

**MOLECULAR CHARACTERIZATION OF RICE VARIETIES (ORIZA SATIVA L.) IN THE DOMINICAN REPUBLIC BY MEANS OF SNP USING ALLELE-SPECIFIC PRIMERS**

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**ABSTRACT**

This project aimed to characterize the twenty most commercially cultivated rice varieties in the Dominican Republic. These varieties are as follows: PROSEQUISA 4; PROSEQUISA 5; PROSEQUISA 10; PROSEQUISA 15; Jaraguá; ISA 40; Juma-57; Juma - 67; Idiaf I; Idiaf II; Idiaf III; Emeraldá; Cristal 100; Yokahu; Puita; Ambar; Palmar 18; Pava Shienn; Quisqueya; and Tempique, which, according to data obtained from Rice Development, Ministry of Agriculture of the Dominican Republic, have a considerable planting area in the country. The methodology that we used for characterizing or "fingerprinting" of these varieties is Single Nucleotide Polymorphism or SNP. These markers are the most abundant and are distributed throughout the genome. For the determination of SNPs, we used the primers developed by different authors we used the protocols they suggested to run the PCR reaction and run 5 µl the product on 2% agarose and compared for each variety. We found differences among some varieties that could be used to part difference in any legal dispute.

**Keywords:** SNP, Rice, Molecular Characterization, Apparent Amylose Content, Grain Size, Rice Yield Rice Resistance.

**1. INTRODUCTION**

*Oryza sativa*, commonly known as Asian rice, contains two main subspecies: the sticky, short-grained japonica or sinica variety and the long-grain indica variety, not sticky. Japonica varieties are generally grown in dry fields, in the temperate zones of East Asia, in the highlands of Southeast Asia and in high elevations of South Asia, while indica varieties are rice mainly from the lowlands, mostly cultivated submerged, throughout tropical Asia.

A third subspecies, which is broad-grained and thrives under tropical conditions, was identified based on morphology, and was initially called javanica but is now known as tropical japonica. Examples of this variety include the cultivars 'Tinawon' and 'Unoy', which are grown on the high-elevation rice terraces of the mountains of the Cordillera of northern Luzon, Philippines (CECAP, PhilRice and IIRR. 2000).

Rice is an annual grass belonging to the genus *Oryza*, native to southern India, where there are very favorable conditions for cultivation (González, 1985 cited by César Moquete. 2010). In this region of India there are many wild species related to cultivation in the riparian areas and the banks of the rivers, where rice was mainly collected (Lu que, 2009 cited by César Moquete. 2010). However, its cultivation began in China, in the fertile valleys of the Rivers Hang-Ho and Yang-

Tse-Kiang, around the fifteenth century BC. Today it is known, thanks to the result of carbon 14 analyses, carried out on rice grains discovered in excavations located in Pengtoushan, that rice was cultivated from the years 8200 - 7800 BC.C. in Hunan (SOS Rice, in the 2009 network cited by César Moquete. 2010).

### **1.1 Molecular Markers**

The analysis of genetic diversity and the relationship between and within different populations, species and individuals is a basic action for many disciplines of the biological sciences (Weising et al. 2005). Over the past three decades, classical strategies for the evaluation of genetic variability, such as comparative anatomy, morphology, embryology, and physiology have been greatly complemented by molecular techniques. These techniques include, for example, the analysis of chemical components (called metabolomics), but more importantly those related to the development of molecular markers. Marker technology based on protein or DNA polymorphisms has catalyzed research in a range of disciplines such as phylogeny, taxonomy, ecology, genetics, and genetic improvement of plants and animals (Weising et al., 2005).

No marker meets all these criteria. However, one can choose from a variety of marker systems, each of which combines some or many of the features mentioned above. For a detailed description of each of the markers consult (Weising et al., 2005).

Normally, the main genetic tools used for the identification and improvement of crops of domesticated species are morphological markers and molecular markers (Tanksley, 1993, cited by Arias et al., 2012). Markers in tropical fruit trees take years to be useful because they cannot be used until the tree exceeds the juvenile stage, in addition to being subject to epigenetic and environmental factors (Arias et al., 2012). Molecular markers, on the other hand, can be used from any tissue and at any time during the growth of the tree, accelerating the process of varietal identification and improvement and thus avoiding the limitations of traditional methods (Azofeifa-Delgado, 2006)

What are genetic markers? These are any monogenic trait (of a single gene) that can be easily analyzed. Genetic markers have been widely used to assist in the management and improvement of agricultural crops. The first source of markers were morphological variants, which have simple Mendelian patterns of inheritance. In the early 20th century, the transmission of morphological markers was used to monitor seed purity and to establish degree of kinship in particular crosses. Unfortunately, these markers have several significant disadvantages. First, very few are available for breeding most crops. Second, many confer some phenotypic disadvantages and their transmission in cultivars may be unwanted. Finally, most are recessive, which greatly limits their usefulness (Clegg et al., 1999).

Genetic markers, heritable entities that are associated with characters of economic importance, have been used in the selection and evaluation of gene banks for different crops (Staub et al., 1982; Darvasi and Soller, 1994). Studies of isoenzymatic polymorphism in germplasm collections have been applied for the knowledge and maintenance of the genetic diversity present in the collections and in the characterization of potentially marker loci, which can be used within genetic improvement programs (Shands, 1990; Arias, 1998). DNA markers are fundamentally

based on the analysis of differences in small DNA sequences between individuals (Asofeifa\_Delgado,2006).

### **1.2 Single nucleotide polymorphism (SNP)**

A single nucleotide polymorphism (SNP, pronounced snip) is a variation in the DNA sequence that affects a single base (adenine (A), thymine (T), cytosine (C), or guanine (G)) of a genome sequence. However, some authors consider that changes of a few nucleotides, as well as small insertions and deletions (indels) can be considered as SNP, being then more appropriate the term Single Nucleotide Polymorphism. (Li et al., 2014). One of these variations must occur in at least 1% of the population to be considered as an SNP. If it does not reach 1%, it is not considered SNP and a point mutation is considered.

SNPs that are located within a coding sequence may or may not modify the chain of amino acids they produced; the former are called non-synonymous SNPs and synonyms (or silent mutations) SNPs are called synonyms (or silent mutations) the latter. SNPs that are in non-coding regions can have consequences on the translation process, especially in processes such as splicing, the union of transcription factors or modifying the sequence of non-coding RNA. In any case, SNPs that alter gene expression in some ways are called SNPs (expression SNPs) and can be found both upstream and downstream of the coding sequence. On the other hand, although they can be both in coding regions and in intronic or intergenic regions, the SNPs that affect the coding regions are the ones that have the most impact on the function of a protein a (although they may not alter the amino acid sequence as a result of the degeneration of the genetic code). On the other hand, any type of SNP can be related to a disease or have an associated observable phenotype, so that:

- Certain SNPs in the non-coding regions of some genes correlate with an increased likelihood of developing cancer (Kimchi-Sarfaty et al., 2007).
- Some SNPs in coding regions consisting of synonymous substitutions, although they do not modify the amino acid sequence of the protein, could alter its function. This occurs, for example, in the case of multiple drug resistance receptor 1 (MDR1), where a silent mutation SNP slows down the translation of the nascent peptide, causing it to fold adopting an alternative conformation less functional than the native three-dimensional structure. Al-Haggar et al., 2012).
- SNPs involving substitution with change of direction alter the amino acid sequence and are the most frequently associated with the occurrence of diseases. An example of this is the SNP 1580G>T in the LMNA gene, which causes the change of arginine for leucine in the protein, a phenotype related to diseases such as progeria or mandibulofacial dysplasia (Cordovado et al., 2012).
- Nonsense SNPs cause the appearance of a premature stop codon that truncates the resulting protein, making it incomplete and normally non-functional. This is reflected in the G542X mutation in the CTFR gene, which causes cystic fibrosis (Altshuler et al., 2000).

