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Molecular characterization of some Upland rice (*Oryza sativa* L.) using RAPD markers

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ABSTRACT
Ten rice accessions commonly grown, adapted to Ekpoma and its environs were selected and analyzed for genetic diversity and yields using RAPD markers. A total of five RAPD markers were used across the ten rice accessions. All the RAPD markers generated polymorphic patterns. The five RAPD primers revealed polymorphism between the rice accessions, of which 3 primers (OPH-09, OPT-07 and OPH-02) were found to be 100% polymorphic and the two remaining primers (OPT-06) and (OPT-20) exhibited 67% and 92% polymorphism respectively. The level of polymorphism with primers differed among the rice accessions. One out of the five primers was subjected to Unweighted Paired Group Method of Arithmetic means (UPGMA) using the Jaccard's similarity coefficient. Cluster analysis revealed the similarity between the rice accessions; it ranged from 35% to 85%. Molecular characterization showed that FAROX 521-E156-1(3.775 t/ha), FAROX 521-H137-1(3.575 t/ha) and FARO 52(3.525 t/ha) had the highest yields while FAROX 521-E285-1 had the lowest value of 3.125 t/ha. Molecular characterization of rice accessions at distance and similarity on yields confirmed and agreed with the morphological characterization.
Keywords: Rice, Molecular characterization, RAPD markers, RAPD primers, polymorphism

INTRODUCTION

Rice (*Oryza glaberrima* Steud and *Oryza sativa* L) belongs to the grass family Poaceae. It is a staple food for half of humanity and more than three billion people depend on rice as a major source of food. Rice, a genetic resource, can be characterized to enable breeders understand and appreciate its wide range of genotypic diversities for relevant crop improvement practices. Characterization of genetic variability of upland rice is therefore important for the effective conservation of rice genetic resources due to their usual features. In genetic terms, characterization refers to the detection of variation as a result of differences in either DNA sequences or specific genes or modifying factors (Kang and Priyadarshan, 2007).

Recently, Molecular markers as technique proven to be more elucidating to evaluate genetic variability in most plant species (Islam, Sattar, Ashrafuzzaman, Saud and Uddin 2012). The efficacies of different classes Of PCR-based markers have been detected when barley and rice cultivars were characterized (Virk, Zhu, Newbury, Bryan, Jackson and Ford-Lloyd, 2000; Saker, Youssef, Abdallah, Bashandy and El-Sharkawy, 2005). DNA sequencing provides the most fundamental measure of diversity because all markers are derived from polymorphisms in the DNA's building blocks.

Polymerase Chain Reaction is a new method that provides an alternative way to amplify a specific gene from any DNA that it contains. Polymerase Chain Reaction (PCR) enables researchers to produce millions of copies of a specific DNA sequence in approximately two hours (Tripathy, Ekka, Lenka, Ranjan, Pal and Mohapatra, 2012). All variations reside in the sequence of DNA molecule and, as such, constitute the genetic code.

A wide variety of DNA-based markers such as Restriction Fragment Length Polymorphism (RFLP), Random Amplified Polymorphic DNA (RAPD), Amplification Fingerprinting DNA (DAF DNA), Arbitrarily Primed Polymerase Chain Reaction (AP-PCR), Sequence Characterized Amplified Region (SCAR), Cleaved Amplified Polymorphic Sequence (CAPS), Inter-Simple Sequence Repeat (ISSR), Amplified Fragment Length Polymorphism (AFLP), Expressed Sequence Tag (EST) and Single Nucleotide Polymorphism (SNP) have been developed. Each marker system has specific advantage and disadvantages, the choice of the marker system to be used is the most important decision (Mahmoud *et al.*, 2005).

The PCR- based RAPD approach requires much less DNA, and is technically simple and cheaper compared to the RFLP. RAPD has already been used in the analysis of rice genotypes by several groups (Moukoumbi *et al*., 2011). Although there have been report of variations in rice accessions in one part of Nigeria (Ogunbayo *et al*., 2005), there has not been reports on molecular characterization of rice accessions in Edo state. Thus this study was carried out to characterize some upland rice accessions grown in Ekpoma, Edo State, Nigeria by using molecular techniques which will enhance improving on the genetic make up for prolific increase in rice yields.

