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Sensory Phenotypic and Molecular Identification of Aromatic Rice Accessions Cultivated in Benin

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Abstract

Rice is one of the most widely cultivated cereals in the world, and its aroma is increasingly in demand. With the advancement of research, a major rice flavor gene has been identified on rice chromosome 8. It encodes non-functional betaine aldehyde dehydrogenase leading to the accumulation of 2-acetyl-1-pyrroline which is the major olfactory compound that confers the fragrant character to rice. The aroma of rice is considered a special trait of enormous economic importance that determines the prime price in world trade. To satisfy the needs of the population and reduce rice imports into Benin, we conducted this study to identify aromatic rice accessions grown in Benin. Seventy-two rice accessions collected across Benin were PCR amplified with three SSR markers RM 7049, Aro 7, and RM 223, linked to the fgr (fragrance of rice) aroma gene. Molecular analysis revealed that 12 of the 72 accessions, namely Bagou 19, Bagou 22, Tchaka 34, Foun 15, Tchaka 41, Nana 32, Kan 61, Kung 69, Kung 67, Bagou 20, Agbab 101 and Koum 55 possess the fgr gene and can be considered as aromatic rice accessions. A sensory phenotypic test using KOH was carried out on rice accessions carrying fgr gene. Of the twelve positives, only one had the smell of aromatic rice, like the Azucena control. These results show that Benin also has aromatic rice varieties that can be sold on national and international markets.

Keywords

Aromatic Rice, 2-Acetyl-1-Pyrroline, SSR Markers, Benin

1. Introduction

Rice is one of the most important staple grains that feed more than 60% of the world's population [1]. Its production in 2020 was estimated at 37,889,802 tons in Africa and 756,743,722 tons worldwide (FAO 2022). It is a very important source of income for many farmers in sub-Saharan Africa. Currently, in Benin, the potential for rice cultivation is estimated at 375,000 ha for producing 374706 tons of paddy rice in 2019 [2] and 411578 tons in 2020 [3]. Although agriculture is the most important sector in terms of contribution to national GDP (36% of GDP) and provides 70% of the country's employment and 75% - 90% of official exports, the arable land potential for rice is insufficiently exploited, even though agro-climatic, edaphic and hydrographic conditions are favorable to the development of this agricultural speculation [4]. After maize, rice is a common daily meal in many households in Benin. Over the years, some people have come to appreciate one quality of rice to the disfavor of others. Aromatic rice is highly valued for its excellent aroma and superior grain quality [5]. Thus, aroma is an important trait for many breeding programs. Most Beninese consumers prefer imported rice for reasons such as the whiteness of the rice, the absence of foreign bodies, and the presence of aroma [4], as well as in India, where this trait is one of the most sought-after characteristics in rice [6]. The preference for aromatic (fragrant) rice implies that it is sold at a higher price than other rice on the market. This high purchase price is explained by the fact that few countries supply these qualities of rice [7]. Given the economic importance of fragrant rice, several research studies have been undertaken. One of the most important has resulted in the genetic mapping of the gene that codes for the flavor of rice [8]. Genetically, it has been proven that the aroma of rice is the phenotypic expression of spontaneous recessive mutations of the Osbadh2 gene also called fgr/osbadh2/Os2AP [7] which leads to the accumulation of 2-acetyl-1-pyrroline (2AP) which is the major olfactory compound that gives aromatic character to rice. These mutations inhibit the flow of y-aminobutylaldehyde to y-aminobutyric acid and consequently, the accumulated y-aminobutylaldehyde is converted to 2-Acetylpyrroline by a non-enzymatic pathway [9]. This mutation, which has led to a natural gift, prevents these rice varieties with aromatic character from being resistant to biotic and abiotic stresses, characterized by a decrease in the yield of these rice varieties and an increase in the market value of these rice qualities. To verify the presence of fragrance in rice in ancient times, phenotypic tests were carried out. These tests consisted of organoleptic [10] [11], sensory, or chemical [12] characterization. Years later, gas chromatography was innovated and made it possible to quantify the 2-Acetylpyrroline content of each variety to classify it

in terms of more fragrant and less fragrant [13]. The sensory and chromatographic testing process was often extremely tedious and costly, as it involved testing all the rice varieties available. Thus, the detailed mapping of the gene has made it possible to identify several molecular genetic markers of the microsatellite type associated with it. Indeed, the advent of PCR-based microsatellite marker technology offers highly efficient and reliable tools for monitoring crop genetic diversity and assessing evolutionary relationships within and between populations, varieties, and plant species [14]. Among molecular markers, microsatellites (SSRs) offer advantages for selection [15]. They are ideally suited for rice characterization given their high reproducibility, simplicity, and co-dominant inheritance [16]. To limit imports, which cause the national economy to lose foreign currency, this study aims to select from our collection of Benin-grown rice accessions those possessing the aromatic trait, using microsatellite and biochemical markers.

