.

4.3.4 Comparison between breeder's seed and certified seed

Certified seed population showed 16 alleles and six genotypes at ten loci. The allele distribution was as follows: loci BM-143, BM-175, BM-188, BM-184 and GATS091 were monomorphic. In contrast, loci BM-172, BM-137, BM-210 and BM-189 exhibited two alleles and BM-199 showed three alleles. The Fisher's exact test revealed that certified seed exhibited changes at genotype frequencies at the loci BM-172, BM-137 and GATS091 when compared with breeder's seed (table 4A).

4.4 Comparison of offtype plants and seeds with breeder's seed

A total of 30 different alleles were identified in the four populations, breeder's seed and the offtype individuals (offtype plants, frijol viterra, and rojo oscuro). On average three alleles per locus were found. Frijol viterra population showed nine monomorphic loci. Breeder's seed, offtype plants and frijol rojo oscuro showed four monomorphic loci. When the H_{exp} and H_{obs} were contrasted it was evident that the observed heterozygocity values in these groups (breeder's seed, 0.00750; offtype plants, 0.0125; frijol viterra, 0.0000; and frijol rojo oscuro, 0.0000) was very low. The average F_{IS} showed that the four groups at the ten loci were highly homozygous, ranging from 0.9592 to 1.0000 (table 7). Five out of ten microsatellites analyzed (BM-175, GATS091, BM-210, BM-137, and BM-188) were showed to be useful in discriminating offtypes plants and seeds from breeder seed, because they possessed at least one new allele (not found in breeder seed, therefore foreign one) in one individual from each group. Four out ten microsatellites (BM-175, BM-137, BM-188 and GATS091) identified alleles not found in the four seed categories (tables 5A and 6A).

Table 7. Number of alleles and F_{IS} index found in breeder's seed and three kind of offtype individuals at 10 microsatellite loci.

Locus	Breeder's seed	Offtypes groups			Mean	Total number of alleles	F_{IS}
		Plants	Frijol viterra	Frijol rojo oscuro			
	Number of alleles						
BM-143	2	1	1	1	1.25	2	1.0000
BM-172	2	1	1	1	1.25	2	1.0000
BM-199	3	1	1	2	1.75	3	1.0000
BM-175	1	3	1	2	1.75	3	1.0000
BM-137	2	3	2	2	2.25	3	1.0000
BM-210	1	1	1	2	1.25	2	1.0000
BM-189	2	2	1	1	1.5	2	1.0000
BM-188	1	2	1	1	1.25	3	1.0000
BM-184	1	2	1	2	1.5	3	1.0000
GATS091	4	4	1	2	2.75	7	0.8197
Average	1.9	2.0	1.1	1.6	1.65	3	0.9820

The test result showed that only frijol viterra was similar to breeder's seed. The F_{ST} values in the different groups described a range between 0.0777 and 0.4343. All the comparisons showed significan differences, with exception of frijol viterra which was similar to breeder's seed and offtype plants (table 8).

Table 8. F_{ST} values between different seed group s within 'INTA ROJO' cultivar based on 10 microsatellite loci.

Groups	Breeder's seed	Offtype plants	Frijol viterra	Frijol rojo oscuro
Breeder's seed	0			
Offtype plants	0.2485*	0		
Frijol viterra	0.0777	0.1593	0	
Frijol rojo oscuro	0.4343*	0.2390*	0.3879*	0

*Significant at $P < 0.05$

The average F_{ST} value was 0.2576. A phylogenetic tree for the four groups showed breeder's seed and frijol viterra as one group, offtype plants in another group, even in the same main branch as breeder's seed and frijol viterra, and finally frijol rojo oscuro was totally a different group than the others (figure 11).