Because SNPs do not change much from one generation to the next (they are inherited very stably), it is easy to follow their evolution in population studies. They are also used in some types of genetic tests and their study is very useful for agricultural research in the development of "fingerprinting" of new varieties. SNPs are considered a form of point mutation that has been evolutionarily successful enough to be fixed in a significant part of a species population and there are SNP markers that detect the change of that single nucleotide.

Exonic SNPs that do not change the amino acid composition of the encoded domain or protein are called SNP synonym (synSNP), while non-synonymous SNP (nsSNP) will change the encoded amino acid. Therefore, nsSNPs can cause the synthesis of a non-functional protein and have an effect on the phenotype. Such SNPs called diagnostics may be associated with certain diseases in humans and with certain agronomic characteristics of plants. For example, a G/T polymorphism was found at the leading site of the 5' splice intron of the waxy gene to control the cooking quality of rice, (Bormans et al., 2002) and two non-synonymous exonic SNPs in the same gene were associated with characteristics of amylase content and viscosity (Larkin and Park, 2003). The detection of diagnostic SNPs is an important goal of many SNP discovery projects.

### **1.3 SNPs in Plants**

In plants, SNP research is still in progress, and SNPs have been rigorously sought in only a few species. These include several important crops such as barley, (Kanazin et al., 2002; Soleimani et al., 2003) rice (Bormans et al., 2002; Hayashi et al., 2004; Larkin and Park, 2003; Nasu et al., 2002) · wheat (Somers et al., 2003; Zhang et al., 2003) maize (Batley et al., 2003; Teneillon et al., 2001) and sugar beet, (Schneider et al., 2001), but also the model plant *Arabidopsis thaliana* (Cho et al., 1999; Drenkard et al., 2000). With about an SNP per 200 to 500 bp, the average density of SNP in plant genomes appears to be relatively high but depends on the species investigated. In maize, analysis of several hundred loci in eight inbred maize lines revealed a very high prevalence of SNPs (one SNP per 83 bp), probably a consequence of free pollination in this species (Bhatramakki et al., 2002). Flanking sequences of microsatellites in maize contain even an SNP per 40 bp, making the estimated total number of SNPs per whole genome at 62 million (Edwards and Mogg, 2001).

In recent years, single nucleotide polymorphisms (SNPs) have become the most popular system of genetic markers in animals and plants. Their extraordinary abundance discovered in several genome sequencing ([http://www.ncbi.nlm.nih.gov/projects/SNP/snp\\_summary.cgi](http://www.ncbi.nlm.nih.gov/projects/SNP/snp_summary.cgi)) projects, combined with recent technological innovations, makes SNP markers very attractive for high-throughput use in marker-assisted selection, EST mapping, and the integration of genetic and physical maps.

Unlike first-generation molecular markers, such as RFLPs (restriction fragment length polymorphisms) and RAPDs (Randomly Amplified Polymorphic DNAs), SNPs can be detected by high-throughput non-gel-based assays, saving time and money (Gupta et al., 2001). Several technologies have been developed for SNP analysis based on various methods of allelic detection and discrimination platforms. Allele-specific hybridization, primer extension, oligonucleotide ligation and invasive pepper from represent four main allelic discrimination reactions that can be

joined with various detection methods, such as fluorescence measurements, luminescence, and mass spectrometry (Massimo et al., 2008). Recently, significant efforts have been attempted towards the characterization of large-scale SNPs in animals and plants with Technology from BeadArray (Illumina [Shen et al., 2005]) and the SNPlex system™ Genotyping (Applied Biosystems Inc., ABI [Tobler et al., 2002]). The selection of an appropriate genotyping method depends on many factors including cost, multiplexing potential and performance, equipment and difficulty of developing assays.

Single nucleotide polymorphisms (SNPs) and insertions/deletions (INDELs) are the most abundant types of DNA sequence polymorphisms and can theoretically be found within each genomic sequence (Rafalsky, 2002a; Rafalsky, 2002b). They can be used as genetic markers for many genetic applications such as crop identification, genetic mapping, genetic diversity assessment, detection of genotype/phenotype associations or marker-assisted selection. (Flint et al., 2005). In addition, the development of high-throughput genotyping methods makes single nucleotide polymorphisms (SNPs) highly attractive as genetic markers (De la Vega et al., 2005).

#### **1.4 SNP Detection**

Public access to the genome sequences of different organisms has allowed the study of sequence variations between individuals, cultivars, and subspecies. These studies reveal that single nucleotide polymorphisms (SNPs) and insertions and deletions (InDels) are very abundant and distributed throughout the genome of several species, including plants (Garg et al., 1999; Drenkard et al., 2000; Nasu et al., 2002; Batley et al., 2003). Comparing the sequences of a japonica rice cultivar with those of an indica variety, e.g. Yu et al. (2002) identified, on average, an SNP every 170 bp and an InDel every 540 bp. The abundance of these polymorphisms in plant genomes makes the SNP marker system an attractive tool for mapping, marker-assisted selection, and mapping-based cloning (Gupta et al., 2001; Rafalski, 2002a; Batley et al., 2003). As suggested by the acronym, an SNP marker is only an individual base change in a DNA sequence, with a usual alternative of two possible nucleotides at a certain position. Therefore, in contrast to all previous methods, allelic discrimination cannot be based on size differences in a gel.

In recent years, a large number of different methods of Genotyping of SNPs and chemicals have been developed based on various methods of allelic discrimination and detection platforms (Rafalski, 2002b; Vignal et al., 2002; Sobrino et al., 2005; Tost and Gut, 2005 for a detailed review). All methods for SNP genotyping combine two elements: firstly, the generation of a specific allele product and secondly the analysis of these. Vignal et al (2002) classify the various methods of SNP detection into two large groups:

1) Direct hybridization techniques.

2) those techniques involving the generation and separation of a specific allele product (restriction enzyme cutting, simple and hetero-duplex DNA strand conformation, primer extension, oligonucleotide ligation test, pyrosequencing, exonuclease or Taq-Man detection, invasive cutting of oligonucleotide probes or invasive assay).

Single nucleotide polymorphisms can be detected by DNA sequencing, a common technique that usually shows high levels of repeatability over time and reproducibility between laboratories.

However, the detection of specific SNPs can be performed by various techniques, many of which are not yet routine. By their nature, in diploid plants, SNPs only have two allelic states, although in polyploids this may vary by dosage effect. The simple structure of SNPs makes their assessment relatively simple and reliable. It also means that, in order to define the profile of a given genotype effectively and efficiently, it will be necessary to analyze a large number of markers, either individually or by combination of several markers. ([http://www.upov.int/edocs/infdocs/es/upov\\_inf\\_17\\_1.pdf](http://www.upov.int/edocs/infdocs/es/upov_inf_17_1.pdf)).

The varieties grown in the Dominican Republic have been mostly obtained in the country and very few (Yokahu, Puita, Tempique, etc.) have a foreign origin. These varieties have been characterized based on their morphology and culinary characteristics, so their molecular characterization ("Fingerprinting") is necessary to protect their identity and the breeder property for the owners of these varieties in case of legal conflicts.

