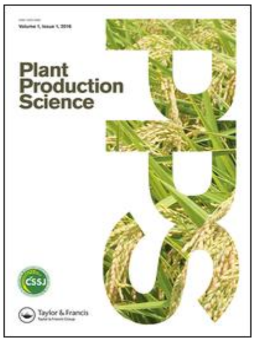

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Characterization of Aroma and Agronomic Traits in Afghan Native Rice Cultivars

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Abstract : In this study, we collected native aromatic rice cultivars from north, east and northeast of Afghanistan, and check cultivars from Japan, Thailand and India. We characterized some important agronomic characters such as plant height, panicle number per plant, grain number per panicle, 1,000-grain weight, grain length and grain width to find the desirable characters for breeding programs. Many of them were classified into tall culm rice according to IRRI index, but had thin and slender grain, and strong aroma which are favorable characteristics in Afghanistan and surrounding regions. The aromatic character was characterized by three methods, 1.7% KOH sensory test, gas chromatography-mass spectrometry-selected ion monitoring (GC-MS-SIM), and polymerase chain reaction (PCR) analysis. These three methods gave similar results. Six out of 10 Afghan native rice cultivars were aromatic and four non-aromatic. Among the check cultivars, Basmati 370, Jasmine 85, Izayoi, Oitakoutou and Jakouine were aromatic and Nipponbare non-aromatic. Improvement of aromatic and high yielding rice and reduction of plant height are the important objectives for rice breeding in Afghanistan. The results showed that Pashadi Konar from Afghanistan has the intermediate plant height, heavier 1,000-grain weight (32 g), longer grain (11 mm) and favorable aroma. Therefore, this cultivar may be a good source of aromatic rice germplasm in Afghanistan. To clarify the genetic nature of aroma in rice, we crossed non-aromatic cultivar Nipponbare with aromatic cultivar Jasmine 85, and examined the aromatic character in the F_2 generation by 1.7% KOH sensory test and PCR analysis. Non-aromatic and aromatic characters were segregated at a ratio of 3:1, showing that aroma is controlled by a single recessive gene.

Key words : 2-acetyl-1-pyrroline, Aromatic rice, Molecular marker, Plant height, Rice (*Oryza sativa* L.), Sensory test.

In Afghanistan, rice is the second among the food crops both in terms of cultivated area and total production, and aromatic rice is a favorable and luxury food for most people. Identifying the genotypic diversities among cultivars, studying genetic behavior of plant characters, and increasing of yield are important for rice breeding in Afghanistan. Agronomic characters are important for characterization of the diversity and improvement of rice. In this study, we characterized some important agronomic characters and aroma nature of Afghan native rice cultivars.

In Afghanistan aroma is characterized by such methods as the 1.7% KOH sensory test (Sood and Siddiq, 1978). Gas chromatography-mass spectrometry-selected ion monitoring (GC-MS-SIM) and PCR analysis may not be available in some conditions. However, comparison among these methods is important for evaluation of aroma nature in rice.

Molecular markers, such as single nucleotide polymorphism (SNP) and simple sequence repeat (SSR) that are genetically linked to aroma have been developed for the quick selection of aromatic rice (Lang and Buu, 2000; Cordeiro et al., 2002; Jin et al., 2003). However, these markers are not highly linked with the aroma gene and therefore are not reliable (Garland et al., 2000; Hien et al., 2005).

More recently, betaine aldehyde dehydrogenase 2 (BAD2) on chromosome 8 of *Oryza sativa* was also identified as probable enzyme of rice aroma expression (Goff et al., 2002; Bradbury et al., 2005a). Non-aromatic rice cultivars possess a full functional copy of the gene encoding BAD2 while aromatic cultivars possess a copy of the gene encoding BAD2 which contains the deletion and SNP, resulting in a frame shift that generate a premature stop codon that presumably disables the BAD2 enzyme.

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Abbreviations : 2-AP, 2-acetyl-1-pyrroline; PCR, polymerase chain reaction; SNP, single nucleotide polymorphism; SSR, simple sequence repeat.