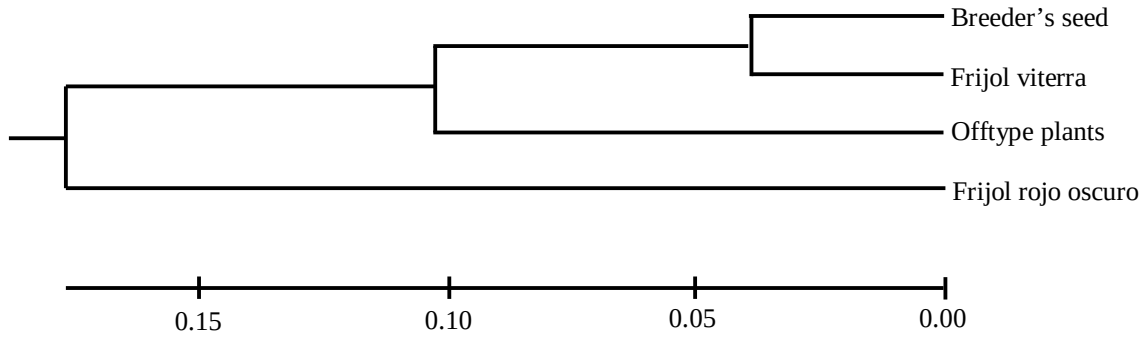

Figure 11. Rectangle cladogram tree showing the relatedness among the three offtype groups and the breeder's seed.

5 DISCUSSION

The study of the genetic composition of a bean bred cultivar has not been targeted in the recent research, were biotechnological tools have been applied. Most of the studies have focused on the quantification of the genetic diversity in landraces along the Mesoamerican and Andean centers of origin (Gomez *et al*., 2004; Gomez *et al*., 2005; Diaz & Blair, 2006; Maras *et al*., 2006; Blair *et al*., 2007; Masi *et al*., 2009). Nonetheless, molecular tools can be incorporated to plant breeding and seed production programs when we lack other precise methodologies and the results will solve a critical situation in a short period of time (Svetleva, *et al*., 2003; Campos *et al*., 2007). The genetic purity of a released cultivar is one of the most important topics to assess. Molecular markers have been successfully used to evaluate the genetic purity of different cultivars (Smith & Register, 1998; Crocket *et al*., 2002; Yashitola, *et al*., 2002; Ilbi, 2003; Mongkolporn *et al*., 2004)

Bean seed production in Nicaragua encourages keeping the genetic purity of cultivars as pure as possible. The bred cultivar 'INTA ROJO' is broadly used and its genetic purity is often questioned during the certification process. This situation drove this research with aim to compare the genetic composition of different seed categories and to confirm the genetic identity of offtype plants and seeds found in this cultivar.

5.1 Genetic changes in different seed categories during seed production

Genotype frequency changes taking place at many loci during seed production, visible as differences as between seed categories, and the clustering of the seed categories as two main groups can be explained by carefully analyzing the genetic population dynamics.

From population genetic point of view, systematic (migration, mutation and selection) and dispersive process (genetic drift) are two agencies that interact jointly changing the genotype frequencies of populations (Falconer & Mackay, 1996). Seed production can be affected by these agencies altering the proportion of different genotypes of the population between generations. However, not all the agencies have the same impact in the population structure. Systematic processes (migration mutation, and selection) in the seed production will be analyzed and their impacts discussed.

Selection as a systematic process during seed production has to be seen as a deteriorative factor in the cultivar already released. However, before presenting the hypotheses, we must define and differentiate three important concepts, artificial selection, natural selection, and varietal depuration.

Basically, artificial selection is the recognition of the target genotypes in a matrix of variable individuals and letting them to increase their descendents in next generations. Thus these genotypes will increase their frequency until achieving a significant change in the population structure (Briggs & Knowles, 1967; Simmonds, 1979; Bos & Caligari, 1995). Natural selection also lets certain genotypes increase in frequency because they have a high fitness. However, the natural forces, such as droughts, diseases, and floods, act on these individuals. Under this scheme of selection many landraces have been improved, achieving high levels of adaptation to variable environments.

In contrast, varietal depuration is an activity that aims to keep a cultivar as genetically pure as possible by removing offtypes plants and seeds from the seed lots. According to Nicaraguan bean seed production normative, NTON-11006 02, Norma Tecnica Obligatoria Nicaraguense para la Produccion de Semilla de Granos Básicos y Soya (MAGFOR, 2002), the varietal depuration is an activity carried out constantly during seed production. Indeed, we can appreciate the difference between these concepts. While selection tends to modify the genetic structure of the population, varietal depuration aims to keep it as original as possible.

