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Grain Quality and Allelic Variation of the *Badh2* Gene in Thai Fragrant Rice Landraces

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Abstract: Fragrance, which plays an important role in determining the economic value of rice to growers and consumers, is known to be controlled by the *Badh2* gene. This study evaluated the grain quality characteristics and allelic variation of the *Badh2* gene in 22 fragrant rice landraces from Thailand. The rice seed samples from farmers' storage facilities in northern, northeastern and southern Thailand, plus two advanced breeding lines and three check varieties, were evaluated for seed morphology and grain quality, and their *Badh2* genes covering intron 4 to intron 8 were re-sequenced. Almost all of the landraces were classified as large grain types, with medium to high gelatinization temperatures. The variation in the *Badh2* gene by haplotype analysis correlated with grain aroma by sensory evaluation. The *badh2*-E7 was found in haplotype 1 with a strong aroma in KH, NDLP, and PLD, as in KDML105 and the moderately aromatic BNM-CMU, BNM4, and SKH, along with PTT1. Three haplotypes had different positions of SNP on the *Badh2* gene with varying results in the sensory test. The present results suggest that some rice varieties could be potentially introduced as genetic resources for fragrant rice breeding programs or could be developed to highly palatable cultivars with geographical indications to increase the income of highland farmers.

Keywords: aromatic rice; local variety; gelatinization temperature; *badh2*-E7 allele

1. Introduction

The eating quality of rice, also known as rice palatability, is a very important factor that determines the economic value of rice for producers and consumers—the aroma of rice plays a significant part of this. Fragrant rice is quality rice with a good taste, softness, and a unique aroma, making it very popular [1]. Aromatic or fragrant rice is the most expensive rice in the global rice market [2–4]. Among the numerous volatile compounds associated with the aroma in rice, the main aromatic compound has been identified as 2-acetyl-1-pyrroline (2AP) [5], produced under the control of the recessive gene *Badh2* [6–8]. In aromatic rice genotypes, there is an eight base-pairs deletion in exon 7 of the *Badh2* gene [9], but with the 2AP concentration and aroma varying greatly depending on the environment [10]. The most widely known aromatic rice are Basmati rice of India and Pakistan [2], and Thailand's Hom Mali [11]. Basmati, with extra-long slender grains of soft and fluffy texture when cooked, belongs to the *japonica* group, have while Hom Mali and other jasmine type varieties, with long slender grains, soft and moist texture when cooked, are more closely with the *indica* group [12]. In spite of their distinctive grain quality features, the *Badh2* gene and 2AP are common to both groups of aromatic rice. Most of India's Basmati rice is grown from the variety Pusa Basmati 1121 [13], while Thai Hom Mali, a worldwide registered trademark [14], is required by law to be grown from the traditional photoperiod sensitive varieties KDML105 and RD15 in the wet season only [15]. However, in addition to these mega-varieties, numerous other fragrant rice varieties are found in rice-growing countries of Asia [2]. The mutations on the *Badh2* gene have led to the introduction of a premature stop codon to produce a protein that disables the *Badh2* enzyme, leading to the accumulation of the 2AP substrate [16,17]. Further studies have revealed that numerous *Badh2* alleles have been detected in diverse rice (*Oryza sativa* L.) germplasms. Fragrant rice accessions have been reported in three different genetic subpopulations of rice, including *Group V* (Basmati and Sadri varieties), *indica* (Jasmine varieties), and *tropical japonica* [18]. There were many kinds of mutations in the *Badh2* gene among different rice varieties, namely the common 8 bp deletion and three single nucleotide polymorphism sites (SNPs) in the 7th exon of the *Badh2* gene (*badh2.1* or *badh2*-E7) [9,19]; a 7-bp deletion in exon 2 (*badh2*-E2.1) [16]; a 7-bp insertion in exon 8 (*badh2*-E8) [20]; an additional eight alleles in exons 1 (*badh2*-E1.1), 10 (*badh2*-E10.1, *badh2*-E10.2, *badh2*-E10.3) 13 (*badh2*-E13.1 and *badh2*-E13.2) and 14 (*badh2*-E14.1 and *badh2*-E14.2) [12]; a 803-bp deletion between exons 4 and 5 (*badh2*-E4-5.2) [21]; a SNP at exon 10 (*badh2*-E10.4); a 75-bp deletion in exon 2 (*badh2*-E2.2); a 806-bp deletion between exons 4 and 5 (*badh2*-E4-5.1) [22]; a 3-bp deletion in the 5′ UTR (*badh2-p-5′ UTR*); an 8-bp insertion in the promoter [23]; an SNP at the exon1–intron1 junction (*badh2*-E1.2) [24]; and a 3-bp deletion in exon 12 (*badh2*-E12) [25].