2. Materials and Methods

2.1. Study Framework and Collection Area

The study involved a collection of seventy-five rice accessions grown in Benin, stored in the Laboratory of Molecular Biology and Bioinformatics Applied to Genomics (BIOGENOM). The different varieties used were collected from all over Benin (**Figure 1**).

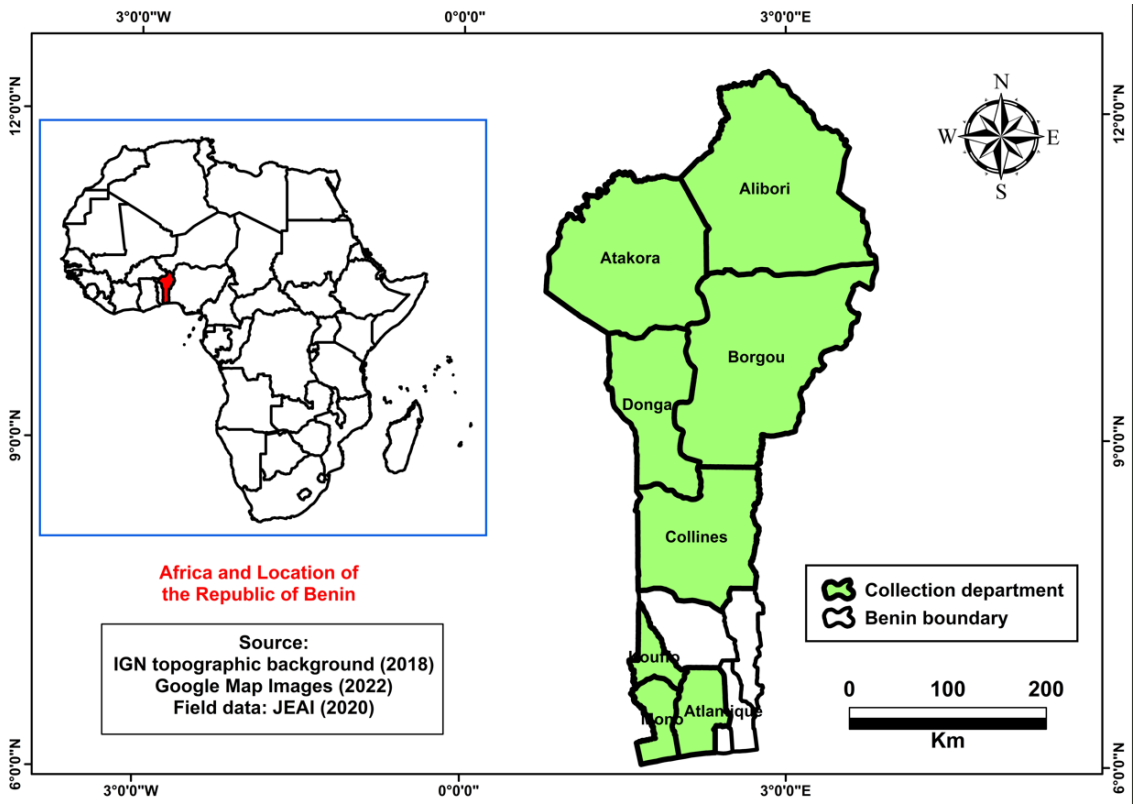

Figure 1. Map showing rice sample collection areas.

2.2. Biological Material

The plant material consists of a collection of 75 samples including seventy-three (72) accessions of rice grown in Benin collected from the North to the South of Benin and three (03) control varieties. The varieties that served as positive controls were Azucena and IR841. Another IR64 variety was used as a negative control for the study. These varieties were chosen as positive and negative controls in the literature reviews which indicate that they are aromatic (Azucena and IR841) and non-aromatic (IR64) rice varieties [17]. They are therefore ideal for identifying other aromatic rice varieties or accessions. Table 1 shows the different accessions used in this study, with their codes, departments, communes, and villages of origin.

