Molecular Diversity Analysis of Traditional Rice Varieties (TRVs) in Nueva Vizcaya, Philippines

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Abstracts: Traditional rice varieties (TRVs) are valued genetic resources because they contain important economic value traits needed to integrate and broaden rice's genetic pool and variability. In this study, twenty-seven TRVs including a check variety (Mindanao – RS25) collected from different locations in Nueva Vizcaya, were subjected to DNA analysis to explore the genetic diversity of the collection and to determine possible aromatic TRVs in the presence of the *fgr*-gene. TRVs demonstrate high genetic diversity based on the relatively low similarity coefficient of 0.12 using 15 robust short tandem repeat (STR) markers. The molecular diversity analysis divided TRVs into two major clusters/groups with eight sub-clusters. Cluster I comprised 15 TRVs along with the check variety, mostly grown in high altitudes/elevation (>500masl) or cooler climatic conditions, the japonica types. Furthermore, in Cluster II, 12 TRVs were commonly cultivated at lower elevations (100-500 masl) or warmer climatic conditions (indica sub-species). Moreover, the presence of the *fgr*-gene allele was observed in TRVs assessed at both production sites. Finally, the province of Nueva Vizcaya had a preliminary characterization and initial conservation of its TRVs, in which these diverse germplasms could offer a valuable gene pool and rare traits for future varietal improvement programs in rice.

Keywords: Molecular Diversity, DNA Marker, Fgr-Gene, Characterization, Polymorphism.

1. INTRODUCTION

Traditional rice varieties (TRVs) are resilient to various abiotic and biotic environmental stressors. Characterization of these germplasms is required for crop improvement and provides valuable information for the development of new rice varieties [1]. The selection and assessment of valuable TRVs based on phenotypes are less and highly influenced by environmental factors [2] and [3]. Molecular markers are used to identify variations among accessions, as they have proven to be powerful tools for assessing genetic variation and in the elucidation of genetic relationships within and among genetically closer organisms [4]. Amongst the several classes of genomic markers, STR markers are of tremendous value and most amenable for several applications in rice including genetic diversity studies due to their multi-allelic nature, high reproducibility, co-dominant inheritance, abundance, extensive genome coverage, and simple reproducible assays [5]. and ease of detection by PCR, the requirement of a small amount of DNA as a starting material, and the ability to act as a universal genetic marker for genetic reagent mapping [6]. Rice fragrance is one of the most important determinants of rice quality [7]. Moreover, scented rice is preferred mostly by consumers because of its aroma. Geneticists and rice breeders have widely investigated aroma gene research and its application in genetic breeding [8]. However, rich in genetic diversity, TRVs in Nueva Vizcaya are differentiated according to the information provided by farmers based on their observations, and none of these genotypes have undergone molecular assessments. This study was conducted to assess the genetic relatedness of TRVs using short tandem repeat (STR) markers and to identify TRVs with fragrant traits using tightly linked markers. Hence, information on the molecular characteristics of TRVs is important for future rice-breeding programs. Likewise, this study would further enhance the on-farm conservation of traditional rice varieties in Nueva Vizcaya, Philippines.

2. MATERIALS AND METHODS

Molecular characterization for the establishment of identity and genetic diversity and determining the absence and presence of traits and resistance in TRVs may provide information to cope with different factors affecting rice production and breeding of improved rice varieties in the future. [9] reported that fingerprinting with molecular markers allows precise, objective, and rapid cultivar identification, and has proven to be an efficient tool for crop germplasm characterization, collection, and management.

2.1. Plant Materials

Seeds of the twenty-seven (27) TRVs, including the check variety (Mindanao – RS25), were collected from 10 municipalities (Figure 1) of Nueva Vizcaya, covering twenty-five (25) rice farmers. Leaf samples were subjected to DNA analysis to explore the genetic diversity and *fgr*-gene detection among the genotypes.

2.2. Experimental Site and Cultural Management

The 27 TRVs were planted on-station at the University Central Experiment Station of Nueva Vizcaya State University (16°28′48″N and 121°08′43″E at 276.4masl), Bayombong, Nueva Vizcaya from October 2018 to March 2019. The on-farm setup was managed by selected farmer cooperators in the community.