In the present study, 1.7% KOH sensory test, GC-MS-SIM (Yoshihashi et al., 2004) and the PCR analysis were applied to characterize aroma in Afghan native rice cultivars. Until now comparative analysis among these three methods has not been reported in Japan.

The objectives of this study were to accurately classify aromatic and non-aromatic rice with characterization of agronomic characters of Afghan native rice cultivars.

Materials and Methods

1. Plant materials

Ten Afghan native rice cultivars, Permel, Sherkati, Luke Qasan, Sela Doshi and Sarda Bala were collected from Baghlan province in the north. Sela Takhar, Lawangi and Germa Bala from Takhar province in the northeast. Pashadi Konar from Konar province and Monda Mashruqi from Laghman province in the east. All these are native Afghan rice cultivars and could be representative of many other native rice cultivars in the country. Sela Doshi, Sarda Bala, Sela Takhar, Lawangi, Germa Bala and Pashadi Konar are the most famous aromatic rice cultivars in Afghanistan.

In addition to the above-mentioned Afghan rice cultivars, Japanese cultivars of Nipponbare (non-aromatic), Izayoi, Oitakoutou and Jakouine (aromatic), and aromatic cultivars Basmati 370 (India) and Jasmine 85 (Thailand) were used in this research. A population of 200 F_2 individuals derived from the cross between Nipponbare and Jasmine 85 were also used for genetic analysis of aroma and agronomic characters.

2. Field experiment

All the plant materials were grown under the same conditions in Honmachi Farm of Tokyo University of Agriculture and Technology (Japan). Rice seeds were sown in plastic trays using the soil treated with fungicide.

The field experiment for varietal evaluation was performed in a completely randomized block design with three replications. Each replication was consisted of 16 plots, and 50 plants per each plot were grown in an area of 2 m^2 (totally 150 plants of each cultivar were grown with three replications). One seedling per hill at three weeks after sowing were randomly transplanted in a space of 20×20 cm. Seventy-five plants of each cultivar were selected for measurement and analysis. All the F_2 plants were used for agronomic character analysis, as well as for aroma evaluation.

Nitrogen (N), phosphorus (P) and potassium (K) at a rate of 70N-40P-70K kg ha^{-1} were applied at three times as follows : One-third of N along with P and K before transplanting, 1/3 of N 20 days after transplanting, and further 1/3 of N 50 days after transplanting.

3. Measurement and analysis of agronomic traits

The plant height was measured in centimeters from ground level to the tip of extended panicle on the main stem after heading stage. Panicle number per plant was recorded after full heading stage. Multi Auto Counter (Daizen Ginken Japan) was used to count the number of grains per panicle. For 1000-grain weight, a random sample of 1,000 well-developed grains was weighed on a precision electronic balance. Grain length and width were measured by using vernier calipers. Grain length was measured in millimeters as the distance from the base of the lowermost sterile lemma to the tip (apiculus) of the fertile lemma or palea, whichever is longer. Grain width was measured in millimeters as the distance across the fertile lemma and the palea at the widest point.

4. Sensory test

In the sensory test, 100 mg of green rice leaves at the heading stage were weighed, cut into small pieces and placed in Petri dishes. Then 10 mL of 1.7% KOH solution was added onto leaves. The Petri dishes were covered and left for one hour at room temperature. The evaluation was done by obtaining the average-score from 0 to 1, and scored from 0 to 0.5 as (–) for non-aroma and from 0.5 to 1 as (+) for aroma, by six panelists.

5. Analysis of 2-acetyl-1-pyrroline

Rice grains were used for quantitative analysis of 2-acetyl-1-pyrroline (2-AP). The grain samples stored at 5°C in a sealed polypropylene bag were hulled with a rice huller (TR-200, Kett, Japan), ground with a 0.5 mm-mesh screen, and put in the vials with a polytetrafluoroethylene (PTFE) septa and screw caps.

From 100 mg of ground grains, 2-AP was extracted in 400 mL of 100% ethanol containing 200 ppb of 2-acetyl-(methyl-^{13}C)-1-pyrroline as an internal standard, and the extract was incubated for 2 hr at 70°C. Then 150 μL of supernatant after centrifugation at 11,100 *g* for 15 min at 4°C was subjected to GC-MS-SIM (Yoshihashi et al., 2004).