MATERIALS AND METHODS

Molecular characterization of *Oryza sativa*

The polymerase chain reaction (PCR) amplification was carried out in NIFOR-CARGS molecular laboratory, Plant Pathology Division, Nigeria Institute for Oil Palm Research (NIFOR), Benin City, Edo State, Nigeria.

Plant materials and Genomic DNA extraction

The plant materials selected for the present study were ten different accessions of rice. These were FAROX 521-E285-1, FAROX 521-H462-1, FAROX 521-A5-1, FAROX 508-3-F4-2-1M, FAROX 521-1137-2 , FAROX 521-H137-1, FAROX 521-A82-1, FAROX 521-E156-1, FAROX 521-A82-2 and FARO 52 collected from National Cereal Research Institute, Badeggi, Niger State, Nigeria.

Healthy seeds of each variety were sown in soil bags containing water under appropriate growth conditions for getting fresh leaves. DNA extraction was carried out from the fresh leaves collected from seedlings used in morphological characterization.

The genomic DNA of the plants was isolated using CTAB method (Doyle and Doyle 1990). The leaf was cut and ground in 600ul of extraction buffer, and it was incubated at 65^0C for 20mins. The sample was removed from the incubator and allowed to cool to room temperature and chloroform was added, the sample was mixed by gentle inversion of the tube several times. Thereafter, the sample was spun at 14,000rpm for 15mins and the supernatant was transferred into a new eppendorf tube and equal volume of cold Isopropanol was added to precipitate the DNA. The sample was kept in the freezer for 1hr and later spun at 14,000rpm for 10mins and the supernatant was discarded and the pellet was washed with 70% ethanol, later the sample was air dried for 30mins on the bench. The pellet was re-suspended in 100µl of sterile distilled water. DNA concentration of all the samples was measured on spectrophotometer at 260nm and 280nm and the genomic purity was determined. The genomic purity was between 1.8 –2.0 for all the DNA samples. The quality of DNA was detected by agarose gel electrophoresis and the size of fragment obtained was about 25kb for all the samples. The genomic DNA was used in PCR amplification using RAPD markers.

Primer test

Five primers of random sequence were screened for amplification of the DNA sequences. A final subset of three primers exhibiting good quality banding patterns and sufficient variability were selected for further analysis.

DNA-Electrophoresis

Agarose gel electrophoresis was used to determine the quality and integrity of the DNA by size fractionation on 1.0% agarose gels. Agarose gels were prepared by dissolving and boiling 1.0g agarose in 100ml 0.5X TBE buffer solutions. The gels were allowed to cool down to about 45^0C and 10ul of 5mg/ml

ethidium bromide was added, mixed together before pouring it into an electrophoresis chamber set with the combs inserted. After the gel has solidified, 3µl of the DNA with 5µl sterile distilled water and 2µl of 6X loading dye was mixed together and loaded into the multi-well crate plates. Electrophoresis was done at 80V for 2 hours. The integrity of the DNA was visualized and photographed on UV light source.

Dilution of DNA for PCR

About 10 µl of each DNA was taken into eppendorf tube and 990µl sterile distilled water was added to make 1000µl. The final concentration became 20-50ng/µl.

PCR reaction mix

The reaction mix was carried out in 20µl final volume containing 60ng - 80ng genomic DNA, 0.1 µM of the primers, 2mM $MgCl_2$, 125µM of each dNTP and 1 unit of Taq DNA polymerase. The thermocycler profiles has an initial denaturation temperature of 3mins at 94^0C, followed by 45 cycles of denaturation temperature of 94^0C for 20 seconds, annealing temperature of 37^0C for 40 seconds and primer extension temperature of 72^0C for 40 seconds, followed by final extension temperature at 72^0C for 5 min was added.

Gel-electrophoresis

PCR amplicon electrophoresis was carried out by size fractionation on 2.0% agarose gels. Agarose gels were prepared by dissolving and boiling 4.0g agarose in 200ml 0.5X TBE buffer solution. The gels were allowed to cool down to about 50^0C and 10µl of 5mg/ml Ethidium bromide was added, mixed together before pouring it into an electrophoresis chamber set with the combs inserted. After the gel has solidified, the PCR amplicon was loaded in the well created. Electrophoresis was done at 100V for 2 hours. The DNA was visualized and photographed on UV light source.