The on-station setup was conducted in two cultivations (irrigated and rainfed), following good management practices for traditional rice. All rice cultivars were planted separately in prepared raised beds and bunds for rainfed lowlands and irrigated rice cultivation, respectively. The sites were prepared to be dry and properly leveled. Plowing and harrowing were performed twice before the layouts. The plot and bund were laid out at 2.5m (L) x 1.5m (W) with alleyways of 0.5m provided to serve as spaces for labeling the plot/bund and used to facilitate the identification of TRVs during data gathering. All the seeds were pre-germinated before sowing to ensure germination. The distance of planting at 30cm x 30cm was followed as a practice by farmers in the area to contain 40 hills per plot/bund. Ten (10) hills of each plot or bund were tagged as sample plants during the early vegetative stage before data gathering.

2.3. Lay-outing and Designing of Treatments

Treatments were grouped based on the geographic elevation in meters above sea level (masl) of the production sites of TRVs in Nueva Vizcaya. The treatments were the following:

Treatment 1 (T1) – UCES-NVSU (276.4masl), On-station

Treatment 2 (T2) – Lower elevation (100 – 300masl), On-farm

Treatment 3 (T3) – Mid-elevation (301 – 500masl), On-farm

Treatment 4 (T4) – Upland elevation (>500masl), On-farm

2.4. Genomic DNA Isolation

A small amount (2 grams) of fresh leaf tissues of rice seedlings were collected from both study sites (on-station and on-farm). Leaf samples were put into labeled glass line bags separately from each other and placed into an insulated water jag packed with ice. These samples were transported immediately to the PhilRice laboratory for DNA extraction. DNA was isolated from the leaf samples following the protocol described and adapted from the PhilRice Manual on DNA Fingerprinting in Hybrid Rice: Its Applications in Varietal Purity Testing [10]. Leaf tissues were ground using a mortar and pestle in liquid nitrogen till fully pulverized. A 750µl of pre-warmed 2x CTAB and 50µl 20% SDS were added to the previously ground leaf samples. Mixed thoroughly by vortexing and incubated in a water bath at 650C for 1 hour. Briefly cooled and added 750µl chloroform and mixed thoroughly using a vortex. The tubes containing the suspensions were centrifuged for 30 minutes at 10,000rpm for 30 minutes (4^0C). The aqueous phase (top phase) was decanted into a new 1.5 ml tube with added 600 µl ice-cold isopropanol and incubated at -20^0C for 1 hour. The tubes were centrifuged at 10,000 rpm for 10 minutes. Isopropanol was decanted and the pellet with 500µl of 70% ethanol. The tubes were centrifuged at 10,000rpm for 3 min, alcohol was discarded, and the tubes were drained by inverting them in a paper towel to remove excess liquid. Pellets were dissolved in 100µl µL TE buffer with RNase and incubated at room temperature for 3 h (pellets were completely dissolved). After DNA extraction, the samples were stored at -20°C.

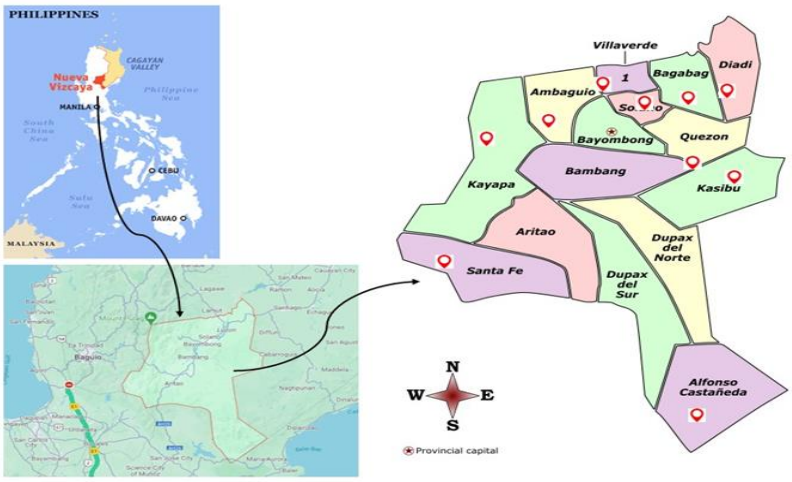

Figure 1. The collection sites of TRVs in Nueva Vizcaya, Philippines.