Detailed studies have been conducted on the *Badh2* gene in the aromatic rice germplasm of Myanmar [26,27], while outstanding commercial potential has been seen with Cambodian Phka varieties [28,29]. Several aromatic rice genotypes have been identified in Thailand, including those with special quality characteristics, e.g., those with pigmented pericarp, Leum Pua [30,31] and Hom-nil [32,33]. Accessions of a fragrant rice landrace, commonly known in some parts of northern Thailand's highlands as Bue Nur Moo (fragrant non-glutinous rice in the Karen language, here referred to as BNM), collected from farmers have been found to range widely both in the content of the aromatic compound 2AP and in the allelic variation of the aroma gene *Badh2* [34]. Two accessions of the landrace were found, with the 2AP content approaching that of KDML105 and the same key deletion of the *Badh2* gene, designated BNM-CMU and BNM4, while others did not have the important deletion, containing little to no fragrance. The BNM accessions also exhibited strong interaction effects of genotype × location on their grain quality characteristics, the head rice yield, gelatinization temperature and 2AP concentration [35]. The dominance of KDML105 in fragrant rice production is a relatively recent development in Thailand, where fragrance was once a common feature among the diversity of grain quality and adaptation traits of local rice landraces [36]. The present study set out to examine the grain quality of fragrant rice landraces from different regions of the country and to explore the allelic variation of the *Badh2* gene.

In Thailand, Hom Mali is grown mostly from the variety Khao Dawk Mali 105 (KDML105), which was judged the world's best rice for two consecutive years at the 9th The Rice Trader (TRT) World Rice Conference 2017 [37]. However, in the following years the title was lost to the Cambodian Malys Angkor, and Vietnam's ST24 [38]. This causes some concern regarding the competitiveness of Thai Hom Mali. With an economic advantage of much higher price than non-aromatic rice, studies of aromatic rice germplasm should contribute towards maintenance and improvement in the rice quality standards.

Thailand is one of the most significant and unique countries for plant genetic resources and crop diversity, especially for rice (*Oryza sativa* L.) [39]. As Thailand lies partly in the center of rice diversity and in the region where rice was originally domesticated [40,41], the characterization of local varieties and landrace collections are critical for the utilization of these resources. The farmers of this region still use their traditional or local cultivars, which not only suit their taste, but also provide crop security. Rice landraces have unique characteristics, including special quality traits such as their aroma, adaptation to the local environment and resistance to biotic and abiotic stress [42]. In addition, local rice contains high levels of genetic diversity, which provides an opportunity for plant breeders to select and improve new cultivars, which include both farmers and breeder's preferred traits. Hence, the development of new modern rice varieties has depended on the availability of genetic diversity [43–46].

Local fragrant rice germplasm of northern Thailand are high-value genetic resources as they contain special qualities. Many fragrant rice varieties of northern Thailand have been collected and selected by Chiang Mai University's breeding team for their high 2AP content in brown rice compared to the famous jasmine rice of Thailand. Initial studies focused on nine populations of one Thai fragrant rice landrace, Buer Ner Moo (BNM), from nine farmers and the two advanced breeding lines of JPD and, BNM-CMU, a cross between BNM and PTT1. The different local fragrant rice varieties showed different levels of 2AP content in brown rice, even within one variety name, due to the variation within landraces. Moreover, *Badh2* allelic variation was found, which illustrated the *badh2-E7* deletion on the *Badh2* gene in BNM4, and BNM-CMU. They had high 2AP contents in brown rice with *badh2*-E7 deletion similar to that in KDML105 and PTT, the popular fragrant rice in Thailand [34]. In addition, a significant correlation was also found between 2AP content in brown rice and elevation. The 2AP content in brown rice of most of the Bue Ner Moo populations significantly increased when grown at high altitude [35]. Therefore, the local rice varieties that profess to be fragrant rice could have the potential to be developed into a new fragrant rice variety with geographical indications to increase income for the highland farmers. However, the studies of the fragrant gene in landrace rice are not well understood. To investigate germplasm resources for crop utilization and improvement programs, it is essential to describe and evaluate the morphological characteristics of existing germplasm resources to effectively identify and differentiate each cultivar, including identification of the interested genes' functional alleles.