Table 1. List of samples.

Number	Code Accession	Department/Municipality/Village
1)	Agbab 101	Hills/Savè/Agbaboué
2)	Ang 16	Alibori/Kandi/Angaradébou
3)	Ang 2	Alibori/Kandi/Angaradébou
4)	Ang 6	Alibori/Kandi/Angaradébou
5)	Ang 1	Alibori/Kandi/Angaradébou
6)	Azucena	GeneBank/IRD-Montpellier/France
7)	Bagou 17	Alibori/Gogounou/Bagou
8)	Bagou 18	Alibori/Gogounou/Bagou
9)	Bagou 19	Alibori/Gogounou/Bagou
10)	Bagou 20	Alibori/Gogounou/Bagou
11)	Bagou 21	Alibori/Gogounou/Bagou
12)	Bagou 22	Alibori/Gogounou/Bagou
13)	Bagou 23	Alibori/Gogounou/Bagou
14)	Bagou 24	Alibori/Gogounou/Bagou
15)	Bagou 25	Alibori/Gogounou/Bagou
16)	Bagou 26	Alibori/Gogounou/Bagou
17)	Bagou 27	Alibori/Gogounou/Bagou
18)	Bagou 28	Alibori/Gogounou/Bagou
19)	Bori 83	Borgou/N'dali/Bori
20)	Bori 84	Borgou/N'dali/Bori
21)	Dev 116	Couffo/Dogbo/Dévé
22)	Doko 122	Atlantic/Abomey-Calavi/Dokomey
23)	Foun 15	Banikoara/Founougo
24)	Gami 74	Borgou/Bembèrèkè/Gamia
25)	Gami 76	Borgou/Bembèrèkè/Gamia
26)	Gami 77	Borgou/Bembèrèkè/Gamia
27)	Gou 10	Alibori/Karimama/Gouroubéri
28)	Gou 11	Alibori/Karimama/Gouroubéri

Continued

29)	Gou 12	Alibori/Karimama/Gouroubéri
30)	IR 64	Atlantique/Zè/Awokpa
31)	IR 841/3	Atlantique/Zè/Awokpa
32)	Can 58	Atacora/Matéri/Kankini-Séri
33)	Can 59	Atacora/Matéri/Kankini-Séri
34)	Can 60	Atacora/Matéri/Kankini-Séri
35)	Can 61	Atacora/Matéri/Kankini-Séri
36)	Kik 96	Bassila/Kikélé-Lokpa
37)	Kotch 70	Atacora/Tanguiéta/Kotchessi
38)	Kotch 71	Atacora/Tanguiéta/Kotchessi
39)	Kotch 72	Atacora/Tanguiéta/Kotchessi
40)	Kotch 73	Atacora/Tanguiéta/Kotchessi
41)	Cold 42	Atacora/Natitingou/Koudengou
42)	Cold 43	Atacora/Natitingou/Koudengou
43)	Cold 44	Atacora/Natitingou/Koudengou
44)	Cold 45	Atacora/Natitingou/Koudengou
45)	Cold 46	Atacora/Natitingou/Koudengou
46)	Koum 47	Atacora/Boukoumbé/Koumadogou
47)	Koum 49	Atacora/Boukoumbé/Koumadogou
48)	Koum 50	Atacora/Boukoumbé/Koumadogou
49)	Koum 51	Atacora/Boukoumbé/Koumadogou
50)	Koum 53	Atacora/Boukoumbé/Koumadogou
51)	Koum 54	Atacora/Boukoumbé/Koumadogou
52)	Koum 55	Atacora/Boukoumbé/Koumadogou
53)	Koung 65	Atacora/Wassa Pehonco/Koungarou
54)	Koung 67	Atacora/Wassa Pehonco/Koungarou
55)	Koung 69	Atacora/Wassa Pehonco/Koungarou
56)	Kpatab 100	Collines/Savalou/Kpataba
57)	Man 118	Mono/Houéyogbé/Manonkpon
58)	Nana 29	Atacora/Cobly/Nanagadé
59)	Nana 30	Atacora/Cobly/Nanagadé
60)	Nana 32	Atacora/Cobly/Nanagadé
61)	NERICA 19	Atacora/Matéri
62)	Okouta 97	Hills/Bantè/Okouta-Ossè
63)	Okouta 98	Hills/Bantè/Okouta-Ossè
64)	ONK 93	Donga/Djougou/Onklou
65)	ONK 93b	Donga/Djougou/Onklou
66)	Tchaka 33	Atacora/Touncoutouna/Tchakalakou
67)	Tchaka 34	Atacora/Touncoutouna/Tchakalakou
68)	Tchaka 36	Atacora/Touncoutouna/Tchakalakou