We can find out that without a careful plan it is not easy to select when we are supposed to depurate. Desai *et al* (1997) mentioned the technique used by the plant breeder and the selection of the diseases as two agents in the varietal deterioration. However, it is important to remark that more than one person take part in the varietal depuration in Nicaragua. Therefore a good plan and the creation of guidelines easy to follow will facilitate this activity. Indeed, the lacking guidelines for this activity and the absence of a specific office with a duty to organize the varietal depuration suggest that in some cases the cultivar can be improved instead depurated, altering their genotypic proportions. If this selection take part in high hierarchical levels, such as breeder's seed and foundation seed, the impact will be greater in certified seed.

It is possible to considerably change the allele or genotype frequencies in few generations. Delaney & Blis (1991) demonstrated that in three generations of selection the allele frequency of loci linked to *phaseolin* content drastically changed in bean seeds. Therefore we can realize that phenotypes (easy to recognize on fields) can be

selected during seed production and their genotype frequencies change in few generations.

Natural selection can also change the genotype frequencies. Seed production takes place in different contrasting regions with very particular environmental conditions. For example, as the seed lots evaluated in this study (table 1), registered seed initially produced in Carazo and after produced in Jinotega and Matagalpa as certified seed should have faced a change in historical precipitations from about 1,451 mm to 1,206 mm and from 24^{o}C to 20.7^{o}C in temperatures (INETER, 2000). Additionally, many diseases and pests are quite specific in these places. Under those conditions the genotype frequencies can change. The production of seed categories in different regions is a common practice in Nicaraguan system (MAGFOR, 2008). Cregan & Busch (1978) stated that natural selection favored the most drought insensitive wheat genotypes (*Triticum aestuvum* L.), when they were cultivated in drought environment. These contrasting conditions altered the genetic constitution of the populations.

Bean populations can change their genotype frequencies depending on the environmental conditions. These changes have been well documented when the bulk breeding method is analyzed through several F_n generations on bean families bulks (Pirola *et al*., 2002; Silva *et al*., 2004; Rodrigues & Santos, 2006).

Migration is the movement of one individual from one population to another. This individual has to be capable to mate with the individuals present in the population and add new alleles to the genetic structure. In seed technology, migration can be exampled as gene flow among cultivars and accidental seed mixtures that take place during seed production. Depending on the quantity of seed that are mixed, the genotype frequencies can change in the seed categories. Even though, the alien individuals are supposed to be removed from fields, it has been possible to detect them in the seed lots in different species accumulating evidence about a migration of individuals from one cultivar to another during seed production (Schuster *et al*., 2004; Ikeda *et al*., 2007).

Mutations can create new alleles in a population. However, their contribution to changes in genotype frequencies is less significant during seed production and in most cases difficult to estimate (at genotype frequency level). Mutants and recombinant individuals can be identified on bean fields, more when their novel features are easily discernible from the wild types. This phenomenon will be explained in more details in the next part, where it has more implications.

As it was mentioned before, it is difficult to estimate changes in genotype frequencies coming from different agents from one seed generation to another. Nonetheless, these

changes have been reported during plant breeding experiments, suggesting that we must consider how the genetic purity has to be idealized within a cultivar. Perhaps one consideration to be stated is the fact that populations within a bred cultivar are supposed to vary the genetic structure without changing their genetic identity. This variation is a response to environmental differences among the seed production areas, some agronomic practices and finally genetic drift derived from sampling.

5.2 Comparisons between offtype individuals and breeder's seed

Even though all the offtype plant and seeds were phenotypically different to breeder's seed, the molecular results clustered rojo oscuro seeds and the offtype plants as different genotypes from 'INTA ROJO', but not frijol viterra which was the same cultivar (figures 11 and 12). These results suggest that at least the removing of the offtype seeds named frijol rojo oscuro and the offtype plants described on this paper (table 3) contribute to the conservation of the genetic purity of the cultivar. As well, the discarding of seed lots due to frijol viterra presence must be reconsidered carefully. The more important remarks derived from this appreciation are discussed in next sections.