Therefore, the objectives of this study were to evaluate the grain qualities, to investigate the allelic variation of the *Badh2* gene in fragrant rice landraces, and to introduce a genetic resource for fragrant rice breeding programs by pure-line selection method or improved variety. This would also provide a basis for the development of other varieties to be consistent with the increasing demand of the market and consumers.

2. Materials and Methods

2.1. Plant Materials

Seed samples of fragrant rice landraces were collected from farmers' seed storages, including nine from the north, eight from the northeast and five from the south of Thailand. Two promising landraces previously identified by a rice breeding program at Chiang Mai University, BNM-CMU and Jow Pluak Dam (JPD), plus two elite fragrant varieties, Khao Dawk Mali 105 (KDML105) and Pathum Thani 1 (PTT1), and one high yielding non-fragrant variety Suphan Buri 1 (SPR1) were also included for comparison (Table 1). The seeds of each population were germinated in petri dishes for 14 days and then transplanted to 30 cm diameter undrained pots, with ten plants per pot, two pots per variety and two replications. The plants were grown as wetland rice, with 15 cm of water maintained above the soil surface at Chiang Mai University, Thailand, in the wet season of 2017/2018. At the tillering stage, leaf samples of each individual were collected and kept at −20 °C for DNA extraction. At grain maturity, seed samples were collected from 10 individual plants from each variety.

Table 1. List of the fragrant rice landraces from three regions of Thailand, the elite varieties, and the advanced breeding lines studied for comparison.

Rice Samples	Source	Code	Rice Variety Name	Source (Province)
Rice landrace	North	BNM1	Buer Ner Moo	Chiang Mai
		BNM2	Buer Ner Moo	Chiang Mai
		BNM3	Buer Ner Moo	Chiang Mai
		BNM4	Buer Ner Moo	Chiang Mai
		BNM5	Buer Ner Moo	Chiang Mai
		BNM6	Buer Ner Moo	Chiang Mai
		BNM7	Buer Ner Moo Pho Phi	Chiang Mai
		BNM8	Buer Ner Moo Pho Phi	Chiang Mai
		BNM9	Buer Ner Moo Phardo	Chiang Mai
	Northeast	EL	E-Leuang	Loei
		KH	Kaow Hawm	Loei
		HS	Hawm Sa-ngium	Loei
		NDLP	Niaw Dam Luem Pua	Loei
		NU	Niaw Ubon	Loei
		PS	Pla Sew	Loei
		PLD	Phayaa Luem Dang	Loei
		SKH	Sew Kliang Hawm	Loei
	South	BH62	Baow Hawm 62	Songkhla
		BH96	Baow Hawm96	Songkhla
		HB	Hawm Baow	Songkhla
		HBK	Hawm Bang Kaew	Songkhla
		HNK	Hawm Na Kaow	Songkhla
Breeding Line		BNM-CMU	Buer Ner Moo-CMU	Department of Agriculture
		JPD	Jow Pluak Dam	Department of Agriculture
Elite rice		KDML105	Khao Dawk Mali 105	Department of Agriculture
(as check varieties)		PTT1	Pathum Thani 1	Department of Agriculture
		SPR1	Suphan Buri 1	Department of Agriculture

2.2. Seed Morphological Characterization

The rice seed morphological characterization was conducted in accordance with the rice descriptor [47]. One hundred seeds of each sample were recorded individually for husk color, pericarp color, and awning. Seed sizes (grain length, width, and thickness) of unhusked seeds were measured by a Digital Vernier caliper (Draper Tools. Ltd., Chandler's Ford, United Kingdom) and classified into shape by the scheme of Matsuo graph [48]. Diversity in seed morphological characteristics was determined by the Shannon–Weaver index (H') [49], defined as:

$$H' = -\sum_{i=1}^{k} pi\ In\ pi \quad (1)$$

where k is the number of phenotypic classes for a trait and $ln\ pi$ is natural log of the proportion of individuals in the ith class of the trait.