Continued

69)	Tchaka 38	Atacora/Touncoutouna/Tchakalakou
70)	Tchaka 39	Atacora/Touncoutouna/Tchakalakou
71)	Tchaka 41	Atacora/Touncoutouna/Tchakalakou
72)	Tchal 89	Donga/Ouaké/Tchalinga
73)	Tot 82	Borgou/Nikki/Getty Images
74)	IR 841(INRAB)	GeneBank/INRAB-Calavi/Beinin
75)	IR 64 (INRAB)	GeneBank/INRAB-Calavi/Beinin

2.3. Sowing and Obtaining Young Leaves

To obtain young leaves for DNA extraction from each sample, five grains of paddy rice from each sample were sown in pots containing potting soil. Each pot was correctly identified and labeled according to the sample it contained. The pots were watered daily until young leaves appeared after two weeks.

2.4. Whole Genomic DNA Extraction

Total genomic DNA from the leaves was extracted using the methodology of Djèdatin *et al*. [18]. The first step of the extraction was to preheat 1000 µl of CTAB extraction buffer (2% hexadecyltrimethylammonium bromide, 1.4 M NaCl, 0.2% B-mercaptoethanol, 20 mM EDTA, 100 Mm Tris-HCl, PH = 8) to 65 C. Next, 200 mg of leaves from each sample were crushed, along with 1 mL (500 µL X2) of the CTAB extraction buffer. The resulting shredded material was decanted into a 2 mL Eppendorf tube followed by the addition of 50 µl of SDS 20%. Each tube containing the shredded material was incubated at 65˚C in the oven for 60 min and then left to cool to room temperature. After cooling, 750 µL of CIA (Chloroform Isoamyl Alcohol) was added to each tube followed by gentle stirring for 5 min, then centrifuged for 15 min at 10,000 rpm. The supernatant of each sample was collected in a 1.5 ml Eppendorf tube. To precipitate the DNA, 800 µL of isopropanol cold at −20˚C was added to the supernatant and gently homogenized by inversion. Centrifugation for 10 min at 10,000 rpm was done and the resulting aqueous solution was removed. The balls obtained were washed with 500 µL of 70% ethanol and centrifuged at 10,000 rpm for 10 min. For the DNA balls to be pure, the washing was done three times successively. After washing, the tubes were dried for a long time. The DNA balls were suspended after drying in 100 µL of pure, sterile distilled water and stored at −20˚C.

2.5. DNA Quality Verification

Verification of the quality and quantity of DNA was done on a 1% agarose gel. A mixture of 2 µL of DNA extract and 8 µL of 2× loading blue was deposited in the gel wells and allowed to migrate at 100 Volts (V) for 15 minutes with a 0.5× of Tris Bromate EDTA buffer (TBE) and then visualized at the transilluminator.

2.6. Amplification Polymerase Chain Reaction PCR

The analytical methods focused on PCR amplification with SSR microsatellite

markers. For this study, two SSR markers were used to confirm the presence of the fgr gene: Aro 7, RM 7049, and RM 223 markers located at 0.57 cM, 1.43 cM, and 3.2 cM respectively of the gene of interest. The amplification was carried out according to the following program: an initial denaturation of 5 min at 95˚C followed by 35 cycles each consisting of a phase of denaturation at 94˚C for 1 min, hybridization of the primer at 54˚C for 2 min and extension to 72˚C for 2 min. The program ends with a final extension phase at 72˚C for 5 minutes. The same program was used for all primer pairs (Table 2).

2.7. Amplification Product Reveal

The amplified products were migrated by electrophoresis on a 3.5% agarose gel in 100 µL of 0.5× TBE buffer for 1 h 30 min at 120 V. The gel containing 1.5 µL of BET was visualized under UV light.