Data Analysis

The gel images were scored using a binary scoring system that recorded the presence and absence of bands as "1" and "0" respectively. From the binary data, the similarity coefficient values between the accessions were derived based on the probability that a particular character of one accession will also be present in another with the Jaccard's correlation analysis using the statistical software "SPSS" version 7.5 for Windows. The statistical analysis is performed using NTSYSpc version 2.1 (Rohlf, 2002). The data matrix was used to construct a phenetic dendrogram using UPGMA (unweighted pair group method of arithmetic averages) (Sneath and Sokal, 1973) in order to cluster the accessions.

RESULTS AND DISCUSSION

The inter-specific genomic polymorphisms in ten rice accessions were evaluated through RAPD-PCR. A total of five RAPD -DNA markers were used to determine the extent of genetic diversity across the ten rice accessions. All the RAPD-DNA markers generated polymorphic patterns. Each polymorphic RAPD marker was considered as a locus so that every locus had two alleles, identified by the presence (1) and absence of the band (0). A total of 40 alleles were amplified, of which 38 (95%) were polymorphic and (5%) were monomorphic. The 5 identified primers were (OPH-09, OPT-06, OPT-07, OPH-02 and OPT-20) (Plate 1-5). The number of polymorphic fragments for each primer varied from 3 to 12 with an average of 7.6 alleles per primer. All five RAPD primers revealed polymorphism between the rice accessions, of which 3 primers were found to be 100% polymorphic and the two remaining primers (OPT-06) and (OPT-20) exhibited 67% and 92% polymorphism respectively. The primer OPT-20 produced the maximum number of polymorphic bands (12), OPT-06 had minimum (3). The level of polymorphism with primers differed among the rice accessions.

The variation in the number of bands amplified by different primers is influenced by variable factors such as primer structure and less number of annealing sites in the genome (Nawrok, 2014). The proportion of polymorphism was higher compared to some previous RAPD analysis in rice e.g. 53.85% in six different rice cultivars (Rahman *et al*., 2007), 85.02% (Rajani *et al*., 2013) and 72.27% (Skaria *et al*., 2011) in some Indian rice varieties. One of the reasons for this high level of polymorphism can be due to intraspecific variation among the rice accessions (Nawrok, 2014). However, the level of polymorphism observed is in accordance with those reported by other researchers (Rabbani *et al*., 2008; Tehrim *et al*., 2012; Islam *et al*., 2013). The average number of polymorphic bands per primer detected agreed with

earlier reports; 7.4 and 7.8 polymorphic bands per primer (Rabbani *et al*., 2008; Saker *et al*., 2005) but higher in the reports with Younan *et al*. (2011), 13.7 polymorphic bands per primer and Rekha *et al*. (2011) , 32.3 polymorphic bands with 15 RAPD primers. Such big discrepancy using RAPD in the average number of polymorphic bands detected might be due to the diverse genotypes used and selection of RAPD primers with scorable bands (Islam *et al*., 2013).

PCR amplification of total genomic DNA using five random 5-mer primers yielded scorable amplification products. This was tested by subjecting the data to unweighted pair group method analysis of arithmetic means (UPGMA). One out of the five primers (Plate 3) with a clearer polymorphism was used to construct the dendogram (Figure 1). The highest similarity matrix revealed were between FAROX 521-H156-1 and FAROX 521-H137-1, FAROX 521-H156-1 and FARO 52 (0.85), while the lowest similarity matrix showed between FAROX 521-A82-1 and FAROX 521-1137-2 (0.35). Cluster analysis revealed the similarity between the rice accessions and it ranged from 35% to 85%. FAROX 521-E156-1 had the highest yields (3.775 t/ha); followed by FARO X 521-H137-1 (3.575 t/ha) and FARO 52 (3.525t/ha). The dendogram divided the rice accessions in to 5 groups or clusters. The increased number of groups can be due to the presence of high variation among the rice accessions. The number of clusters was higher compared to a previous study, e.g., two clusters in some Indian rice varieties (Rajani *et al*., 2013). Higher yield accessions with genetic similarity clustered together. While also, low yield accessions with genetic similarity clustered together. High yield accessions distance genetically from low yield accessions.

Molecular characterization of rice accessions at distance and similarity co-efficiencies on yields confirmed and agreed with the morphological characterization.