2.3. Grain Qualities

Alkali spreading assay was performed to determine the gelatinization temperatures [47,50]. One hundred whole grains of milled rice from each experimental unit were placed in a petri dish, with 20 grains per dish. Subsequently, 10 mL of 1.7% KOH was added to the petri dishes and kept at room temperature for 23 h. After the 23 h incubation, the seeds were evaluated visually and given a score in accordance with the following seven point scale: (1) grain not affected; (2) grain swollen; (3) grain swollen, collar incomplete and narrow; (4) grain swollen, collar complete and wide; (5) grain split or segmented, collar complete and wide; (6) grain dispersed, merging with collar; and (7) grain completely dispersed and intermingled [51]. The alkali spreading value corresponded to the gelatinization temperature as follows: 1–2, high temperature (74–80 °C) and hard when left to cool after cooking; 3–5, intermediate temperature (70–73 °C) and medium hardness when left to cool after cooking; and 6–7, low temperature (<70 °C) and soft when left to cool after cooking [52]. KDML105 (low gelatinization temperature) and RD4 (high gelatinization temperature) were used as checks.

The fragrance status of each cultivars was identified by tasting the milled grain. One milliliter of 1.7% potassium hydroxide (KOH) solution was applied to ten de-husked grains of each sample for 10 min at room temperature. The presence or absence of aroma was scored from 0–3: 0 for non-aromatic, (1) for slightly aromatic, (2) for moderately aromatic, and (3) for strongly aromatic. Each individual sample was inspected multiple times by 10 trained persons to confirm the phenotype (modified from the method of [53]).

2.4. DNA Extraction and Badh2 Sequences

In order to understand the genetic basis of the fragrance in Thai rice landraces, genomic DNA was extracted from leaf samples of each individual landraces using the CTAB method [54]. The DNA of each sample was examined and sequenced for the allele *Badh2/badh2* in exon 7 of *Badh2* gene by using primers Badh2P5 (F: 5′-CCTCCGTGTTAATGCAGCTC-3′, R: 5′-CATAGCAAGTGGCATGTACC-3′) and Badh2P6 (F: 5′-GGTTGGTCTTCCTTCAGGTG-3′, R: 5′-GTCCTTCCTAACTGCCTTCC-3′) [21]. Each 50 μL reaction contained 5 μL 10X PCR buffer, 2.5 μL 2.5mM MgCI2, 0.4 μL 0.2mM Deoxyribonucleotides (dNTP), 5 μL of each primer (10 ng/ μL), 0.4 μL 0.5U Taq DNA polymerase (Thermo scientific), 40 μL dH2O and 5 μL genomic DNA (50 ng/ μL).

The amplification consisted of 94 °C/2 min, followed by 40 cycles of 94 °C/45 s, 50 °C/45 s, and 72 °C/1 min, ending with 72 °C for 5 min as the final extension. Amplified products were genotyped using 1.5% agarose gel electrophoresis. Then, staining was carried out with MaestroSafe^{TM} Nucleic Acid Stains (MAESTROGEN, Xiangshan, Hsinchu, Taiwan) and visualized under a UV transilluminator (BioDoc-It^2 imaging systems, Analytik Jena, Upland, CA, USA) before samples were sent for sequencing at Macrogen, Inc. (Seoul, South Korea). The sequence size was 1323 bp, from intron 4 to intron 8 of the *Badh2* gene.

The sequences were assembled using the DNA baser assembler v5.15.0 trial version (Heracle BioSoft S.R.L., Arges, Romania). The resulting contigs were used as BLAST queries using the data of *Badh2/badh2*, which were reported in the GenBank database of National Center for Biotechnology Information (NCBI, Bethesda, Maryland, USA) including the fragrant rice variety, SuYuNuo, and the non-fragrant rice variety, Nanjing11 (accession numbers EU7703020.1 and EU710319.1, respectively). The aligned sequences were imported into MEGA7 software [55] to compare sample sequences. Haplotype data were generated in DNAsp 5.10.01 [56]. The relationship between haplotypes was investigated by constructing median networks using Network version 5.0 [57]

2.5. Statistical Analysis

Analysis of variance was used to determine the significant difference between the different morphological characteristics at $p < 0.05$ by statistical analysis STATISTIX 8.0 (Tallahassee, FL, USA). Least significant difference (LSD) was used to indicate the mean differences in the grain size, weight, and shape between the varieties in regards to grain morphology. *T*-tests were performed between the mean sensory test score of the varieties in each haplotype pool versus the score of each check line (high and low separately).