2.8. Sensory Testing of Aromatic Varieties

Rice accessions having the aromatic gene were subjected to a sensory test with the KOH to assess the presence of the aroma phenotypically. The method described by Sun *et al*., 2008 [19] was used. 1 g of a tillering leaf of varieties with the aromatic gene was cut into small pieces and incubated with 2.5 ml of 1.7% KOH solution in petting dishes at room temperature. 15 minutes after incubation, the boxes were opened one by one and the smell was smelled by 10 people. For each variety, the test was done twice and the smell was compared to that of the aromatic positive control, Azucena.

3. Results and Discussions

The aroma of rice is genetically controlled by genes in the rice cell nucleus [22] [23]. PCR amplification using the RM 7049, Aro 7, and RM 223 markers revealed bands of 159 bp, 302 bp, and 165 bp, respectively, marking the presence of the gene encoding the desired aromatic trait. For our study, among the rice accessions screened with the RM 223 marker, we obtained eight accessions that presented the 165 bp band as a positive Azucena control characteristic of the gene of interest. With the RM 7049 marker, four accessions presented the desired band of 159 bp. concerning the Aro 7 marker, all accessions showed the 302 bp band characteristic of the gene of interest. Thus, only accessions with the

Table 2. Characteristics of the markers used.

Marker	Sequence	Expected Band	Chromosome	Reference
RM 7049	F: 5'AACCTAGATCTAATCCGTGG 3' R: 3'CATCTCTGAGTTGAGCAAAC5'	159 bp	8	[19]
RM 223	F: 5'GAGTGAGCTTGGGCTGAAAC 3' R: 3'GAAGGCAAGTCTTGGCACTG5'	165 bp	8	[20]
Aro 7	F: 5'ATTTGCCTCCTGAGTCTG 3' R: 3'GAGGATGGGGAAGATAAA5'	302 bp	8	[21]

159 bp and 165 bp bands were considered to have aromatic character, *i.e.* 16.66% of the accessions collected. In addition, among the three markers used, two were found to be polymorphic and discriminating. These are RM 223 and RM 7049 markers (**Figure 2**). The Aro 7 marker cited in the literature as polymorphic on acrylamide gel [24] [25] was found to be monomorphic on agarose gel in this study. Acrylamide gel was not used because we do not have the necessary material to handle this carcinogen gel in our laboratory. Several studies have been conducted to identify aromatic rice varieties using SSR microsatellite markers. In 2018, Manisha *et al*. assessed genetic variability between aromatic rice varieties using RM 7049 and Aro 7 markers. The Aro 7 marker that was shown to be monomorphic on agarose gel in this study was shown to be polymorphic on agarose gel for the differentiation and molecular classification study conducted by Manisha *et al.* in 2018 with a polymorphism rate of 44.44%. It can therefore be concluded that the polymorphism of a marker could vary depending on the study. In our case, the aromatic varieties identified would therefore not show any genetic variability likely to be revealed by this marker. The improved rice variety IR 841 is known for its aromatic character. However, in this study, it (sample N˚73) was not found to be aromatic with the RM 7049 marker, *i.e.* it did not present the 159 bp band like the Azucena control. This result could be due to the fact that there would have been a mutation in the sequence of this marker in this harvested variety. However, in our study, screening with the RM 223 marker revealed the presence of a second a specific band in the positive control Azucena that the aromatic detected accessions did not present. Since the Azucena variety is a naturally aromatic rice variety, the presence of a second band, probably a second allele revealed by the RM 223 marker, may lead to the conclusion that there is another allele or QTL involved in the expression of the aromatic character of the rice. Therefore, further studies will need to be carried out to identify the origin of this allele and its role. Through this revelation, we considered that the RM 223 marker could be an ideal marker for a study of diversity within aromatic rice varieties or accessions. Our results tend to be similar to those of Golestan Hashemi *et al*. [25], who showed that in an F2 population resulting from a cross between an aromatic and a non-aromatic variety of rice, 50% of F2 individuals with a high aroma carried a B allele of RM 223, L06, NKSbad2, FMbadh2-E7, BADEX7-5, Aro 7, SCU015RM and RM 515, which was closely related to putative QTL on chromosome 8, compared to non-aromatic progeny with this allele. Peng *et al*. [26] consequently asserted that the odorous characteristics of the rice grain can also be regulated by other genes or introns. In a study by Kumar *et al*. [27], while the majority of newly designed primers generated only one amplified product in each genotype tested, a few primers were able to amplify two bands in a particular aromatic rice DNA sample as is the case in our study for the Azucena sample.