M 1 2 3 4 5 6 7 8 9 10 M

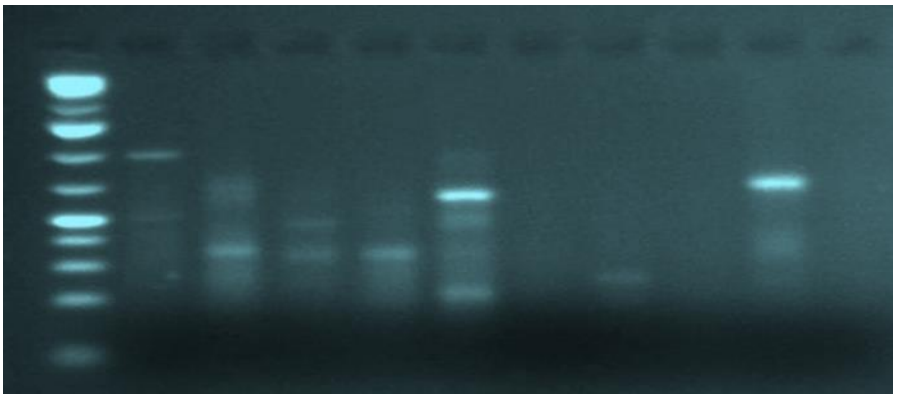


Plate 1: Electrophoresis gel image of the primer OPH-09

1=FAROX 521-1137-2, 2=FAROX 521-A82-1,3=FAROX 521-E156-1, 4=FAROX 521-A82-2, 5=FAROX 521-E285-1, 6=FAROX 521-H462-1,7=FAROX 52, 8= FAROX 508-3-F4-2-1-M, 9=FAROX 521-H137-1 and 10=FAROX 521-A5-1

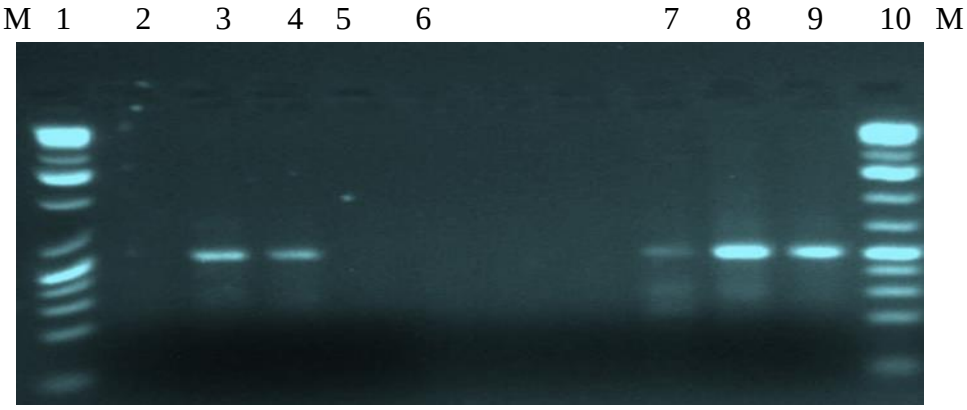


Plate 2: Electrophoresis gel image of the primer OPT-06

1=FAROX 521-1137-2, 2=FAROX 521-A82-1,3=FAROX 521-E156-1, 4=FAROX 521-A82-2, 5=FAROX 521-E285-1, 6=FAROX 521-H462-1, 7=FAROX 52, 8= FAROX 508-3-F4-2-1-M, 9=FAROX 521-H137-1 and 10=FAROX 521-A5-1