6. PCR experiment

Total DNA from green leaves was extracted by the rapid DNA extraction method using cetyl trimethyl ammonium bromide (CTAB) method (Doyle and Doyle, 1987). PCR (ASTEC, Gene Amp PC system 320, Japan) was performed with 25 μL of reaction components including 15.4 μL of double distilled water (DDW), 1 unit of *Taq* DNA Polymerase (TaKaRa, Japan), 20 ng of genomic DNA, 2.5 μL of 10X buffer (Fermentas Inc®), 2 μL of 50 mM $MgCl_2$ (Fermentas Inc®), 2 μL of dNTPs [5 mM], 10 pM of each primer, external sense primer (ESP) 5´-TTGTTTGGAGCTTGCTGATG-3´, internal fragrant antisense primer (IFAP) 5´-CATAGGAGCAGCTGAA

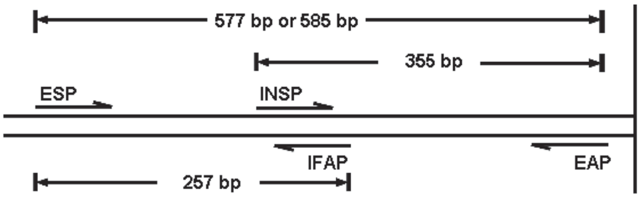

Fig. 1. Relative positions of PCR primers used for determination of aromatic and non-aromatic rice. External antisense primer (EAP) and external sense primer (ESP) generate a fragment of approximately 580 bp as a positive control for each sample. Internal non-fragrant sense primer (INSP) and the corresponding external antisense primer (EAP) produce a 355 bp fragment from a non-aroma allele. Internal fragrant antisense primer (IFAP) and the corresponding external sense primer (ESP) produce a 257 bp fragment from an aroma allele (Bradbury et al., 2005b).

ATATATACC-3´, internal non-fragrant sense primer (INSP) 5´-CTGGTAAAAAGATTATGGCTTCA-3´ and external antisense primer (EAP) 5´-AGTGCTTTACAAAGTCCCGC-3´, to distinguish between aromatic and non-aromatic rice (Fig. 1), (Bradbury et al., 2005b).

The PCR conditions were as follows : denaturation at 94°C for 2 min followed by 30 cycles at 94°C for 30 s, at 58°C for 30 s, at 72°C for 30 s, and final elongation step at 72°C for 5 min. PCR products were separated by electrophoresis on a 1% agarose gel. For the estimation of fragment size, we used 1 Kb MassRuller™. The gel was stained with ethidium bromide for 20 min, and then photographed under UV light.

Results and Discussion

1. Agronomical characterization

In the present study, important agronomic characters of aromatic and non-aromatic native rice cultivars from Afghanistan and other countries were evaluated. The plant height of Afghan cultivars, Permel, Sherkati, Sarda Bala, Sela Doshi, Sela Takhar, Germa Bala, Lawangi, and Indian cultivar Basmati 370 ranged from 138.7±7.2 to 194.2±21.4 cm (mean±standard deviation). According to the IRRI index (IBPGR-IRRI, 1980), this range belongs to the tall plant group. The plant height of Afghan cultivar Pashadi Konar, and Japanese cultivars Izayoi and Nipponbare ranged from 110.8±2.6 to 125.2±3.1 cm, which belong to the intermediate plant group. The plant height of Afghan cultivars Monda Mashruqi and Luke Qasan, and Thai cultivar Jasmine 85 ranged from 89.3±0.4 to 101±8.9 cm, which belong to the short plant group (Fig. 2).