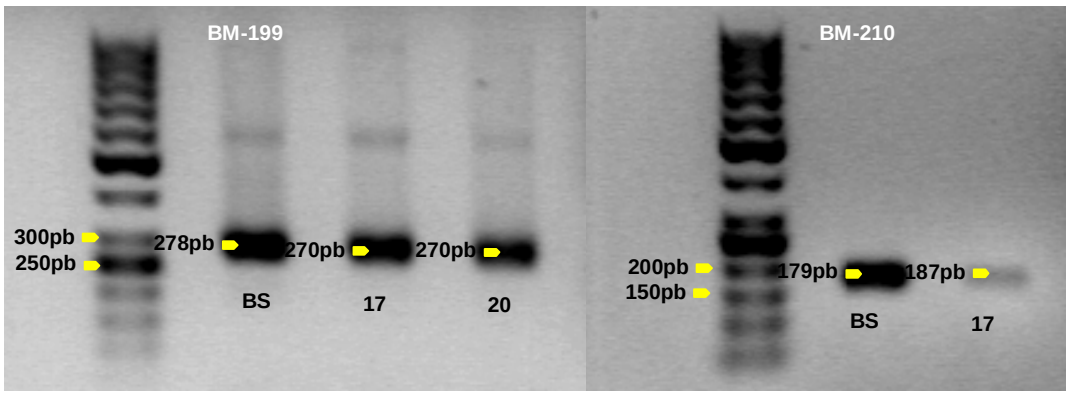

Figure 12. Even thought almost indistinquible at naked eye, differences between breeder's seed (BS) and frijol rojo oscuro (17, 20) at two microsatellites. Agarose gel (1%) ran at 100 V for 45 min.

5.2.1 Non-differentiation of frijol viterra

Frijol viterra was considered offtype because it is bigger than the normal seed and it has light red-colored seeds (table 4). However, frijol viterra was quite similar to breeder's seed when contrasted at ten loci. When Wilhem Johannsen in 1909 stated the pure line theory, he evaluated the selection on different seed weights, obtaining selection gains in each generation, until the pure lines were stable for this trait (Roll-hansen, 1989). However, after this notable discovery many other studies have shown that seed size or seed weight is determined by quantitative gene action, something strongly influenced by the environment and difficult to select in most of the cases (Falconer & Mackay, 1996). Nowadays, we know that during the domestication process two main origin centers gave birth to two kinds of different beans with seeds differing in size, among other features, Andean beans being bigger than Mesoamerican ones. Within these groups, Nicaraguan bean cultivars can exhibit differences in sizes (Carballo & Jenkins, 2002; Marenco & Montserrat, 2003; Suárez & Solis, 2006; MAGFOR, 2004). For this reason, Nicaraguan certification authorities have paid attention to this trait in 'INTA ROJO' and reported the frijol viterra as offtype.

We must consider that even though it is possible to select pure lines for seed weight; the heritability of this trait is strongly influenced by the environment. Therefore, it is difficult to know at least based on weight differences if it is the same cultivar or not, even though we know the expected seed weight for each cultivar. We can state at least under the conditions of the current research that the frijol viterra seeds analyzed have the same genetic identity than 'INTA ROJO' and the differences in weight are likely the response to environmental conditions or agronomic practices.

Bean plants respond to different plant densities. When the densities are higher than recommended, bean plants decrease seed weight during seed filling. Contrary, if plant densities are lower bean plants increase seed weight considerably (Shimada & Arf, 2000; Njoka *et al*., 2005). If we consider that non-uniform plant densities are some of the problems that bean farmers face in Nicaragua (INTA, 2004), we can find seeds than differ in weight (produced from different plants at non-uniform densities on fields).

Indeed, the environmental effects can alter the phenotype of plants and seeds and not all the putative offtypes have to be real offtypes. Schuster *et al*., (2004) after testing eleven soybean [*Glycine max* (L.) Merr.] seed lots labeled as genetically contaminated found that only four were really contaminated with other cultivars. That study

demonstrated the usefulness of the SSRs for detecting seed mixtures when environmental effects difficult a phenotypic test.