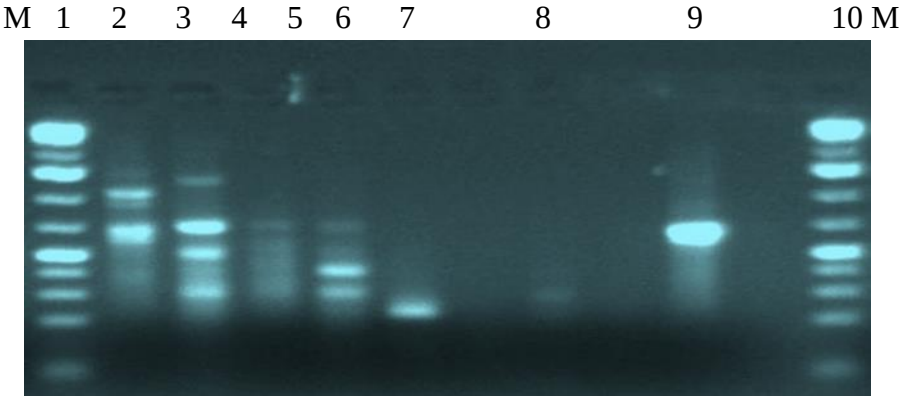


Plate 3: Electrophoresis gel image of the primer OPT-07

1=FAROX 521-1137-2, 2=FAROX 521-A82-1,3=FARO X 521-E156-1, 4=FAROX 521-A82-2, 5=FAROX 521-E285-1, 6=FAROX 521-H462-1, 7=FAROX 52, 8= FAROX 508-3-F4-2-1-M, 9=FAROX 521-H137-1 and 10=FAROX 521-A5-1

M 1 2 3 4 5 6 7 8 9 10 M

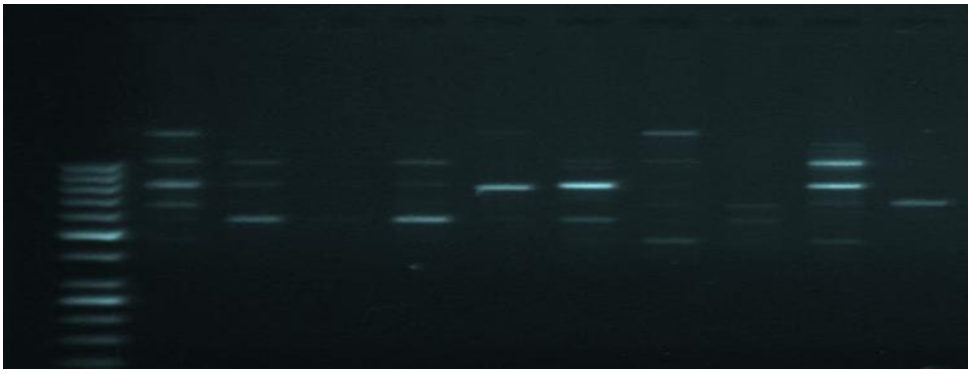


Plate 4: Electrophoresis gel image of the primer OPH 02

1=FAROX 521-1137-2, 2=FAROX 521-A82-1,3=FAROX 521-E156-1, 4=FAROX 521-A82-2, 5=FAROX 521-E285-1, 6=FAROX 521-H462-1, 7=FAROX 52, 8= FAROX 508-3-F4-2-1-M, 9=FAROX 521-H137-1 and 10=FAROX 521-A5-1