3. Results

3.1. Seed Morphological Variation of Thai Fragrant Rice Landraces

The seed morphological traits of 22 Thai fragrant rice landrace populations are moderately heterogenous (Table 2 and Figure 1). The variation within populations was found in husk color and seed awning. A variation of husk color was found in BNM1, BNM2, BNM6, BNM7, HB, HBK, and HNK with straw colored husks mixed with brown furrows on straw. The Shannon–Weaver index (H') varied from 0.991 in HB to 0.098 in BNM2 and BNM6 while the rest of the landraces including BNM4 and BNM-CMU and three check varieties had uniform straw-colored husks. Ten Thai fragrant rice landrace populations, BNM-CMU and PTT1 had varying seed awning, from short awn and partly awn, while the remaining ten rice landrace populations, JPD, KDML105 and SPR1 were awnless. No variation was found within populations in the pericarp color; 26 rice populations had colorless pericarp except NDLP which had a purple pericarp (Table 3 and Table S2).

Table 2. Grain size and shape of paddy rice of 22 fragrant rice landrace accessions, 2 breeding lines, and 3 elite variety checks.

Varieties	Grain Length (mm)	Grain Width (mm)	Grain Thickness (mm)	Grain Shape [#]
BNM1	9.87	3.11	2.18	Large type
BNM2	10.20	3.03	2.10	Large type
BNM3	10.34	3.25	2.21	Large type
BNM4	10.41	3.12	2.15	Large type
BNM5	10.41	3.10	2.19	Large type
BNM6	10.29	3.29	2.21	Large type
BNM7	10.40	3.23	2.19	Large type
BNM8	11.08	3.28	2.18	Large type
BNM9	10.38	3.24	2.14	Large type
EL	11.08	3.50	2.22	Slender type
HS	9.98	2.51	1.79	Large type
KH	11.25	3.91	2.45	Slender type
NDLP	11.07	3.53	2.15	Slender type
NU	10.65	2.62	1.94	Large type
PLD	11.22	4.04	2.51	Slender type
PS	10.72	3.09	2.16	Slender type
SKH	10.79	3.13	1.89	Slender type
BH62	9.23	2.50	1.77	Large type
BH96	9.06	2.40	1.90	Large type
HB	9.08	2.12	1.70	Slender type
HBK	9.91	2.48	1.75	Large type
HNK	9.86	2.64	1.94	Large type
BNM-CMU	10.83	2.96	2.12	Large type
JPD	10.05	3.05	2.09	Large type
KDML105	10.52	2.49	1.88	Slender type
PTT1	10.56	2.49	1.94	Slender type
SPR1	9.60	2.38	2.06	Slender type
Mean	10.33	2.98	2.07	
SD	0.62	0.48	0.20	
CV (%)	6.16	5.77	6.46	
F-test	***	***	***	
LSD $_{(0.05)}$	0.32	0.09	0.07	

CV (%), Coefficient of variation; LSD, least significant difference; ***, Significant at the 1% level; [#] classified with the scheme of Matsuo (1952).

Table 3. Seed morphology of 22 landrace fragrant rice varieties, 2 breeding line varieties, and 3 check varieties.

Varieties	**Husk Color**		**Pericarp Traits**	**Awning**	
	Phenotype	***H′***		**Phenotype**	***H′***
BNM1	Straw and brown furrows on straw	0.135	Colorless	Short and partly awned	0.135
BNM2	Straw and brown furrows on straw	0.098	Colorless	Short and partly awned	0.227
BNM3	Straw	0	Colorless	Short and partly awned	0.135
BNM4	Straw	0	Colorless	Short and partly awned	0.168
BNM5	Straw	0	Colorless	Short and partly awned	0.456
BNM6	Straw and brown furrows on straw	0.098	Colorless	Absent	0
BNM7	Straw and brown furrows on straw	0.168	Colorless	Short and partly awned	0.325
BNM8	Straw	0	Colorless	Short and partly awned	0.423
BNM9	Straw	0	Colorless	Short and partly awned	0.254
EL	Straw	0	Colorless	Absent	0
HS	Brown furrows on straw	0	Colorless	Absent	0
KH	Straw	0	Colorless	Absent	0
NDLP	Brown furrows on straw	0	Purple	Absent	0
NU	Straw	0	Colorless	Absent	0
PLD	Straw	0	Colorless	Absent	0
PS	Straw	0	Colorless	Short awned	0
SKH	Straw	0	Colorless	Absent	0
BH62	Straw	0	Colorless	Absent	0
BH96	Straw	0	Colorless	Short awned	0
HB	Straw and brown furrows on straw	0.991	Colorless	Short and partly awned	0.683
HBK	Straw and brown furrows on straw	0.637	Colorless	Absent	0
HNK	Straw and brown furrows on straw	0.673	Colorless	Short and partly awned	0.598
BNM-CMU	Straw	0	Colorless	Short and partly awned	0.135
JPD	Black	0	Colorless	Absent	0
KDML105	Straw	0	Colorless	Absent	0
PTT1	Straw	0	Colorless	Short and partly awned	0.199
SPR1	Straw	0	Colorless	Absent	0