5.2.2 Differentiation of frijol rojo oscuro and offtype plants

Rojo oscuro beans and offtype plants showed to be a confirmed offtypes and probably other cultivars distinct to 'INTA ROJO'. The phenotypic analysis showed frijol rojo oscuro was already more contrasting compared with breeder's seed, and it was molecularly confirmed. The microsatellite markers used on this study showed to be useful for identifying bean cultivars, because they found in these offtypes alleles not identified on the array of individuals in 'INTA ROJO' along the four seed categories. Yashitola *et al*., (2002) also reported that in hybrid rice production, it was possible to identify offtypes in the seed lots using one or two very discriminating microsatellite loci.

The study of offtype individuals within a cultivar has relevance from two points of view. First, they represent an undesired variation that affects the certification process when their frequencies are higher than those allowed. Second, some of these offtypes can be new genotypes for a further plant breeding program if their characteristics are (extremely) novel and useful.

There are some examples of cultivars originating from offtypes individuals. The soybean cultivar 'CEA-CH-86' was identified as a single offtype plant in a seed lot in the Brazilian bred cultivar 'CRISTALINA'. After several cycles of selection the new cultivar was successfully released and currently broadly cultivated in Nicaragua (Villalobos & Camacho, 2003). The rusty leaf peanut genetic stock registered by Branch (1999) was originated as an offtype plant found in a foundation seed lot in the cultivar 'VIRGINIA BUNCH 67'. Its name comes from the fact that it has a pale green leaf color with small white speckled areas on the youngest leaves, mimicking rusty plants.

It is difficult to estimate the origin of the offtypes analyzed in this study, because they can come from different sources. Nonetheless, we discovered that they were not formally other cultivars, at least the registered ones in the national varietal register (MAGFOR, 2009). Therefore, even discussed, seed mixtures are not considered as the main candidate source. Their origin can be rooted by mutations or crosses between different cultivars. These possible factors will be examined carefully below.

Accidental seed mixtures are undesirable during seed production, but they arise at some point in the system. These seed mixtures can take place from harvest to the storage, more when the cultivars involved are quite similar in phenotypic features.

Based on our experience, bean plants are harvested as small groups on fields and left to dry on the ground. After plants are dried, they are placed into a bag and hit until the pods deliver the seeds into the bag. The harvester should be careful in placing the right cultivar in the labeled bag, because sometimes more than one cultivar is harvested at the same time during the production of high seed categories. Some seeds can pass from one lot to another one without being noticed at all.

After drying the bean seeds, they are submitted to a manual selection whose objective is to remove inert matter, damaged seeds, small seeds and offtypes seeds. During this activity field personnel screen the whole seed lot looking for impurities. All putative offtype seed has be removed from the lot. Even though some offtype seeds escape, they can be roughed out in the next seed generation from the fields as offtype plants.

According to MAGFOR (2002), during all these activities one seed inspector from MAGFOR has to supervise the proper following of the guidelines, guaranteeing an acceptable genetic purity. However, it is not always possible to inspect the bean fields during all the stages, because lacking funds and scarce personnel. Therefore, many of the tasks rest on INTA-CNIAB which manages the seed increasing at highest levels in the seed production system (figure 2). Additionally, the seed increasing is not always carried out under the supervision of the same personnel and in the same geographical area.

Despite this situation, a proper manual screening of the seeds is a key stage that must give confidence about the purity of the materials. Otherwise, the effect of divergent criteria or lacking supervision increases the number of offtype plants and seeds into the populations. The level of accidental seed mixtures is difficult to estimate accurately because it implies human errors at bean seed processing something that varies depending on the planning and farming conditions.

Common bean is a self-pollinated species which out-crossing rate is thought to be very low, because bean floral morphology in which stamens are quite close to the stigmas and fertilize them before the floral buds open (Webster *et al*, 1977; Adams *et al*, 1985). Nevertheless, Webster *et al* (1977) stated that one different pistil which stigma remains receptive after the floral bud opens is able to cross pollinate in presence of insects. Unfortunately, information about this variation in Nicaraguan bean cultivars is not available to date.