The sensory test performed with the twelve varieties with the gene showed that only one had a smell of fragrant rice after comparison with the smell experienced in the aromatic control Azucena. The absence of favorable conditions

during the growth phase of aromatic rice in different environments has a direct impact on the expression of the aroma gene. In this way, our result may be due to environmental factors that would have prevented gene expression in the other accessions, as claimed by some authors following several studies showing that temperature, water, salinity, shade, submergence, and ultraviolet irradiation, which can reduce grain quality and affect rice aroma quality [28] [29] [30]. They also reported that the low 2-acetylpyrroline content of these varieties may explain the absence of smell. It would therefore be important to perform gas chromatography to be able to draw a sound conclusion on these aromatic varieties obtained in this study. The results of this study, more or less satisfactory, with a rate of 16.67% of aromatic rice accessions, enabled us to gain an insight into the aromatic rice accessions available in our country, which could be used at a later date to develop one or more high-quality aromatic rice varieties for the Beninese market. However, the accessions detected as aromatic during this study will need to undergo further testing for aroma content before they can be used in future breeding programs.

4. Conclusion

Aromatic rice is one of the rice varieties that occupies an important place in the rice trade. With the help of SSR molecular genetic markers, we were able to identify aromatic rice accessions (16.66%) in rice cultivated in Benin. It will therefore be possible to recommend these varieties of rice to producers and agricultural entrepreneurs operating in the field so that they can meet the needs of the population. For future studies, it would be important to check if the gene is

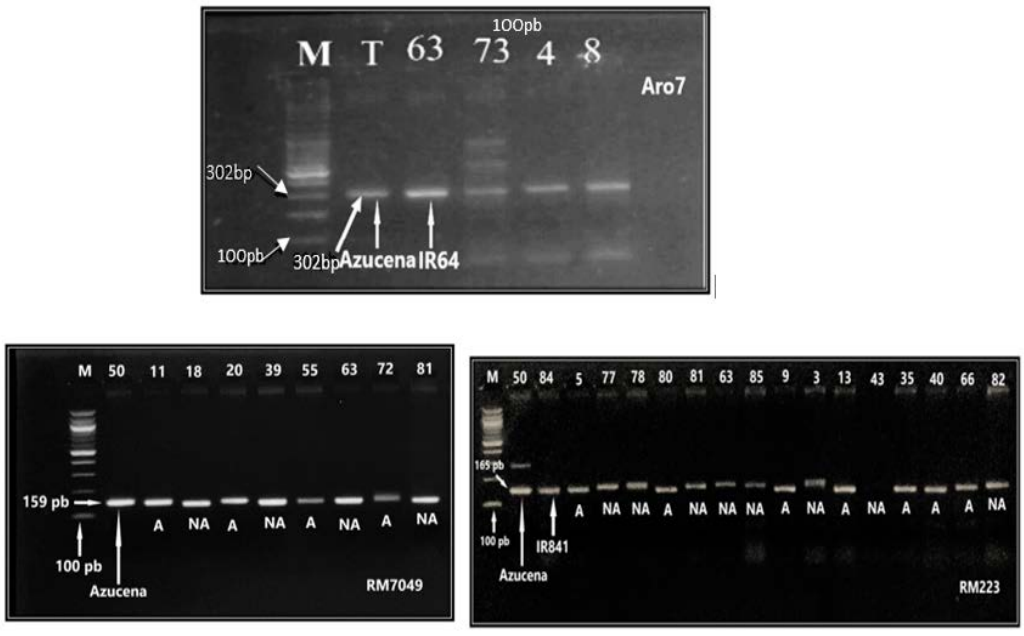

Figure 2. Representative gel pictures showing amplification patterns generated by different microsatellite markers used in the study. **Legend: M:** NA: Non-aromatic; A: Aromatic. Azucena: Positive Control; 159 pb: RM7049 marker positive size; 165 pb: RM223 marker positive size.

expressed in these varieties, to identify the conditions that favor the increase of aroma in rice, and to conduct the same study using other aromatic markers. It would also be important to look in other departments for aromatic varieties or those that will have the gene in a heterozygous state. These heterozygotes, which will be identified, will be crossed with each other to obtain homozygous recessive individuals that will have this trait. Heterozygotes will also be crossed with homozygotes possessing the gene to obtain pure lines. This work will make it possible to obtain a new variety of aromatic rice of Beninese origin.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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