} The main objective of this research work was the molecular characterization or "fingerprinting" of the twenty (20) varieties of rice shown in the previous table using the SNP technique. In the Dominican Republic, there are no studies concerning the classification or characterization by any method of commercially grown rice varieties. At IIBI an attempt was made to characterize about five varieties grown in the Cibao region using the microsatellite technique, but this work did not produce conclusive data. The SNP technique has not been worked on in the Dominican Republic so this will be a good opportunity to train Creole personnel in this one.

## **2. MATERIALS AND METHOS**

The works that involve the development of the first objective of this project, contemplate the execution and regular fulfillment of the following activities:

- a) Collection of biological material under study.
- b) Extraction, qualification, and quantification of DNA.
- c) Application of the Molecular Characterization Technique (SNPs).
- d) Analysis, comparison and determination of genetic distances and similarity index from the patterns obtained with the molecular technique used.

### **2.1 Sample Collection**

The samples for this study were collected at the Rice Experimental Station located in Juma, Bonaó, Monseñor Nouel Province. The seeds collected at the Experimental Station were sown in pots with sterilized soil under shade from where the leaves were collected for DNA extraction.

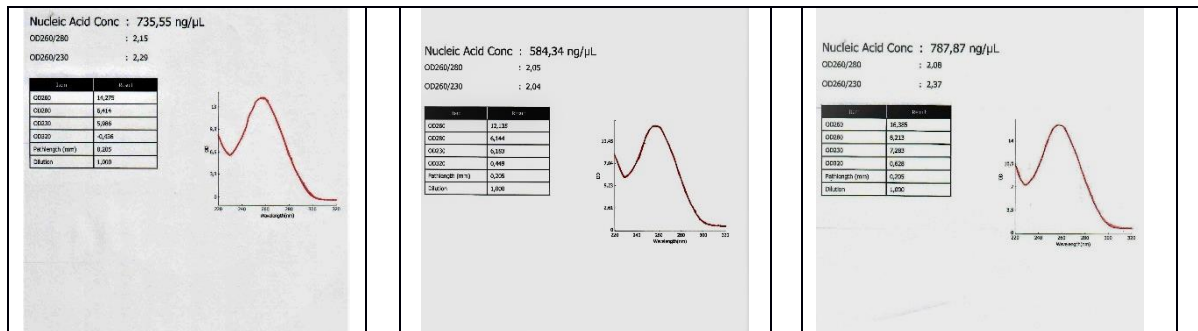
### **2.2 DNA extraction**

#### **Plant DNA Extraction Protocol Using CTAB**

Adapted from: Daniel L. Nickren: *Molecular Methods in Plant Biology*, Second Edition, 2006. Department of Plant Biology Southern Illinois University.



DNA extraction was done by the CTAB method commonly used at CEBIVE. Many of the extractions were of very good quantity and quality. In the following figure you can see in Figure, 1 the quantity and quality of three varieties whose DNA was extracted by the CTAB method. And quantified with a Shimadzu **BioSpec-nano** Spectrophotometer, Shimadzu Corporation, Kyoto Japan



**Figure 1.** Three examples of DNA samples extracted from varieties 20, 19 and 17. Pictures generated with Shimadzu Spectrophotometer BioSpec-nano. Shimadzu Corporation, Kyoto Japan.

### 2.3 SNP Techniques

The methodology used in the determination of SNPs was described by each of the authors who developed the different SNPs.

### 2.4 Primers

The primers used were those specified for each of the genes/alleles that were analyzed. These genes should be common to all varieties.

E.g., disease resistance/susceptibility genes, rice fragrance genes; starch content genes; etc.

## 3. RESULTS AND DISCUSSION

### 3.1 Fragrance

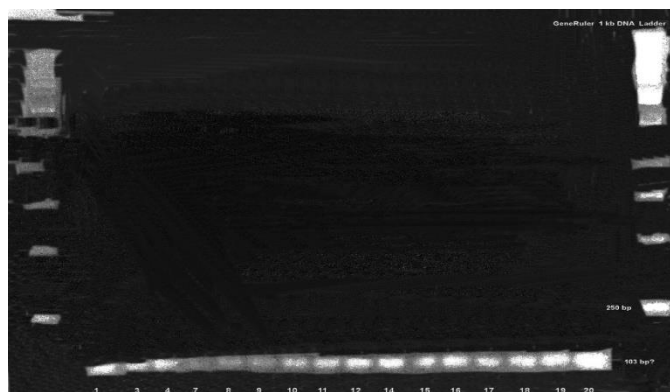
One of the most important characteristics related to the quality of rice is the fragrance (Asadollah et al, (2010). The fragrance of cooked rice is composed of more than one hundred volatile components such as alcohols, aldehydes, ketones, acids, esters, phenols, and others (Yajima et al. (1978); Maga (1984); Takashi et al. (1980); Paule and Powers (1989), cited by Asadollah et al. (2010).

The aromatic trait of rice has previously been mapped on chromosome 8 by both qualitative and quantitative methods (Lorieux et al. (1996)).

### 3.2 PCR and electrophoresis condition

The polymerase chain reaction was performed in a volume of 45 µl containing 2.25 µM/l from each primer. The master mix was prepared according to the volume to be used. The pair of primers used called Arm1 were **F: 5'-TCCTCTCAATTACATGGTTTATTG-3'**; **A: 5'-TTGGAAACAAACCTTAACCATAG-3'**. The reaction conditions for CRP were as follows; 94°C for 5 minutes, followed by 35 cycles of 94°C for 1 minute, 55 ° C for 1 minute 72° C for 2 minutes and finally 72 ° C for 7 minutes in the final extension(Asadollah et al. (2010).

Electrophoresis was run in 2% horizontal agarose gelatin and stained with ethidium bromide 1mg/ml. The running time varied depending on the electrophoresis chamber.



**Figure 2.** PCR band patterns generated by arm1 primer in 2.0% agarose gelatin

List of varieties analyzed.

Variety 1 (Aceituno) was a substitute of Ambar because it did not germinate at all.

1=Aceituno 12 = Pava Shien

3 = Cristal 100 14 = PROSEQUISA 5

4 = IDIAF II 15 = PROSEQUISA 10

7 = Jaragua 16 = PROSEQUISA 15

8 = Juma 57 17 = Quisqueya

9 = Juma 67 18 = Puita

10 = Juma 68 19 = Tempique

11 = Palmar 18 20 = Trabuco

The alleles produced by this primer for the fragrance trait were around 103bp. Varieties 7, 8 and 9 (Jaragua, Juma 57 and Juma 67) showed little fragrance compared to the rest of the varieties analyzed.

### 3.4 Starch content

The apparent amylose content (AAC) is a key factor indetermining the organoleptic and culinary quality of rice (*Oryzasativa*). The AACes are mostly controlled by the Wx gene encoding a granule-bound starch synthase (GBSS).



A single nucleotide changes from G to T at position 144 of the *Wx* gene (the +1 position of intron 1) has been reported to be strongly associated with amylose content (Ayres et al. (1997) cited by Popluechai et al. (2012).

To determine the SNPs of the *Wx* gene, two primers were used (*Waxy In1*: F: 5'-ACCATTCCTTCAGTTCTTTGTCT-3'; R: 5- TAGCATGTATGAGACTACTTGTA-3': and the specific allele primer *Ex6*: F: 5'- CCC ATA CTT CAA AGGAAC ATA-3': R: - GGT TGG AAG CAT CAC GAG TT-3').