2.5. Genotyping Using STR Markers

Extracted genomic DNA was subjected to a polymerase chain reaction (PCR) using 15 sequences or short tandem repeat (STR) markers to assess the genetic diversity of the collected samples. The reaction was carried out in 7.5µl of master mix per sample containing sterile distilled water, 5 × PCR buffer, 25 mM MgCl2, 5 mM dNTPs, 10µm Forward Primer, 10µm Reverse Primer, commercial Taq polymerase, and template DNA (approximately 100 ng/µL). The PCR conditions were as follows: initial denaturation at 94°C for 5 min, followed by 35 cycles of final denaturation at 94°C for 30 s, primer annealing at 55°C for 1 min, and extension at 72°C for 1 min. The final extension was performed at 72°C for 5 min and held at 4°C. The PCR products were electrophoresed for 90 min at 100 volts in an 8% non-denaturing polyacrylamide gel, stained with Gel Red, and viewed using a Gel Documentation (Gel Doc) System.

The reaction was performed on a labeled 96-well PCR plate. Two (2) µl of diluted DNA solution (working DNA stock diluted 10 1:20) were dispensed into each well. The cocktail mix was prepared in 1.5 ml or 2 ml microcentrifuge tubes by dispensing the different components as follows:

PCR profile using commercial Taq.

Components of cocktail	1 Reaction (µl)
Sdh_20	1.12
5x PCR buffer	2.00
25 mm $MgCl_2$	0.60
5 mm dNTPs	0.40
10 µm Primer FR	0.80
BSA	0.53
Taq Polymerase	0.05
DNA	2.00
Total volume	7.50

The cocktail was thoroughly mixed and aliquot 8 µl to each pre-dispensed DNA sample in the PCR plate. The mixture was overlaid with a drop of mineral oil. The PCR plate was covered with adhesive tape (or sealing film) and labeled with the name of the researcher, date of preparation, primers used, and used entries or rice samples. The plate was placed in a thermal cycler (PCR machine) programmed using the following profiles:

Step	Temperature	Time
1	94^0C – initial denaturation	5 min
2	94^0C – denaturation	1 min
3	55^0C – annealing	1 min
4	72^0C – primer extension	2 min

5	35 cycles to step 2	
6	72^0C – final extension	5 min
7	10-15^0C – soaking	

After PCR cycles were completed, the plate was removed and stored in a refrigerator. Table 1 shows the fifteen (15) optimized primers used in this study. These robust STR primers were adapted from PhilRice, which is usually used in DNA profiling systems for rice-based studies.

2.6. Trait Association of Gene Markers

Important character traits, such as fragrance or aroma, were selected and observed using trait-associated markers in all collected TRVs in the study's on-station and on-farm sites. Also, Table 1 presents the different markers used for the detection of *fgr*-gene traits in rice studies. The relative positions of the markers used for the determination of aromatic and non-aromatic rice are shown in Fig. 2. The External Sense Primer and EAP generated a fragment of approximately 580 bp, which served as a positive control for each sample.

Table 1. Target traits and DNA marker information used in molecular diversity and characterization analyses of TRVs in Nueva Vizcaya, Philippines.