M 1 2 3 4 5 6 7 8 9 10 M

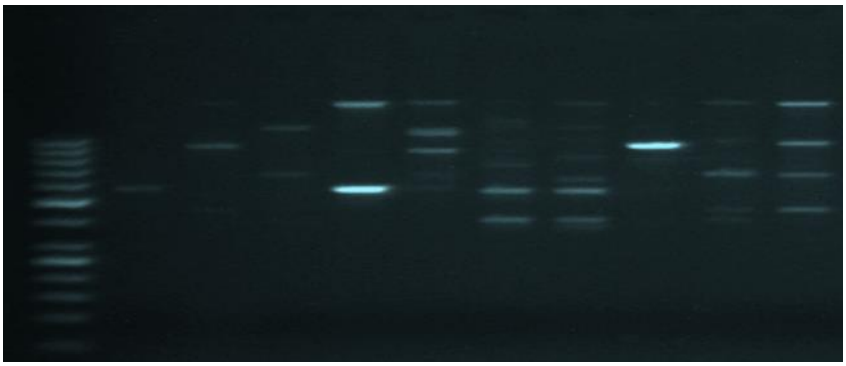


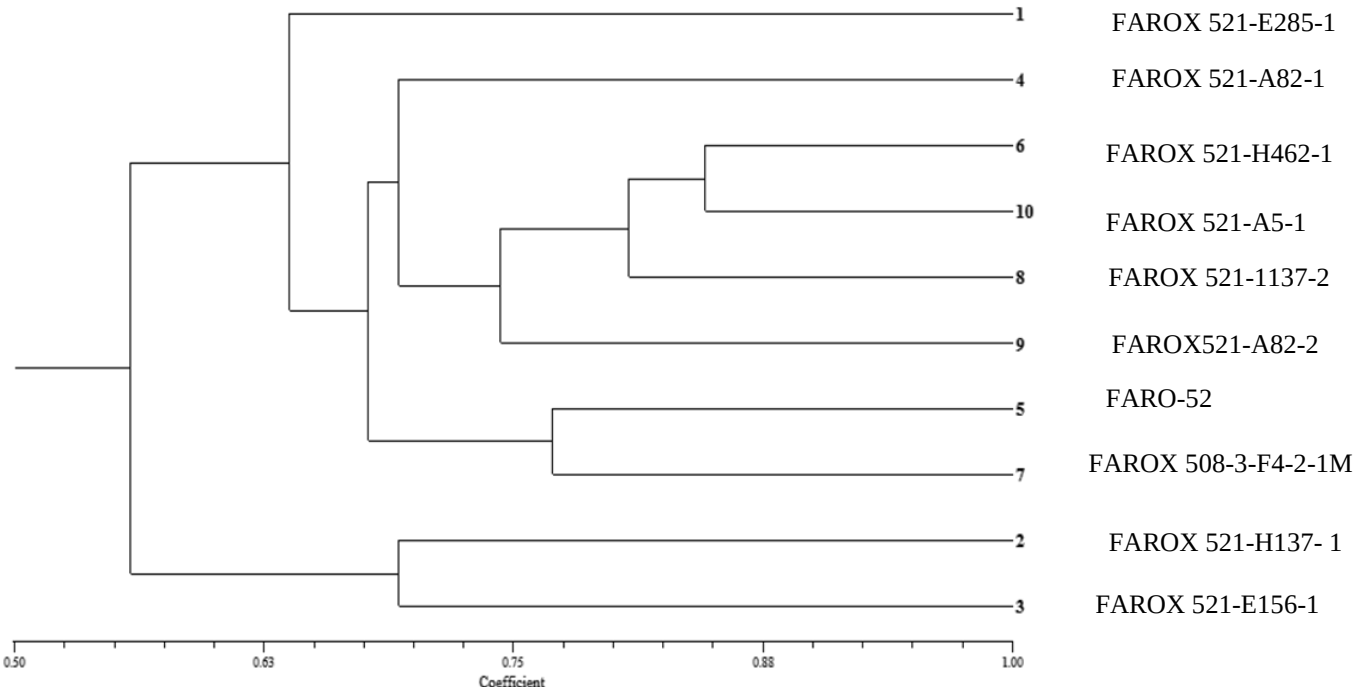
Plate 5: Electrophoresis gel image of the primer OPT 20

1=FAROX 521-1137-2, 2=FAROX 521-A82-1,3=FAROX 521-E156-1, 4=FAROX 521-A82-2, 5=FAROX 521-E285-1, 6=FAROX 521-H462-1, 7=FAROX 52, 8= FAROX 508-3-F4-2-1-M, 9=FAROX 521-H137-1 and 10=FAROX 521-A5-1

Table 1. Primer sequences and percentage polymorphism

Primer Names	Sequences	No of Monomorphic markers	No of Polymorphic markers	Total no of markers	% Polymorphism
OPH-09	TGTAGCTGGG	Nil	07	07	100
OPT-06	CAAGGGCAGA	01	02	03	67
OPT-07	GGCAGGCTGT	Nil	09	09	100
OPH 02	TCGGACGTGA	Nil	09	09	100
OPT 20	GACCAATGCC	01	11	12	92
TOTAL		02	38	40	

Figure 1: Dendogram for the DNA samples



CONCLUSION

The level of polymorphism with primers differed among the rice accessions. The variation in the number of bands amplified by different primers was observed to be influenced by factors such as primer structure and number of annealing sites in the genome. The highest Jaccard's similarity matrix were between

FAROX 521- E156 and FAROX 521-H137-1, FAROX 521-E156-1 and FARO 52 (0.85), while the lowest similarity was between FAROX 521-A82-1 and FAROX 521-1137-2 (0.35). FAROX 521-E156-1(3.775 t/ha), FAROX 521-H137-1(3.575 t/ha) and FARO 52 (3.525 t/ha) had the highest yields. While FAROX 521-H462-1 and FAROX 521-A5 -1 respectively had the lowest value of 3.275 t/ha. The use of DNA-based technique for evaluating genetic variability is highly recommended as it cannot be influenced by environmental factors and growth practices unlike morphological and biochemical analysis.

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