The conditions for the PCR run for this pair of primers were as follows: the reaction was run in a volume of 45 µl with 2.25 µM/l of each primer. The master mix was prepared according to the volume to be used. The PCR amplification reaction was carried out in a thermocycler brand Applied Biosystems Model ProFlex PCR System.

The running conditions were 94° C for 5 minutes, followed by 30 cycles of 94° C 45 seconds, 58° C 45 seconds and 72° C for 30 seconds and a final cycle of 72° C for 7 minutes. The PCR product was run in a 2.0% agarose gelatin and stained with ethidium bromide 1 mg/ml.

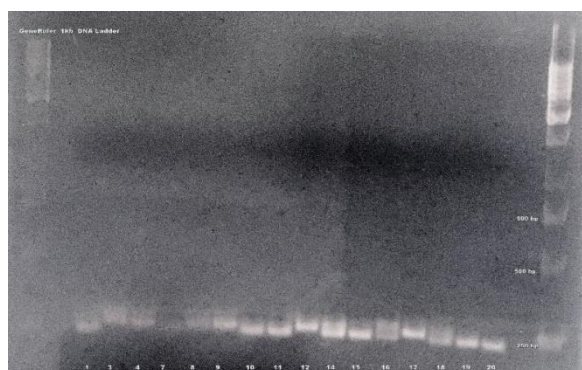


Figure 3a. Pattern of primer bands *Waxy In1*

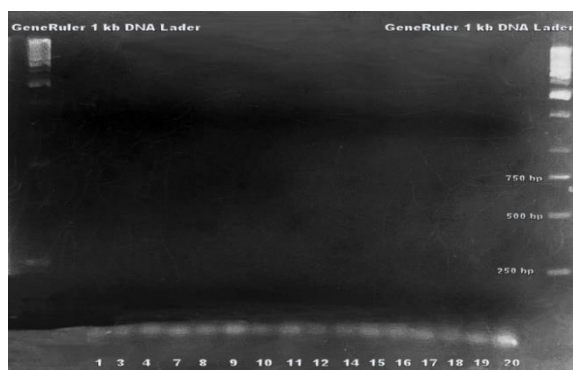


Figure 3b. Pattern of primer bands *Ex6*

The PCR product amplified with *waxy-F* and *Waxy-R* primers showed bands around 250 kb. About 50% of the varieties analyzed showed a low amylose content. These varieties were 1, 3, 4, 7, 8, 16, and 18 (Aceituno, Crystal, IDIAF II, Jaragua, Juma 57, PROSEQUISA 15, and Puita). The apparent amylose content, analyzed with the *Ex6* primer showed a low amylose content in all the varieties analyzed. The only varieties that could be differentiated from the rest were the Juma 67 and the Trabuco that showed more notable bands in the electrophoresis run.

The apparent amylose content (AAC) is controlled by the *Waxy* gene which regulates the synthesis of amylose in the developing grain which is regulated by the enzyme Granule-Bound Starch I Synthase (GBSSI) (Biselli et al. 2014). The apparent amylose content (AAC) is the primary key to rice's culinary qualities, according to Biselli et al. (2014). According to these authors, varieties with a high amylose content are cooked loose with grains (grained), while varieties with a low amylose content are cooked pasty.

Figure 3b (*Ex6*) shows the s-band patterns for this marker which revealed a large presence for

varieties 9 and 20 (Juma 67 and Trabuco). Other varieties were less prominent, such as 7, 11, 15, 16, 17 and 19 (Jaragua, Palmar 18, PROSEQUISA 10, PROSEQUISA 15, Quisqueya and Tempique). Other varieties were practically absent with this marker, 1, 8, 10, 12, 14 and 18 (Aceituno, Juma 57, Juma 68, Pava Shien, PROSEQUISA 5 and Puita). The Waxy gene is located on chromosome 6 and consists of 13 introns and 12 exons (Biselli et al. 2014).

The primers used to determine AAC through the GBSSI were:

<b>GBSSI Amplicon 1</b>	<b>F</b>	<b>CTAGCAGCACAGAGGCACAG</b>
	<b>R</b>	<b>CACAAGCAGAGAAGTGAAGCA</b>
<b>GBSSI Amplicon 2</b>	<b>F</b>	<b>GGACCGGGTAAAATGTGTTG</b>
	<b>R</b>	<b>ATAAATATCCTGGCCGTTGC</b>
<b>GBSSI Amplicon 3</b>	<b>F</b>	<b>CCGAGTTGGTCAAAGGAAAA</b>
	<b>R</b>	<b>GATCTTCTCACCGGTCTTTCC</b>
<b>GBSSI Amplicon 4</b>	<b>F</b>	<b>ACCAGTACAAGGACGCTTGG</b>
	<b>R</b>	<b>ATGAGCTCCTCGGCGTAGTA</b>
<b>GBSSI Amplicon 5</b>	<b>F</b>	<b>GAGGGCAGGAAGATCAACTG</b>
	<b>R</b>	<b>CCCTGCAGCTGGATGAGT</b>
<b>GBSSI Amplicon 6</b>	<b>F</b>	<b>GAGGGCAGGAAGATCAACTG</b>
	<b>R</b>	<b>ATTGGCTATAGCTACAAATT</b>

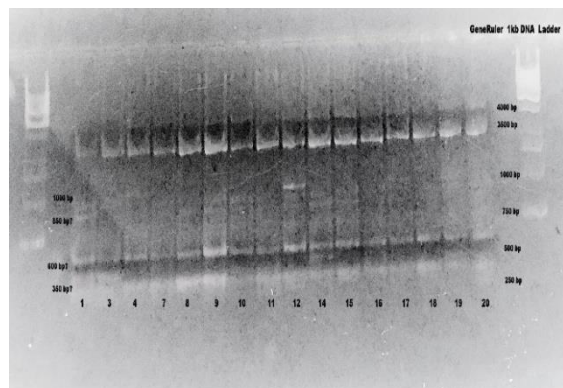
The conditions for PCR run for these amplicons were the same as those used for the previous two pairs of primers. That is: 94° C for 5 minutes, followed by 30 cycles of 94° C 45 seconds, 58° C 45 seconds and 72° C for 30 seconds and a final cycle of 72° C for 7 minutes.

The PCR was carried out in a thermocycler brand Applied Biosystems Model ProFlex PCR System.

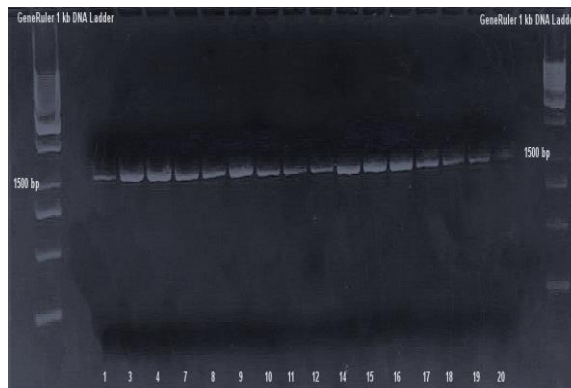
The PCR product was run in a 2.0% agarose gelatin and stained with ethidium bromide 1 mg/ml.