Bean seed production has to be carried out on isolated fields. MAGFOR (2002) states that the distance between two seed lots from different cultivars must be five meters. However, many studies have shown that this out-crossing rate is higher (from 0.71 to 39.3%) and it depends on the environmental conditions and the cultivar (Wells *et al*., 1988; Brunner & Beaver, 1989; Ibarra *et al*., 1996; Ibarra *et al*., 1997; Royer *et al*., 2002; Hoc *et al*., 2006; Ferreira *et al*., 2007). It is difficult to estimate a value because it can change drastically from one region to another. Ferreira *et al*., (2000) found no hybrid individuals in their study remarking that at least at Austurias conditions (Northern Spain) common bean out-crossing was about 0.74%.

In consequence, taking into account the flowering correspondence among bean cultivars in Nicaragua: 'INTA ROJO', 36-38 days; 'INTA CARDENAS', 35-40 days; 'INTA NUEVA GUINEA', 34-36 days; INTA MASATEPE', 32-34 days; and 'INTA CANELA', 36-38 days (PROMESA, 2002; Rosas *et al*., 2004), it is possible that a higher out-crossing rate in some regions is contributing to genetic segregations among bean cultivars, producing these offtypes difficult to identify as registered cultivars. Unfortunately, the out-crossing rate for Nicaraguan conditions remains unknown.

Spontaneous mutations arise at very low rates in most organisms (10^{-5} or 10^{-6} per generation in most loci). That means about one in 100,000 or one in 1,000,000 gametes (Falconer & Mackay, 1996; Acquaah, 2007). Even though natural mutation rates are very low in plants, they have been an important agent for the occurrence of offtype plants on bean fields. Additionally, these mutations have helped to understand the gene action related to seed traits such as color, size and shapes.

McClean *et al*., (2002) constructed a gene map for the genes that interacts in seed color and color patterns. They described one single locus that determines the presence of color in the seed coat (*P_*) or the absence (*pp*), three possible genotypes for flower colors: purple (*V_*), pink (V^{lae}) and white (*vv*), and about nine genes interacting for different coat patterns (*Gy, C, Z, R, J, G, B, Rk, T, L, Bip*). After that Bassett & Miklas (2007) proposed the gene *bic* with pleiotropic relation between flower and seed coat

colors. Recently, the recesive allele *j* was ascribed to the postharvest darkening in seeds (Junk-Knievel *et al*., 2008). Some mutations have risen in these genes showing strange seed coat patterns never seen before. Ernest & Kelly (2005) found in a bean foundation seed strange offtype seeds with seed patterns suggesting a recessive mutation at *T* locus. This mutation conferred a self colored seed coat and masking the expression of the gene *z* and others. We can find out the usefulness and importance of these mutations for different applications.

Frijol rojo oscuro can result from crossing among cultivars, spontaneous mutations in the genes above described, or both. These offtypes appear in 'INTA ROJO' seed lots often. As well, the occurrence of violet flowers (instead white) in the same 'INTA ROJO' seed lots suggests that they could be related in some way (Aurelio Llano, personal communication). Unfortunately, during our field sampling no violet-flowered plants were detected. However, the field personnel were willing to mark those plants and harvest the seeds (separated) for further analysis.

In the same way, Ikeda *et al.*, (2007) carried out a study where different rice offtypes were analyzed into NERICA varieties. They suggested that the main offtype sources were: mechanical seed mixtures, segregations, out-crossing among cultivars, and natural mutations. These results and conclusions are in concordance with the findings and hypothesis presented on this paper.

Additionally, many seed farmers have expressed (unpublished information) the presence of other offtypes with attractive features, such as higher number of pods per plant, resistance to environmental stresses, and high yield in the Northern Nicaragua. These occurrences must be studied carefully because they can represent new genotypes highly adapted to specific conditions.

6 CONCLUSIONS

The changes detected in the genotype frequencies of the microsatellite loci showed that the genetic composition of the common bean is affected during seed production. These changes are associated to selection processes imposed during the seed certification and varietal depuration, and the changes in the environmental conditions when the seed production is moved from one region to another. These results and the available literature suggest that we must reconsider this variation when evaluating the genetic purity in a seed lot, taking into account the contrasting environments where seed production is carried out.