TRAIT	MARKER NAME	TYPE OF MARKER	5' PRIMER SEQUENCE 3'	REFERENCE
A. List of STR markers used for rice genotype identification				
STR markers used for genetic diversity analysis	RM206	Microsatellite	Fwd. TGTAAAACGACGGCCAGCCCA Rev. CGTTCCATCGATCCGTATGC	[12]
	RM44	Microsatellite	Fwd. TGTAAAACGACGGCCAGTGCTT Rev. TAGCAGCACCAGCATGAAAG	
	RM324	Microsatellite	Fwd. TGTAAAACGACGGCCAGCTGAT Rev. GATTCCACGTCAGGATCTTC	
	RM154	Microsatellite	Fwd. TGTAAAACGACGGCCAGGACG Rev. CGATCTGCGAGAAACCCTCTCC	
	RM169	Microsatellite	Fwd. TGTAAAACGGCCAGTGGC Rev. TCCCGTTGCCGTTCATCCCTC	
	RM3412	Microsatellite	Fwd. TGTAAAACGACGGCCAGAAAG Rev. CCCATGTGCAATGTGTCTTC	
	RM547	Microsatellite	Fwd. TGTAAAACGGCCAGTAGG Rev. GTCAAGATCATCCTCGTAGCG	
	RM5	Microsatellite	Fwd. TGTAAAACGACGGCCAGTGCA Rev. GCATCCGATCTTGATGGG	
	RM490	Microsatellite	Fwd. TGTAAAACGACGGCCAGATCTG Rev. AGCAAGCAGTGCTTTCAGAG	
	RM536	Microsatellite	Fwd. TGTAAAACGACGGCCAGTCTCT Rev. ACACACCAACACGACCACAC	
	RM234	Microsatellite	Fwd. TGTAAAACGACGGCCAGACAG Rev. CACGTGAGACAAAGACGGAG	

Table 1. Continue …

TRAIT	MARKER NAME	TYPE OF MARKER	5' PRIMER SEQUENCE 3'	REFERENCE
	RM202	Microsatellite	Fwd. TGTAAAACGACGGCCAGCAGA Rev. CCAGCAAGCATGTCAATGTA	
	RM592	Microsatellite	Fwd. TGTAAAAACGACGGCCAGTCTTT Rev. AGAGATCCGGTTTGTTGTAA	
	RM589	Microsatellite	Fwd. TGTAAAACGACGGCCAGATCAT Rev. CAGGTTCCAACCAGACAGTG	
	RM566	Microsatellite	Fwd. TGTAAAACGACGGCCAGACCC Rev. CTCCAGGAACACGCTCTTTC	
B. Primers for analysis of fragrance in TRVs				
Fragrance (Aroma)	External Sense Primer (ESP)	Allele-Specific Amplification (ASA)	TTGTTTGGAGCTTGCTGATG	[11]
	Internal Fragrant Antisense Primer (IFAP)	Allele-Specific Amplification (ASA)	CATAGGAGCAGCTGAAATATA TACC	

	Internal Non-fragrant Sense Primer (INSP)	Allele-Specific Amplification (ASA)	CTGGTAAAAAGATTATGGCTTCA	
	External Antisense Primer (EAP)	Allele-Specific Amplification (ASA)	AGTGCTTTACAAAGTCCCGC	

Internal Non-fragrant Sense primers and corresponding external (EAP) primers produce a 355bp fragment from a non-aroma allele or non-aromatic genotype. Internal Fragrant Antisense Primer and corresponding external (ESP) primers produce a 257bp fragment from an aroma allele or fragrant genotype [11].

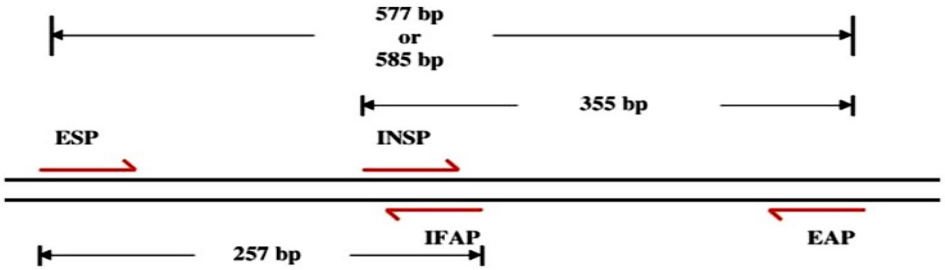

Figure 2. Positions of primers used in fragrance gene detection in rice [11].

Phenotypic and molecular data in both setups were compared to identify the extent of the existence or absence of the *fgr*-gene in all genotypes. Whatever information was given on the phenotypic data of aroma was based on the experience of farmers, while the actual sensory evaluation through smell during the growing period of rice plants was observed on-station by the researcher. According to [13], the f*gr*-gene starts expressing in the early seedling stage of the rice plant and continues even after the grain harvest, and compound 2 acetyl-1-pyrroline (2AP) content was highest in mature grains of rice, followed by the booting stage [14].