The band patterns of these primers are shown in the following figures:

**Figure 4.** Band patterns shown by GBSSI primers in 2.0% agarose gelatin.



**Figure 4a. GBSSI Amplicon 1**



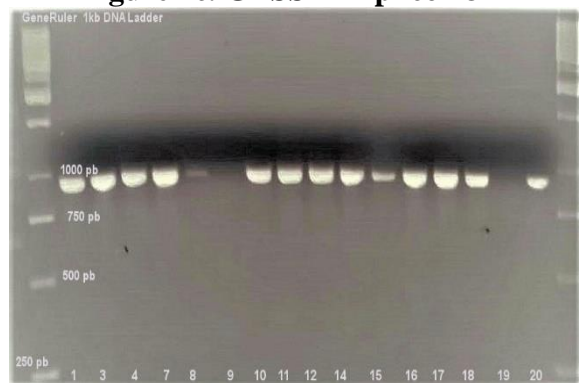
**Figure 4b. GBSSI Amplicon 2**



**Figure 4c. GBSSI Amplicon 3**



**Figure 4d. GBSSI Amplicon 4**



**Figure 4e. GBSSI Amplicon 5**



**Figure 4f. GBSSI Amplicon 6**

Figure 4a shows a series of well-defined bands around 3500 bp with varieties 1, and 10 (Aceituno and Juma 68) being the least defined. Variety 12 showed a band at around 1000 bp, being the only variety to produce band in this range, while all varieties showed a band at around 600 bp. Varieties 8, 9, 12 and 18 showed the brightest band in sterling It should be noted that varieties 3, 4, 7, 8, 9, 11, 12, 15, 16 and 18 (Cristal 100, IDIAF II, Jaragua, Juma 57, Palmar 18, Pava Shienn, PROSEQUISA 10, PROSEQUISA 15, and Puita).

The Amplicon 2 of Figure 4b shows us a single series of bands in the range of around 1500 bp

being the varieties 1, 18, 19, and 20 (Aceituno, Pita Tempie and Trabuco) the weakest.

Amplicon 3 did not develop very clear bands although a band around 650 bp can be noticed for variety 12 (Pava Shien). This same variety showed another pale band around 200 bp. All varieties, except for 8, 10, 14, 16 and 19 showed clear bands around 95 bp.

Varieties 1, 3, 4, 7, 12, 14, 16, and 18 showed bands with Amplicon 4 around 1500 bp, while varieties 1, 3, 4, 8, 9, 15, 16, and 18 showed weak bands below 250 bp ( $\approx 100$  bp).

Except for varieties 8, 9 and 19, (Juma 57, Juma 67 and Tempique) all varieties analyzed with Amplicon 5 showed great activity for the GBSSI gene (*Waxy*, as shown in Figure 4e, with very clear bands around 1000 bp.

The Amplicon 6 showed bands for all varieties except for varieties 4 and 7. These bands were shown around 750 bp showing great activity of the waxy gene for amylase synthesis.

### 3.5 Grain Size and Yield

Grain size is one of the most effective traits in rice yield (*Oryza sativa* L.); while this is a very important quality trait as well (Jahani et al. 2012). Grain size and grain number are the most affective characters in yield. In addition, grain size was also a target of artificial selection during domestication, where grain size or weight is important in the evolution of cereal crops because humans tended to select for large seed size during the early domestication process (Harlan 1992: Doganlar et al. 2000) cited by Jahani et al. 2012).

Three main traits (grain size, grain number per panicle, and number of panicles per plant) are directly associated with rice grain productivity, and these traits depend heavily on the genetic potential of rice. However, these traits are complex and quantitative in nature (Kim et al. 2016).

With quantitative analysis of trait loci (QTL) with fine tracing or positional cloning using rice mutants, about 20 genes that are implicated in yield-related traits have been isolated in rice (Wang and Li 2005; Xing and Zhang 2010; 2011; Huang et al. 2013; Miura et al. 2011; Huang et al. 2013; Liu et al. 2015). As far as the genes that control grain size are concerned, six genes can be considered in rice breeding programs. The GW2 encoding type RING E3 ubiquitin ligase regulates the width of the grain. The loss of GW2 function by a premature stop codon caused by a 1 bp deletion in the fourth exon increases grain width, resulting in increased yield (Song et al. 2007), cited by Kim et al. 2016.

### Primers

To determine the size of the grain, the primers used by Jahani et al. 2012 were used. These were:

Name of the primer	Primer sequence
Grain Size E F	AGGCTAAACACATGCCCATCTC
Grain Size E R	CCCAACGTTTCAGAAATTAAATGTGCTG
Grain Size I F	AACAGCAGGCTGGCTTACTCTCTG
Grain Size I R	ACGCTGCCTCCAGATGCTGA

The PCR reaction was performed in a 45  $\mu$ l volume containing 1x PCR buffer, 0.5 mM of each primer, 3 U of Taq polymerase and 120 ng of DNA. The PCR amplification reaction was carried

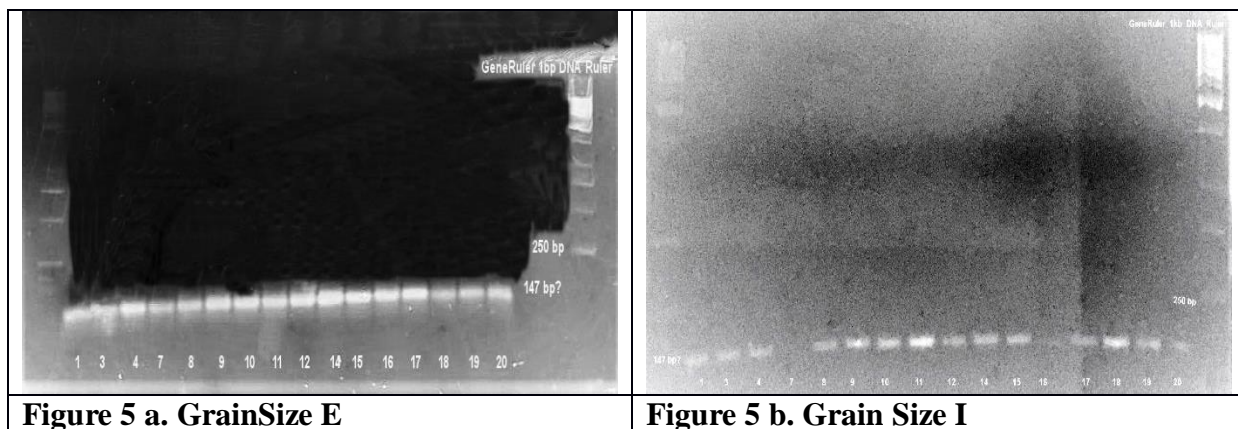


out in a thermocycler brand Applied Biosystems Model ProFlex PCR System.

The amplification conditions were 94° C for 4 min followed by 30 cycles of 94° C for 30 sec 69° C by 1 min and finally 1 cycle of 72° C for 7 min.

The PCR product was run in a 2.0% agarose gelatin and stained with ethidium bromide 1g/ml. The band patterns of this gelatin can be seen in the figures below.

**Figure 5.** Band patterns shown by Grain Size E and Grain Size I primers, in 2.0% agarose gelatin.



The Grain Size E primer (Figure 5 a) produced a band line at approximately 147 bp with a marked intensity indicating a fairly uniform grain size for all the varieties analyzed with no indication of difference between them in this category. The Grain Size I primer (Figure 5b) showed that there is variability in this allele. Some varieties (7 and 16) do not have this allele according to these results).

*Gn1a* alleles were used to determine the yield of the analyzed varieties. The primers used were those of Kim et al. 2016. To determine the number of spikes, which is another performance factor, the *SPL14* primers used by these same authors were used, as well as the *SPIKE* primers.