Although a short rice plant does not guarantee a high degree of lodging resistance (Ookawa and Ishihara, 1992), most of the Afghan native rice cultivars with tall culm were susceptible to lodging after heading stage. Thus, breeding for reduction of the

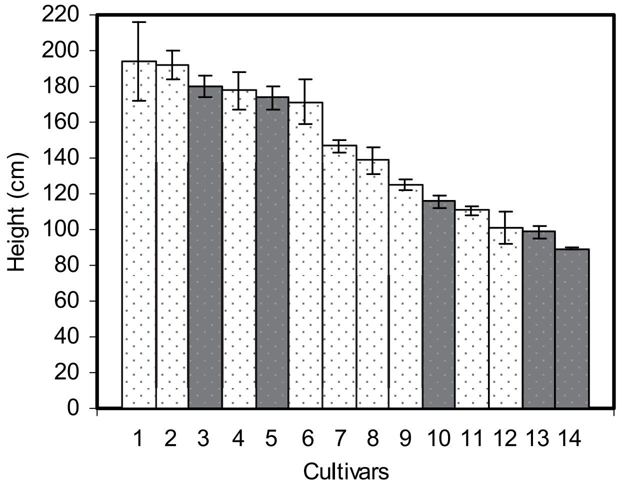

Fig. 2. Distribution of plant height among aromatic (white columns) and non-aromatic rice cultivars (black columns). The values express means±standard deviations. Seventy-five plants per cultivar were used for measurement of the plant height. 1, Basmati 370; 2, Germa Bala; 3, Sherkati; 4, Lawangi; 5, Permel; 6, Sarda Bala; 7, Sela Takhar; 8, Sela Doshi; 9, Pashadi Konar; 10, Nipponbare; 11, Izayoi; 12, Jasmine 85; 13, Monda Mashruqi; and 14, Luke Qasan.

plant height is an important objective in Afghanistan. Clearly, linkage was not found between aroma and plant height (Fig. 2).

The yield of paddy rice is mainly determined by three components, panicle number per plant, grain number per panicle, and 1,000-grain weight (Hoshikawa, 1989; Kang et al., 2007; Haryanto et al., 2008). In the present study, panicle number per plant ranged from 8±1.5 to 13±1.3, and it was not correlated with grain number per panicle, because Luke Qasan and Germa Bala with the largest number of panicles per plant had the lowest number of grains per panicle, while, Sela Takhar, Sela Doshi and Pashadi Konar with the appropriate number of panicles per plant had the largest number of grains per panicle. Grain number per panicle in Afghan native rice cultivars widely ranged from 69 to 169. Most Afghan cultivars had more than 100 grains per panicle, and this character is desirable for breeding programs. The 1,000-grain weight ranged from 20 to 32 g and Pashadi Konar from Afghanistan had the heaviest grain (32±3.5 g) (Table 1).

Basmati rice has extremely thin and slender grains (Singh et al., 2000). Grain length and width determine the grain shape that is preference character to consumers with aroma. Grain length in Germa Bala, Sherkati, Lawangi, Permel, Sarda Bala, Sela Takhar, Sela Doshi and Pashadi Konar from Afghanistan ranged from 9.4±0.4 to 11.0±0.3 mm, while Basmati 370 had 10.1±0.2 mm grain length. Grain width in the above-mentioned Afghan cultivars ranged from 2.2±0.2 to 2.7±0.2 mm, but that in Basmati 370 was 2.4±0.0 mm (Table 1).

Table 1. Comparison of yield components and agronomic characters among native Afghan rice cultivars and check cultivars.

Cultivars		Panicle number per plant [a]	Grain number per panicle [a]	1000-grain wt. (g) [a]	Grain length (mm) [b]	Grain width (mm) [b]
1	Basmati 370 (Check)	8±1.5	153±16.4	24±1.5	10.1±0.2	2.4±0.0
2	Germa Bala	13±2.6	74±11.5	29±1.5	10.1±0.3	2.6±0.2
3	Sherkati	9±2.5	151±23.0	32±0.4	10.8±1.0	2.4±0.1
4	Lawangi	11±0.4	123±28.0	27±1.1	9.3±0.3	2.2±0.2
5	Permel	11±2.0	99±25.2	30±2.3	10.7±0.2	2.7±0.2
6	Sarda Bala	14±3.6	144±15.5	26±1.5	10.4±0.4	2.5±0.0
7	Sela Takhar	11±1.5	169±53.9	25±1.5	10.6±0.4	2.3±0.0
8	Sela Doshi	11±1.5	157±26.2	24±2.1	9.4±0.4	2.5±0.0
9	Pashadi Konar	9±0.3	148±21.0	32±3.5	11.0±0.3	2.5±0.1
10	Nipponbare (Check)	9±1.5	125± 8.6	25±2.0	7.0±0.1	3.2±0.2
11	Izayoi (Check)	9±1.5	175±59.4	24±1.3	9.7±0.8	3.0±0.0
12	Jasmine 85 (Check)	10±2.0	149±32.6	27±1.3	10.2±0.2	2.6±0.0
13	Monda Mashruqi	11±0.6	114±19.2	20±0.7	8.2±0.3	2.5±0.0
14	Luke Qasan	13±1.3	69±10.8	20±3.1	7.3±0.3	3.6±0.3