2.7. Data Analysis

All data recorded in the study on TRVs, including the check variety for molecular characterization, were subjected to analyses. Unambiguously amplified amplicons from the gel images were scored for the presence or absence of bands for each primer. The scores were obtained in the form of a matrix with '1' and '0,' which indicated the presence and absence of bands, respectively. A binary data score was used to construct the dendrogram. The genetic associations between genotypes were evaluated by calculating Pearson's Correlation Coefficient for pairwise comparisons based on the proportions of shared bands produced by the primers/markers. A similarity matrix was generated using NTSYS statistical software, version 2.0. Similarity coefficients were used for cluster analysis based on a simple matching coefficient. The dendrogram was constructed using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) and sequential agglomerative hierarchical non-overlapping (SHAN) clustering in the NTYSYSpc program [15].

3. RESULTS AND DISCUSSION

Molecular profiling of rice genotypes is important for both genetic and breeding studies [9]. The study characterized 27 TRVs that were managed both on-station and on-farm using STR markers.

3.1. Genetic Diversity Analysis of TRVs

The genetic diversity of the 27 rice cultivars was determined using STR markers. The coefficient of genetic distance ranged from 0.12 to 0.93 which generates two clusters with eight sub-clusters. Both Clusters are

composed of four sub-clusters. The Mindanao – CV3 (RS25) check variety belonged to sub-cluster 2 under Cluster I (japonica-type variety). Based on the phenotypic and molecular characterization of rice cultivars, the study observed that genotypes in Cluster I and Cluster II most likely belonged to the japonica and indica subtype varieties of rice, respectively (Table 2).

Eight TRVs grouped into 4 were found to be closely related with a similarity coefficient higher than 0.85, namely Binuggon (RS6), Bongkitan (RS13), Balasang (RS21), Bongkitan (R32), Guyabano (RS17), Sta. Maria (RS20), Guyabano (RS24), and Tuddoy (RS18). Balasang, Sta. Maria and Guyabano were the most likely to be similar to the 0.933 similarity coefficient. No TRVs in the population were similar to the 1.00 similarity coefficient. Altogether, the genetic diversity of the 27 collected TRVs in Nueva Vizcaya, including the check variety, depicted a highly diverse population with a relatively low similarity coefficient of 0.12. The genetic diversity among the TRVs was analyzed using STR markers (Figure 3).

Japonica rice is found in the cooler zones of the subtropics and upland areas. It has narrow, dark green leaves. It is low-tillering and has a tall plant stature. Grains are short, and roundish, spikelets are awnless to long-awned, grains do not shatter easily, and have 0-25% amylose content. Further, *indica* rice is the major type of rice grown in the tropics and subtropics. It has broad to narrow, light green leaves and tall to intermediate plant stature (except for the semi-dwarf). These plants produce tillers profusely, having grains that are long to short, slender, and somewhat flat, and the spikelets are awnless. Indica grains shatter more easily and have 23-31% amylose content [16].

Wide genetic variability is expected in rice among its wild relatives providing opportunities for future crop improvement [17]. Traditional rice landraces possess a wide or broad genetic base valuable for rice breeding and suitable for subsistence farming in a given community [18]. [19] concluded that the utilization of STR markers in the molecular analysis of rice revealed a remarkably higher level of genetic polymorphisms, which allowed unique and unambiguous differentiation and identification of landraces of rice and aromatic varieties.

Table 2. Distribution of rice cultivars to different clusters based on the UPGM methods.