Name of the primer	Primer sequence
<b>Gn1a-17SNP-OP (Yield)</b>	<b>F: 5'- TCGCAGGCACTGCACTTCA-3'</b> <b>R: 5'-GCCACCCTAGGTTTGATTCC-3'</b>
<b>Gn1a-17SNP-A (Yield)</b>	<b>F: 5'- CATACTAGCGTTCTATGCTGA-3'</b> <b>R: 5'-GGAAGATAAAGAAATTTACATAACC-3'</b>
<b>Gn1a-indel3 (Yield)</b>	<b>F: 5'- GATCTAGATGCTCCAAAGTCC-3'</b> <b>R: 5'- CTGTACGTACGTGCACGTAG-3'</b>
<b>SPL14-04SNP (Yield)</b>	<b>F: 5'- TAGCCATAGCTTCTGCGTGA-3'</b> <b>R: 5'- ACCGTGCTTACCGCCTGG-3'</b>
<b>SPL14-12SNPC</b>	<b>F: 5'- ACCGACTCGAGCTGTGTTC -3'</b>

(Yield)	R: 5'-CAAGTGAGACTTCATGTGGT-3'
SPIKE-03SNP (Yield)	F: 5'-CTACTCGACCGTCTGGAAC-3' R: 5'-TGGCTCGAAGATCTCTTCTAC-3

The conditions for the PCR run for these primers were as follows: the reaction was run in a volume of 45 µl with 2.25 µM/l from each primer. The master mix was prepared according to the volume to be used. The PCR amplification reaction was carried out in a thermocycler brand Applied Biosystems Model ProFlex PCR System.

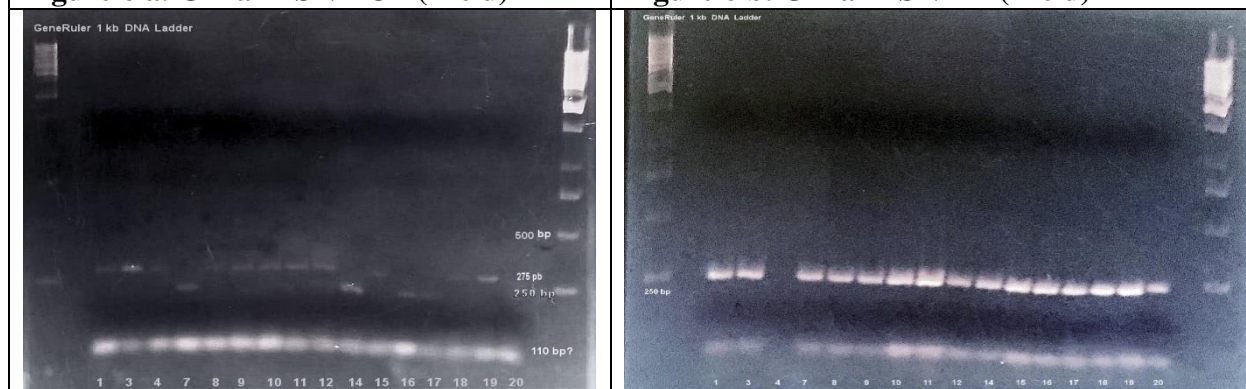
The running conditions were 94° C for 3 minutes, followed by 35cycles of 95° C 25 seconds, 55° C 25 seconds and 72° C for 35 seconds and a final cycle of 72° C for 5 minutes The PCR product amplified with these markers was run in an electrophoresis of 2.0% agarose was run in a 2.0% agarose gelatin and stained with ethidium bromide 1 mg / ml. The following figure shows the band patterns obtained in the electrophoresis of these markers.

**Figure 6.** Band patterns obtained in 2.0% agarose gelatin dyed with ethidium bromide 1 mg/ml.



**Figure 6 a. Gn1a-17SNP-OP (Yield)**

**Figure 6 b. Gn1a-17SNP-A (Yield)**



**Figure 6 c. Gn1a-indel3 (Yield)**

**Figure 6 d. SPL14-12SNPC (Yield)**



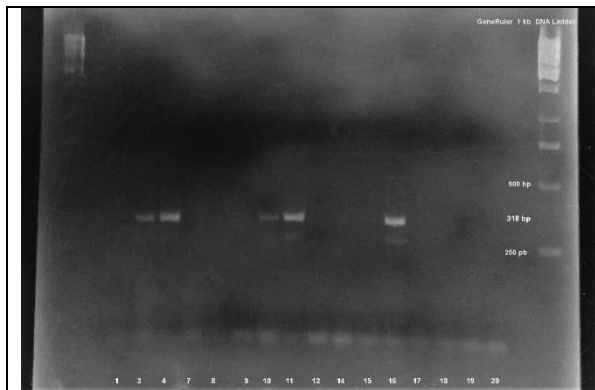


Figure 6e. SPIKE-01SNP (Yield)

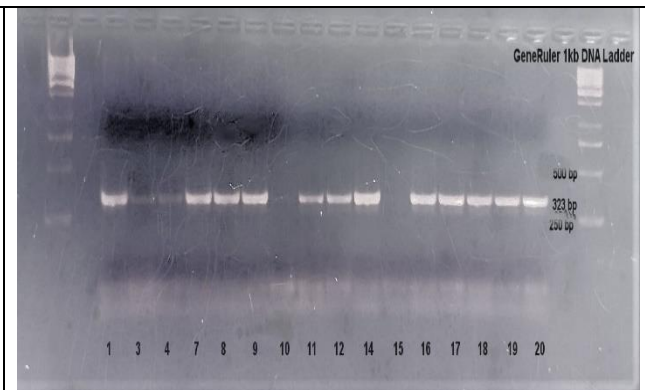


Figure 6 f. SPIKE-03SNP (Yield)

6 markers related to rice yield were analyzed for the 16 varieties in question.

### **Gn1a Markers**

Three of the performance markers were of the *Gn1a* class whose results are shown in Figures 6 a, 6 b, and 6 c.

Marker 6 a (**Gn1a-17SNP-OP(Yield)**) produced a series of bright bands around 532 bp with variety 15 being the least notable. The marker in Figure 6 b (**Gn1a-17SNP-A(Yield)**) produced a marked band line around 321 bp. Varieties 1, 4, 7, 10, 12 and 19 produced very bright bands while varieties 14 and 15 produced no bands at all. The rest of the varieties produced bands with intermediate intensity with this marker. The marker **Gn1a-indel3(Yield)** (Figure 6c) produced a series of bands in the 275 bp for varieties 1, 3, 4, 8, 9, 10, 11, 12 and 19 the most notable being varieties 3 and 19 the rest of the varieties of this group produced very faint bands. In the 250 bp four bands were produced being the varieties 7 and 14, the most notable. Varieties 16 and 17 produced very pale bands in this region. This marker. It produced bands for all varieties in the 110 bp approximately. The varieties 3 and 17 produced much less intense bands than the others with this marker in this region.

### **SPL14 Markers**

Of these markers, Kim et al. (2016) only one was used in this study, **SPL14-12SNPC (Yield)**.

This marker (Figure 6 d). produced two lines of bands, one very bright in the vicinity of 250 bp and the other less bright below 250 bp (95-110 bp?). This position is difficult to determine until the fragments obtained by PCR are sequenced.