Frijol rojo oscuro seeds were really another unknown cultivar mixed with 'INTA ROJO'. The origin of these seeds seems to be rooted in mutations or natural segregations more than in seed mixtures. Frijol viterra was the same 'INTA ROJO' genotype. Therefore, the phenotype differences were associated to environmental effects on the seed weights.

7 FUTURE PERSPECTIVES

Bean seed production is a very important activity in Nicaraguan agriculture. Many of the current efforts point to improve the quality of the seeds that are distributed to the bean farmers. To improve genetic quality imposes several challenges. The lacking information and the scarce use of biotechnological tools make this activity more difficult. The study of the genetic quality of the cultivar 'INTA ROJO' was the start point of this improvement.

National regulations must be reconsidered taking into account the real conditions where the seed production is carried out and the challenges and changes that global warming represents. In addition, it is necessary to create reliable varietal depuration protocols adjusted to the nature of the cultivars that are multiplied in the national seed system. The methods described in this paper can be integrated to the seed quality control in order to quantify the genetic purity of the cultivars, at least at higher level in the seed categories, to avoid wrong estimations derived from phenotypic methods.

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10 APPENDICES

Table 1A. Microsatellites analyzed in this study.

Locus	LG	Size	Motif	Repeat N°.	Primer Sequence_FORWARD	Primer Sequence_REVERSE
BM053	b01	287	CT	21	AACTAACCTCATACGACATGAAA	AATGCTTGCACTAGGGAGTT
BM143	b02	143	GA	35	GGGAAATGAACAGAGGAAA	ATGTTGGGAACTTTTAGTGTG
BM172	b03	107	GA	23	CTGTAGCTCAAACAGGGCACT	GCAATACCGCCATGAGAGAT
BM199	b04	304	GA	15	AAGGAGAATCAGAGAAGCCAAAAG	TGAGGAATGGATGTAGCTCAGG
BM175	b05	170	GA	19	CAACAGTTAAAGGTCGTCAAATT	CCACTCTTAGCATCAACTGGA
BM137	b06	155	CT	33	CCGTATCCGAGCACCGTAAC	CGCTTACTCACTGTACGCACG
BM210	b07	166	CT	15	ACCACTGCAATCCTCATCTTTG	CCCTCATCCTCCATTCTTATCG
BM189	b08	114	CT	13	CTCCCACTCTCACCCTCACT	GCGCCAAGTGAAACTAAGTAGA
BM188	b09	177	CA	18	TCGCCTTGAAACTTCTTGTATC	CCCTTCCAGTTAAATCAGTCG
BM212	b10	214	CA	13	AGGAAGGGATCCAAAGTCACTC	TGAACTTTCAGGTATTGATGAATGAAG
BM184	b11	160	AC	11	AGTGCTCTATCAAGATGTGTG	ACATAATCAATGGGTCACTG
GATS091	b02	229	GA	17	GAGTGCGGAAGCGAGTAGAG	TCCGTGTTCCTCTGTCTGTG

LG=Linkage group. (Gaitán *et al.,* 2002; Blair *et al*., 2003)

Table 2A. Comparison between breeder's seed and foundation seed at genotype frequency level

Locus	Breeder's seed						Foundation seed						P value
	Genotype frequencies						Genotype frequencies						
	A_1A_1	A_1A_2	A_1A_5	A_2A_2	A_3A_3	A_4A_4	A_1A_1	A_1A_2	A_1A_5	A_2A_2	A_3A_3	A_4A_4	
BM-143	39	0	0	1	0	0	40	0	0	0	0	0	1.0000
BM-172	26	0	0	14	0	0	31	0	0	9	0	0	0.3232
BM-199	33	0	0	6	1	0	35	0	0	4	1	0	0.8661
BM-175	40	0	0	0	0	0	40	0	0	0	0	0	1.0000
BM-137	22	0	0	18	0	0	24	0	0	16	0	0	0.8212
BM-210	40	0	0	0	0	0	40	0	0	0	0	0	1.0000
BM-189	39	0	0	1	0	0	37	0	0	3	0	0	0.6153
BM-188	40	0	0	0	0	0	40	0	0	0	0	0	1.0000
BM-184	40	0	0	0	0	0	40	0	0	0	0	0	1.0000
GATS091	1	2	1	35	1	0	0	0	0	24	0	16	0.0000*

*= Statistically significant values from Fisher's exact test.