[a], Seventy-five plants of each cultivar were used for analysis; [b], Twenty grains of each cultivar were used for analysis; Check, Standard cultivars, the values express means±standard deviations.

Almost all Afghan native rice cultivars were the same as Basmati 370 in grain number per panicle, 1,000-grain weight, and grain length and width. Thus, Afghan native rice cultivars had thin and slender grains, like Basmati 370, but had a larger panicle number per plant than Basmati 370. Pashadi Konar from Afghanistan had an intermediate plant height, desirable yield components having thin and slender grain, and favorable aroma. Thus, this cultivar could be suitable for further improvement of aromatic rice in breeding programs.

We analyzed the plant height in the F_2 generation of the cross between Nipponbare (P_1) and Jasmine 85 (P_2). The height of P_2 plants ranged from 100 to 110 cm and that of P_1 plants and the F_1 generation ranged from 110 to 120 cm. The distribution of plant height in the F_2 population ranged from 80 to 130 cm (Fig. 3). The plant height exhibited a continuous segregation in the F_2 population. We selected plant height for characterization, because it is one of the most important characters in breeding programs.

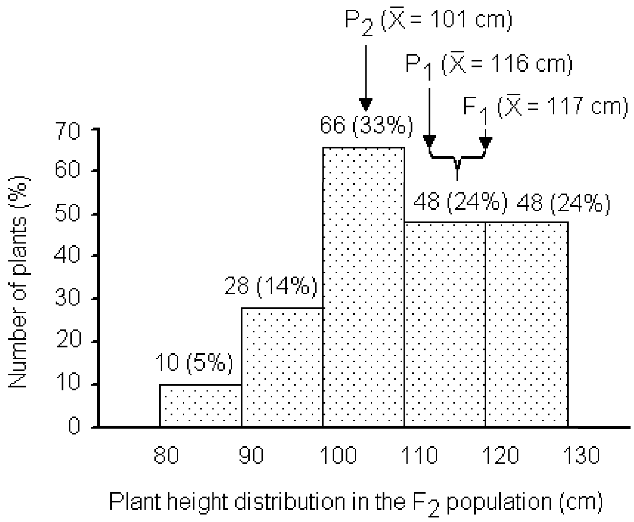

Fig. 3. Frequency distribution of plant height in the F_2 (200) individuals derived from the cross between Nipponbare and Jasmine 85 was analyzed. The average of plant height in Nipponbare (P_1), Jasmine 85 (P_2) and F_1 are shown on the top of their belonging histogram.

2. Chemical and molecular characterization of aroma in rice

In this study, sensory, GC-MS-SIM and PCR methods were applied to distinguish between aromatic and non-aromatic rice.

In the sensory test, leaf tissue after the reaction with 1.7% KOH (Sood and Siddiq, 1978) was sniffed. The results showed that six Afghan native rice cultivars, Germa Bala, Lawangi, Sarda Bala, Sela Takhar, Sela Doshi and Pashadi Konar, were aromatic and four Afghan cultivars, Permel, Sherkati, Luke Qasan and Monda Mashruqi were non-aromatic (Table 2).

The aroma of Basmati and Jasmine types of rice is associated with increased levels of 2-acetyl-1-pyrroline (2-AP) (Buttery et al., 1983; Lorieux et al., 1996; Widjaja et al., 1996; Yoshihashi, 2002). In this study, therefore, the concentration of 2-AP was analyzed by the GC-MS-SIM method (Yoshihashi et al., 2004). The results showed that the concentration of 2-AP ranged from 0 to 324 ppb, and that the rice with a high level of 2-AP had strong aroma and that containing no 2-AP

Table 2. Identification and characterization of aromatic and non-aromatic rice cultivars by three methods.