CLUSTER	NUMBER OF GENOTYPE	NAME OF RICE CULTIVAR
Cluster I (*Japonica* type)		
Sub-cluster 1	1	Mindoro (RS4)
Sub-cluster 2	9	Binuggon (RS6), Bongkitan (RS13), Balasang (RS21), Bongkitan (RS32), Mimis (RS39), Kotse (RS11), Ketnel (RS28), Mindanao - CV3 (RS25), Palawan (RS10)
Sub-cluster 3	4	Guyabano (RS17), Sta. Maria (RS20), Guyabano (RS24), Tuddoy (RS18)
Sub-cluster 4	2	California (RS27), Palawan (RS36)
Cluster II (*Indica* type)		
Sub-cluster 5	5	Red C4 (RS5), Patata (RS29), PBB410 (RS34), Pilit (RS35), Imelda (RS9)
Sub-cluster 6	3	R5 (RS15), Wag-wag (RS16), Raminad (RS40)
Sub-cluster 7	2	Wag-wag (RS38), AG5 (RS41)
Sub-cluster 8	1	Elmer (RS7)

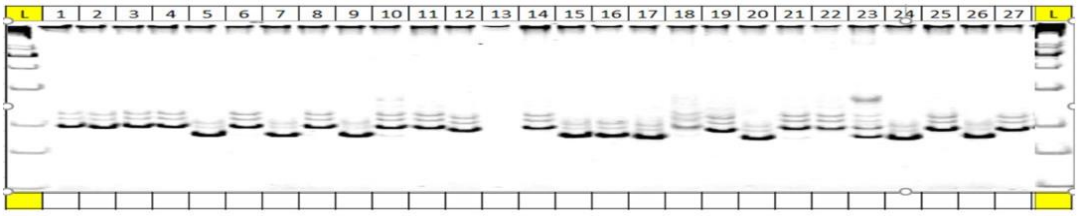

Figure 3. High DNA polymorphism among the 27 rice varieties was viewed in non-denaturing polyacrylamide gel electrophoresis using the RM154 marker.

3.2. Detection of *fgr*-gene Presence in TRVs

This study screened rice varieties for the presence of the *fgr*-gene using an Allele-Specific Amplification (ASA) PCR fragrance assay composed of four primers, including two external primers (ESP and EAP) and two internal primers (IFSP and IFAP). Under the on-station setup, 5 aromatic TRVs were detected (287bp) namely, Mindoro (RS4), Elmer (RS7), and Sta. Maria (RS20), Mindanao – CV3 (RS25), and PBB410 (RS34). The remaining were non-fragrant and unidentified rice cultivars (Figure 4a).

Meanwhile, 6 TRVs were found to have positive alleles for a fragrance like Mindoro (RS4), Elmer (RS7), Tuddoy (RS18), and Sta. Maria (RS20), Mindanao – CV3 (RS25), and Pilit (RS35), 17 were non-fragrant, and 3 were unidentified for the *fgr*-gene for the on-farm setup (Figure 4b). Comparison of the phenotypic and genotypic geographic distribution of TRVs showed that the Pilit cultivar detected non-fragrant on-station (276.4masl), in contrast to being aromatic on-farm (307masl). The majority of the evaluated TRVs were invariable in their traits across locations based on the DNA markers used in the study.

Comparison of both phenotypic and genotypic data of the 27 TRVs with fragrant traits across locations. Only the Pilit (RS35) cultivar demonstrated in the molecular characterization that non-fragrant in on-station but aromatic when cultivated on-farm. But still, 6 TRVs were detected aromatic in both phenotype and genotype evaluation over production sites namely, Mindoro (RS4), Elmer (RS7), Tuddoy (RS18), Sta. Maria (RS20), Mindanao-CV3 (RS25), and PBB410 (RS34).