Table 3A. Comparison between breeder's seed and registered seed at genotype frequency level

Locus	Breeder's seed							Registered seed							P value
	Genotype frequencies							Genotype frequencies							
	A_1A_1	A_1A_2	A_1A_5	A_2A_2	A_3A_3	A_4A_4	A_5A_5	A_1A_1	A_1A_2	A_1A_5	A_2A_2	A_3A_3	A_4A_4	A_5A_5	
BM-143	39	0	0	1	0	0	0	40	0	0	0	0	0	0	1.0000
BM-172	26	0	0	14	0	0	0	34	0	0	6	0	0	0	0.0691
BM-199	33	0	0	6	1	0	0	32	0	0	4	0	4	0	0.1470
BM-175	40	0	0	0	0	0	0	36	0	0	4	0	0	0	0.1155
BM-137	22	0	0	18	0	0	0	14	0	0	26	0	0	0	0.1151
BM-210	40	0	0	0	0	0	0	36	0	0	1	3	0	0	0.1155
BM-189	39	0	0	1	0	0	0	37	3	0	0	0	0	0	0.2405
BM-188	40	0	0	0	0	0	0	40	0	0	0	0	0	0	1.0000
BM-184	40	0	0	0	0	0	0	39	0	0	1	0	0	0	1.0000
GATS091	1	2	1	35	1	0	0	6	0	0	27	0	5	2	0.0018*

*= Statistically significant values from Fisher's exact test.

Table 4A. Comparison between breeder's seed and certified seed at genotype frequency level

Locus	Breeder's seed						Certified seed						P value
	Genotype frequencies						Genotype frequencies						
	A_1A_1	A_1A_2	A_1A_4	A_1A_5	A_2A_2	A_3A_3	A_1A_1	A_1A_2	A_1A_4	A_1A_5	A_2A_2	A_3A_3	
BM-143	39	0	0	0	1	0	40	0	0	0	0	0	1.0000
BM-172	26	0	0	0	14	0	38	0	0	0	2	0	0.0015*
BM-199	33	0	0	0	6	1	34	0	0	0	5	1	1.0000
BM-175	40	0	0	0	0	0	40	0	0	0	0	0	1.0000
BM-137	22	0	0	0	18	0	3	0	0	0	37	0	0.0000*
BM-210	40	0	0	0	0	0	39	0	1	0	0	0	1.0000
BM-189	39	0	0	0	1	0	39	1	0	0	0	0	1.0000
BM-188	40	0	0	0	0	0	40	0	0	0	0	0	1.0000
BM-184	40	0	0	0	0	0	40	0	0	0	0	0	1.0000
GATS091	1	2	0	1	35	1	0	0	0	0	40	0	0.0000*

*= Statistically significant values from Fisher's exact test.

Table 5A. Fragment sizes in basepairs (bp) of the different alleles found at ten microsatellite loci in four seed categories.

Allele	Locus									
	BM-143	BM-172	BM-199	BM-175	BM-137	BM-210	BM-189	BM-188	BM-184	GATS091
	Fragment size (bp)									
A_1	153	76	278	186	100	179	104	150	159	252
A_2	155	78	280	158	98	187	102	-	-	254
A_3	-	-	270	-	-	177	-	-	-	258
A_4	-	-	-	-	-	267	-	-	-	250
A_5	-	-	-	-	-	-	-	-	-	256

Table 6A. Fragment sizes in basepairs (bp) of the different alleles found at ten microsatellite loci in offtype individuals.

Allele	Locus									
	BM-143	BM-172	BM-199	BM-175	BM-137	BM-210	BM-189	BM-188	BM-184	GATS091
	Fragment size (bp)									
A_1	153	76	278	186	100	179	104	150	159	252
A_2	155	78	280	158	98	187	102	380*	-	254
A_3	-	-	270	160*	88*	177	-	-	-	258
A_4	-	-	-	-	-	267	-	-	-	250
A_5	-	-	-	-	-	-	-	-	-	256
A_6	-	-	-	-	-	-	-	-	-	246*

*****= New alleles not found in the four seed categories.