	Cultivars	Origin	1.7% KOH test	2-AP (ppb)	Molecular marker
1	Basmati 370 (Check)	India	+	148	A
2	Germa Bala	Afgh	+	145	A
3	Sherkati	Afgh	–	0	N
4	Lawangi	Afgh	+	160	A
5	Permel	Afgh	–	0	N
6	Sarda Bala	Afgh	+	150	A
7	Sela Takhar	Afgh	+	250	A
8	Sela Doshi	Afgh	+	214	A
9	Pashadi Konar	Afgh	+	120	A
10	Nipponbare (Check)	Japan	–	0	N
11	Izayoi (Check)	Japan	+	95	A
12	Jasmine 85 (Check)	Thailand	+	212	A
13	Monda Mashruqi	Afgh	–	0	N
14	Luke Qasan	Afgh	–	0	N
15	Jakouine (Check)	Japan	+	324	A
16	Oitakoutou (Check)	Japan	+	118	A

Check, Standard cultivars; Afgh, Afghanistan; –, Non-aroma; +, Aroma; 2-AP, 2-acetyl-1-pyrroline; N, Non-aroma; A, Aroma.

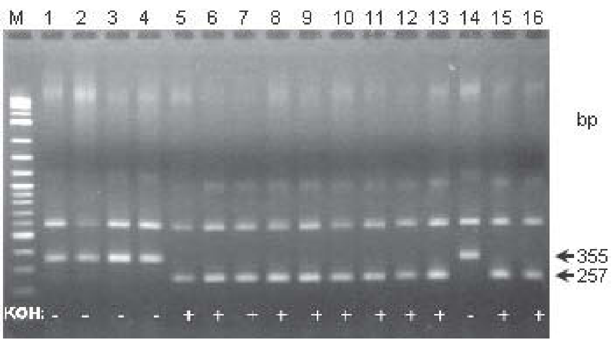

Fig. 4. Estimation of aromatic and non-aromatic rice cultivars by PCR method (355 bp band, Non-aroma; 257 bp band, Aroma) and 1.7% KOH sensory test (+, Aroma; –, Non-aroma). M, Marker; 1, Permel; 2, Sherkati; 3, Luke Qasan; 4, Monda Mashruqi; 5, Pashadi Konar; 6, Sarda Bala; 7, Sela Doshi; 8, Sela Takhar; 9, Germa Bala; 10, Lawangi; 11, Jasmine 85; 12, Basmati 370; 13, Izayoi; 14, Nipponbare; 15, Jakouine; and 16, Oitakoutou.

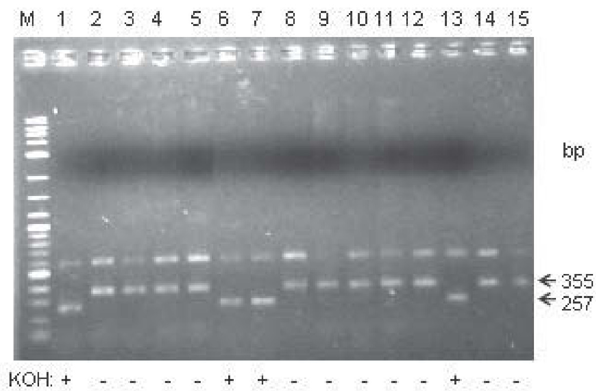

Fig. 5. Segregation of aromatic and non-aromatic rice in the F_2 individuals derived from the cross between Nipponbare and Jasmine 85 by PCR method (355 bp band, Non-aroma; 257 bp band, Aroma) and 1.7% KOH sensory test (+, Aroma; –, Non-aroma). M, Marker; Lane 1–15, F_2 individuals.

had no aroma. The highest concentration of 2-AP (324 ppb) was observed in Jakouine and the lowest (95 ppb) in Izayoi. The concentration of 2-AP in four Afghan cultivars, Permel, Sherkati, Luke Qasan and Monda Mashruqi, was 0, while it ranged from 120 to 250 ppb in the others (Table 2). The concentration of 2-AP in these Afghan native rice cultivars was high enough for breeding programs.