### **SPIKE Markers**

The *SPIKE* allele is related to the increase in the number of grains which leads to the increase in rice yield (Fujita et al. 2013; Zhang et al. 2014). In this work, two *SPIKE* markers were used to determine the differences between the varieties analyzed with respect to this character. The markers used were **SPIKE-01SNP (Yield)** (Figure 6 e) and **SPIKE-03SNP (Yield)** (Figure 6 f). The **SPIKE-01SNP(Yield)** marker produced clear bands for varieties 3, 4, 10, 11 and 16 at 318 bp, while the **SPIKE-03SNP(Yield)** marker produced clear bands for most varieties, except for varieties 10 and 15 (Juma 68 and PROSEQUISA 10) which did not produce bands with this

marker at 323 bp. Varieties 3 and 4 (Cristal 100 and IDIAF II) produced very pale bands in this phase of the scale.

### 3.6 Rice Blast

Rice blight, caused by the fungus *Mangaloregrisea*, causes significant annual economic losses in yield globally and has been one of the worst rice diseases due to its pathogenic complexity, host and microclimate (Jeong-Soon Kim et al. 2010).

For this study, seven specific SNP markers for rice blight resistance were used. The primers for these markers are as follows:

Name of the Primer	Sequence	Reference
Rice blast Piz z4792	F: 5'-CGCACGCTTTCCGAACTACTCCCGCT-3' R: 5'-AGTGTGTGCGGCACGTGCTTTG-3'	Jeong-Soon Kim et al. (2010)
Rice blast Piz, Piz-t60510	F: 5'-GGAGTTGGTGCACGGTGCCGTTAC-3' R: 5'-GCGCGGACCGGCCAGCTAGTTGAC-3'	Jeong-Soon Kim et al. (2010)
Rice blast Piz-t zt6057	F: 5'-GAAGCTCAAACCTAGGAACGTGACGC-3' R: 5'-ACTGGAAGTCCCTCTATATGCCC-3'	Jeong-Soon Kim et al. (2010)
Rice blast Pik-p k39575	F: 5'-GGTGTTTGGGAACCTGAACCCTA-3' R: 5'-TTTCTGTTCGTCGGATGCTC-3'	Jeong-Soon Kim et al. (2010)
Rice blast Pit t256	F: 5'-GGATAGCAGAAGAAGCTTGAGACTA-3' R: 5'-CATGTCTTTAACATAAGAAGTTCTC-3'	Jeong-Soon Kim et al. (2010)
Rice blast Pik k6415	F: 5'-GCATTAGATAGTGATGAAAGCCGG-3' R: 5'-TGTTTCATCCAGGCAATTGGC-3'	Jeong-Soon Kim et al. (2010)

The PCR reaction was prepared in a volume of 45 µl containing 112.5 ng of genomic DNA, 11.5 pmol of each of the pairs of primers and 2.5 units of TaqDNA polymerase. The other components of the reaction were added according to the prepared volume. The PCR amplification reaction was carried out in a thermocycler brand Applied Biosystems Model ProFlex PCR System.

The parameters for PCR were as follows: 95° C per 4 min 1 cycle; 40 cycles of 94° C for 30

sec, 52°C for 30 sec and 72°C per 1 min; 1 final cycle of 72°C per 10 min.

Seven markers were analyzed to determine rice blight resistance in the 16 selected varieties.

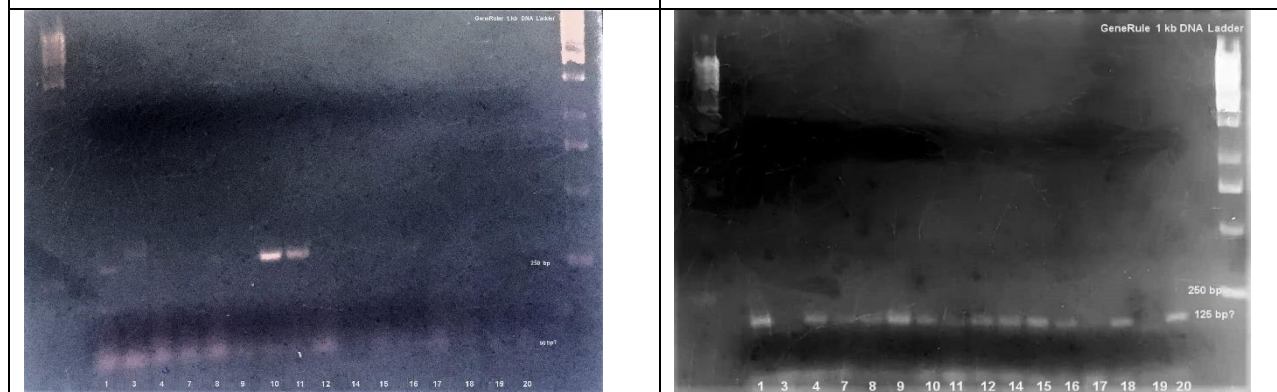
PCR products were run in 2.0% agarose gelatin and stained with ethidium bromide 1mg/ml. These results are shown in Figure 7 below.

**Figure 7.** Patterns of electrophoresis bands of PCR products for the varieties analyzed for resistance to Rice Blight.



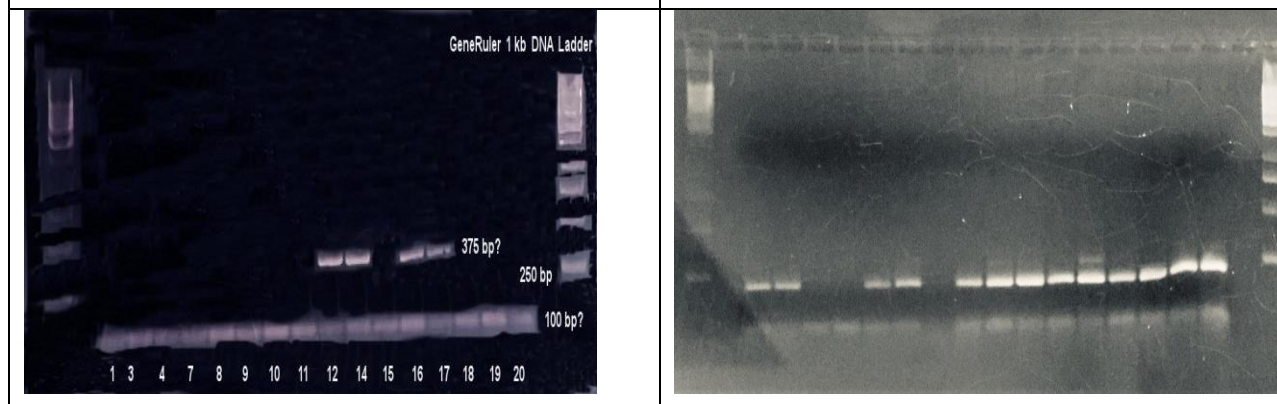
**Figure 7 a.** Rice blast Piz z4792

**Figure 7 b.** Rice blast Piz, Piz-t60510



**Figure 7 c.** Rice blast Piz-t zt6057

**Figure 7 d.** Rice blast Pik-p k39575



**Figure 7 e Rice blast Pit t256**

**Figure 7 f. Rice blast Pik k6415**

Figure 7 a. (**Rice blast Pizz4792**) shows the bands produced by this marker. Four of the varieties analyzed showed resistance to blight: 1, 8, 14 and 16 (Aceituno, Juma 57, PROSEQUISA 5 and PROSEQUISA 15). It should be noted that most varieties showed weak bands below 100 bp indicating some resistance to this allele of the gene.