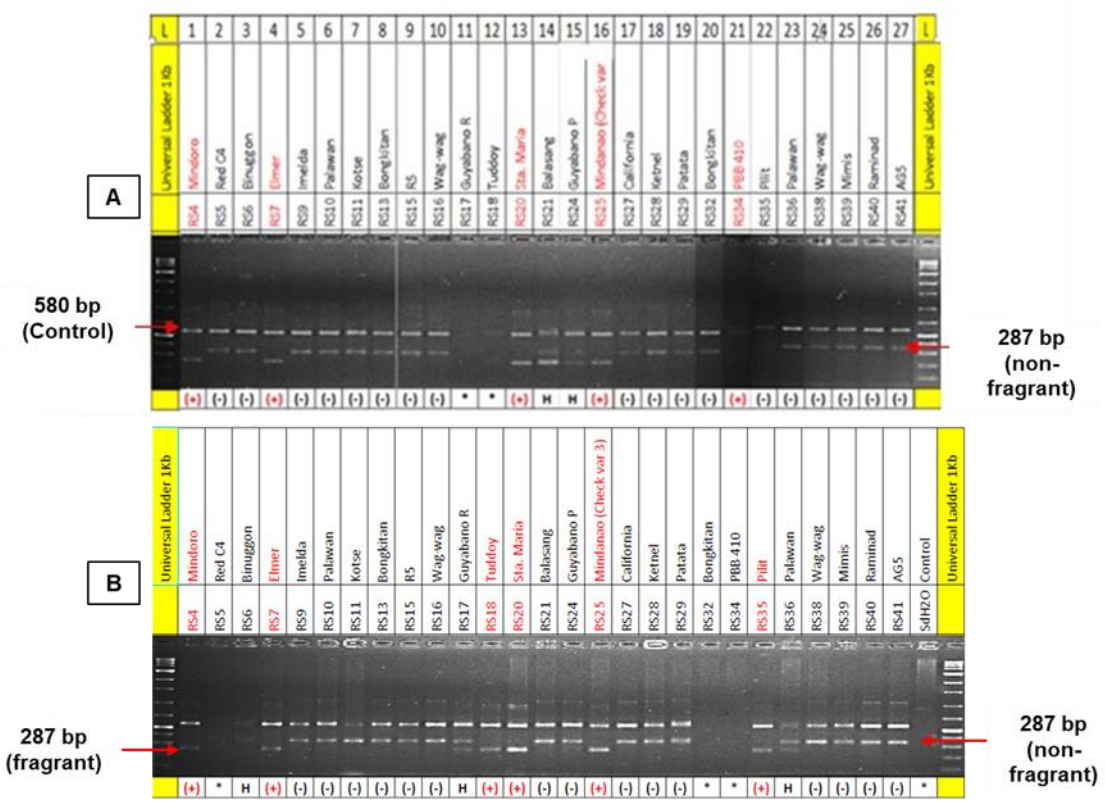

Figure 4. PCR profile generated from 27 genotypes of TRVs using *fgr* markers: A) On-station set-up and B) On-farm set-up.

Legend: (+) Homozygous fragrant; (*) Unidentified; (H) Heterozygote non-fragrant; (-) Homozygous non-fragrant.

Environmental factors and crop management practices may be the possible reasons that can affect 2-AP biosynthesis and gene expressions on aroma formation in rice. Nitrogen fertilizer affects aroma formation in aromatic rice and shows a significant relationship with 2-AP biosynthesis. Also, the effects of temperature, irrigation regimes, drought stress, salinity, planting density, harvesting time, and storage conditions could influence the accumulation and biosynthesis of 2-AP [20].

With the rapid development of rice functional genomics and sequencing technology, great progress has been made in understanding the aroma in rice, such as the determination of functional and useful markers that have been utilized for screening and identification of genes associated with the aroma in rice and eventually used to develop fragrant rice varieties [8]. Through molecular mapping, the process of selection would accelerate the efficiency of aromatic rice breeding programs [21].

The aromatic trait in rice has a significant role in consumer acceptability. [22] reported that scented rice is preferred by most consumers due to its flavor and palatability, and consumers are willing to pay a premium price for fragrant kinds of rice [11]. Aromatic varieties have comparable or superior nutritional values and better amino acid profiles (having a higher lysine, phenylalanine, leucine, and methionine content) than non-aromatic varieties [21].

CONCLUSIONS

Molecular markers revealed differences among rice genotypes providing a more direct, reliable, and efficient tool for germplasm characterization, conservation, and management. It provided a positive assessment of the ability of the STR market to produce unique DNA profiles of TRVs. Some characterized TRVs were aromatic based on phenotype and molecular evidence. These genotypes were identified to have fragrant genes (*fgr*) for further evaluation. The output would facilitate and recommend the potential utilization of TRVs in rice breeding programs and other molecular classification activities of rice to develop unique germplasms that complement the existing varieties in Nueva Vizcaya, Philippines.

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