Bradbury et al. (2005b) reported on PCR analysis using the four primers to distinguish among homozygous aromatic, homozygous non-aromatic and heterozygous non-aromatic rice. Here, we applied the PCR analysis using these four primers to evaluate Afghan native rice cultivars, and confirmed that non-aromatic rice cultivars produced a 355 bp fragment and aromatic rice cultivars a 257 bp fragment, and found that six out of ten Afghan native rice cultivars were characterized as aromatic and four cultivars as non-aromatic (Fig. 4). The correlation among 1.7% KOH sensory test, GC-MS-SIM method and PCR analysis was high enough, and will allow us to select the Afghan native aromatic rice cultivars for the breeding program in Afghanistan (Table 2).

F_2 individuals of the cross between non-aromatic

Table 3. Genetic analysis of aromatic character by using 1.7% KOH sensory test and PCR analysis in the F_1 and F_2 generations derived from the cross between Nipponbare (non-aromatic) and Jasmine 85 (aromatic).

Cultivars	Non-aroma	Aroma	Segregation ratio	χ^2 -value	P-value
Nipponbare (♀)	15	0	1 : 0		
Jasmine 85 (♂)	0	15	0 : 1		
F_1 (N×J)	2	0	1 : 0		
F_2 (N×J)	148	52	3 : 1	0.1	0.70 - 0.80

N, Nipponbare; J, Jasmine 85; ♀, Female; ♂, Male.

Nipponbare and aromatic Jasmine 85 were analyzed for aromatic character by using the 1.7% KOH sensory test along with the PCR analysis. The sensory test and PCR analysis gave similar results. The results showed that 148 F_2 individuals were non-aromatic and 52 aromatic (Fig. 5).

The observed segregation ratio between non-aromatic and aromatic rice in the F_2 population was tested by χ^2 analysis against the expected ratio for a single gene. A 3 : 1 segregation between non-aromatic and aromatic (df=1, χ^2=0.1, P=0.70–0.80) was observed in the F_2 population (Table 3).

One recessive gene codes for aroma, and the gene is located on chromosome 8 (Berner and Hoff, 1986; Ahn et al., 1992). Pinson (1994) reported two complementary recessive genes. Although many researchers reported that one gene controls the aroma in rice, in some articles the possibility of control by plural genes has also been reported. In the present study, Afghan native aromatic rice cultivars produced the same PCR band as Jasmine 85 and F_2 segregated for aroma, while Afghan native non-aromatic rice cultivars produced the same PCR band as Nipponbare and F_2 segregated for non-aroma. Thus, our results are also confirmed that aroma is controlled by a single recessive gene.

PCR and GC-MS-SIM are not widely available in developing countries including Afghanistan. In the present study, the results of the 1.7% KOH sensory test closely correlated with those obtained by GC-MS-SIM method and PCR analysis, supporting the reliability of the 1.7% KOH sensory test. Further comparative analysis of these three methods is needed to establishing the sensory test as a method for characterization of aromatic rice.

Conclusion

In this study, original materials from Afghanistan were used to evaluate agronomic and aroma characters. The obtained results can help breeders for the improvement of aromatic rice in Afghanistan and in other countries.

Aromatic rice cultivars such as Basmati 370 and Jasmine 85 were introduced originally from India and Thailand. This study showed that native Afghan rice cultivars such as Germa Bala, Sarda Bala, Sela Takhar, Sela Doshi, Pashadi Konar and Lawangi, which have favorable aroma and the desirable agronomic characters such as thin and slender grains, can be used for further breeding of aromatic rice. Demand for the above-mentioned characters is very high in Afghanistan and surrounding regions.

In the present study, aromatic and non-aromatic rice were analyzed by using 1.7% KOH sensory test, PCR analysis and GC-MS-SIM method. The 1.7% KOH sensory test was useful for distinguishing between aromatic and non aromatic rice in Afghanistan. GC-MS-SIM and PCR methods will be useful for breeders to streamline the breeding process and reduce the development time for new cultivars in Afghanistan in the future.

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