Figure 7 b (**Rice blast Piz, Piz-t60510**) shows two well-defined band patterns. A very clear and defined one in the vicinity of 375 bp for varieties 1, 3, 10, 16 and 20 (Aceituno, Crystal 100, Juma 68 PROSEQUISA 15 and Trabuco). The other band pattern occurred well below 250 bp for all the varieties analyzed indicating that there is a resistance to this allele of rice blight in all the varieties analyzed, but much more marked in the five varieties indicated above.

Figure 7 c (**Rice blast Piz-t zt6057**) shows two bands defined in the 250 bp for varieties 10 and 11 (Juma 68 and Palmar 18) a very pale band can be noticed for variety 1 (Aceituno) in this same area (250 bp). Another very pale band can be seen for variety 3 (Cristal 100) above 250 bp. Below 250 bp (90bp) less defined bands were recorded for varieties 1, 3, 4, 7, 8, 9, 12, 15 and 17 (Aceituno, Cristal 100, IDIAF II, Jaragua, Juma 57, Juma 67, Pava Shien, PROSEQUISA 10 and Quisqueya). The other varieties showed no resistance to this allele of the rice blight gene.

Figure 7 d. (**Rice blast Pik-p k39575**) shows bands in the region approximate to 125 bp for most varieties with the exception of varieties 3, 17 and 19 (Cristal 100, Quisqueya and Tempique) did not produce bands with this marker indicating that there is no resistance to this allele of the rice blight gene. Other varieties such as 7, 8 and 11 (Jaragua, Juma 57 and Palmar 18) showed a weak resistance to this allele according to the intensity of the bands produced.

The **marker Rice blast Pit t256** (Figure 7 e) produced two sets of bands, one very clear and bright at approximately 375 bp, for varieties 12, 14, 16 and 17 (Pava Shien, PROSEQUISA 5, PROSEQUISA 15 and Quisqueya) indicating that they are the most resistant varieties to this allele of the rice blight gene. The other set of bands was produced in about 100 bp for all varieties. These bands were of lower intensity than the anteriores, but there is still an indication of resistance to this allele of the rice blight gene.

The last marker analyzed and shown in Figure 7 f (**Rice blast Pik k6415**) produced a series of bands in the 250 bp herds for all varieties except for varieties 4, 7 and 10 (IDIAF II, Jaragua and Juma 68), however 4 and 7 produced pale bands in the vicinity of 100 bp indicating some resistance to this allele of the rice blight resistance gene.

**4. CONCLUSIONS**

**4.1 Aroma**

Compared to the rest of the varieties analyzed, the Jaragua, Juma 57 and Juma 67 varieties showed little fragrance according to the data shown in Figure 2.

**4.2 Starch content**



The varieties Aceituno, Cristal, IDIAF II, Jaragua, Juma 57, PROSEQUISA 15, and Puita are varieties of low starch production according to the analysis with the *waxyInI* marker. All varieties analyzed with the *Ex6I* marker showed low apparent amylose content (AAC) with the exception of Juma 67 and Trabuco varieties.

The analysis with the markers GBSSI (Amplicons) The varieties Aceituno and Juma 68 differed from the other varieties analyzed by producing faint bands 3500 bp. The Pava Shien variety differed from the other varieties by producing a defined band in the 1000 bp. The Trabuco variety differed from the other varieties by not producing a defined band in the 1500 bp with the Amplicon 2. Amplicon 3 did not produce very defined bands but it can be seen that the Juma 57 and Tempique varieties did not produce bands in any area while the Pava Shien variety produced bands at approximately 650 bp and 200 bp. The Amplicon 4 produced bands in the 1500 bp for the varieties Aceituno, Cristal 100, IDIAF II, Jaragua, Pava Shien, PROSEQUISA 5, PROSEQUISA 15 and Puita. This Amplicon also produced bands in the 100 bp approximately for the varieties Cristal 100, Juma 57, Juma 67, PROSEQUISA 10 and PROSEQUISA 15. The only varieties that did not produce bands with Amplicon 5 were Juma 57, Juma 67 and Tempique, differing in this category from the other varieties analyzed. Likewise, the IDIAF II and Jaragua varieties differed from other varieties analyzed with Amplicon 6 by not producing visible bands...

#### **4.3 Grain Size and Yield**

Analyzing the varieties with the **Grain Size E** marker (Figure 5 a) no difference could be found between the 16 varieties analyzed, while the analysis with the **Grain Size and I** marker showed that the Jaragua variety and the PROSEQUISA 15 variety are different from the other varieties because they do not produce bands in the 147 bp (Figure 5 b).

The PROSEQUISA 10 variety produced the least bright band with the **Gn1a-17SNP-OP** marker. The other ranges produced very bright bands indicating grains of very good size. The varieties PROSEQUISA 5 and PROSEQUISA 10 differ from the other varieties by not producing bands with the marker **Gn1a-17SNP-A** in the region of 321. The analysis with the **Gn1a-Indel3 ma** read or did not detect a difference between the varieties analyzed. The same happened with the varieties analyzed with the **Gn1a-Indel1** marker

The analysis with the **SPL-14-04** marker produced very bright bands around 267 bp, with the Tempique variety being the only one that could be differentiated from the others by producing a less bright band (see Figure 6 e). The IDIAF II variety differed from all other varieties by not producing any band when the **SPL14-12NPC** marker was analyzed as shown in Figure 6 f.

The analysis with the **Spike-01** marker did not produce if the last series of bands produced below 250 bp is not considered, but when taking this last series of bands into account we see that the only variety not to produce band was Juma 57 (see Figure 6 g). Figure 6 h shows the band patterns generated by the **Spike-03** marker where very clear bands can be seen for most of the varieties analyzed except for the Cristal 100 and IDIAF II varieties that produced less defined bands. The varieties Juma 68 and PROSEQUISA 10 did not produce bands in this area of 323 bp.

The Juma 67 Palmar 18 varieties differ from the other varieties by not producing bands in the 87 bp region while the Pava Shien and Quisqueya varieties did not produce bands in the 63 bp region making them different from the other varieties analyzed.

#### 4.4 Rice Blast

The varieties Aceituno, Juma 57, PROSEQUISA 5 and PROSEQUISA 15 showed a good resistance to rice blight, so they were different from the other varieties analyzed with the marker Riceblast **Pizz4792** (Figure 7 a). The analysis with the marker **Rice blast Piz, Piz-t60510** (Figure 7 b) showed bands event to 5 of the varieties analyzed in the 375 bp (Aceituno, Crystal100, Juma 68 PROSEQUISA 15 and Trabuco) differentiating them from the other varieties. The Juma 68 and Palmar 18 varieties clearly differ from the other varieties by producing very bright bands at 250 bp when analyzed with the Marker Riceblast **Piz-t zt6057** (Figure 7 c). Analysis with the Marker **Rice blast Pik-m k 6441** (Figure 7 d) differentiated the Tempique variety from the others by producing a weak band at 325 bp that was not produced by any of the other varieties. The analysis with the marker in Figure 7 e (Riceblast **Pick-p k39575**) differentiated the varieties Cristal 100, Quisqueya and Tempique by not producing visible bands at 125 bp. Four varieties (Pava Shien, PROSEQUISA 5, PROSEQUISA 15 and Quisqueya) differed from the other varieties by producing bright bands in the 375 bp region. The other varieties did not produce visible bands in this region when analyzed with the marker **Rice blast Pitt256** (Figure 7 e). The varieties IDIAF II, Jaragua and Juma 68 did not produce bands when they were analyzed with the marker in the 250 bp **Rice blast Pikk6415** (Figure 7 f differing in this way from the other varieties analyzed.)

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