Sample = 100 seeds (n = 100).

The grain size of the paddy rice was found to vary between the different rice samples collected ($p < 0.05$) (Table 2 and Table S1). The grain length, width, and thickness were found to vary at 10.33 ± 0.62 mm, 2.98 ± 0.48 mm, and 2.07 ± 0.20 mm, respectively. The range of the grain length, width, and thickness of landrace rice were found to be 9.06–11.25 mm, 2.12–4.04 mm, and 1.70–2.51 mm, respectively. The two advanced breeding lines and two elite checks had ranges of 9.06–10.83 mm grain length, 2.38–2.96 mm width, and 1.88–2.12 mm thickness, and classification by the Matsuo (1952) scheme found fifteen of the rice landrace varieties along with the advanced breeding lines BNM-CMU and JPD belong to the large grain type, and the remaining seven as slender type, similar to the elite varieties, KDML105, PTT1 and SPR1 (Figure 1).

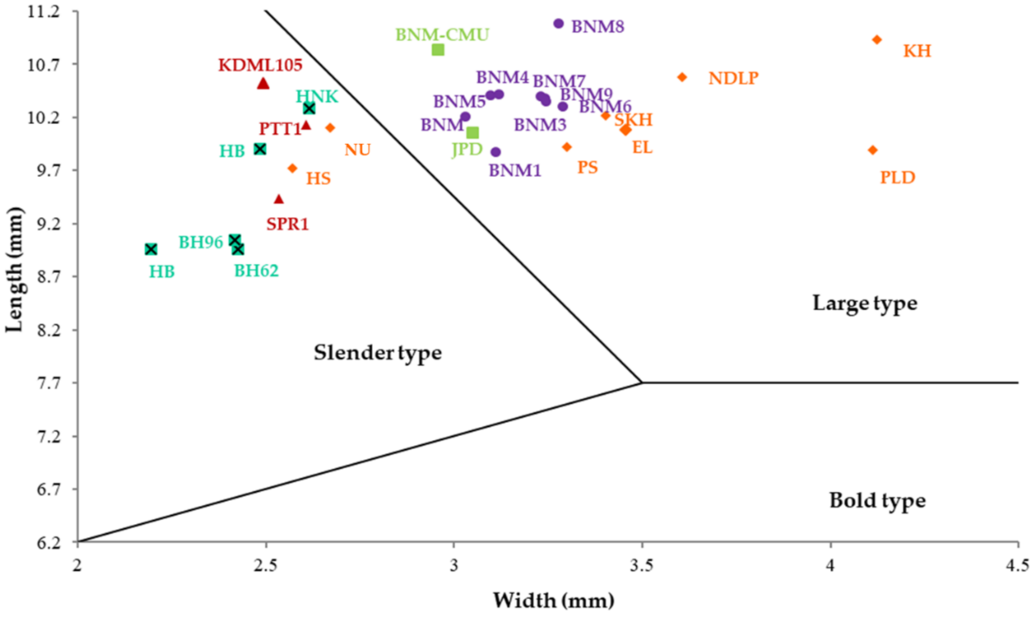

Figure 1. Shape of unhusked grains of 22 fragrant rice landrace varieties, with 2 breeding line varieties, and 3 check varieties, classed with the scheme of Matsuo (1952). Different colors and symbols identify different regions and rice groups: The nine purple circles, northern; the eight orange diamonds, the northeastern; the five blue squares, the southern; the two light green squares, promising landraces; and the three red triangles, elite fragrant varieties.

3.2. Grain Quality

Of the landraces locally recognized as fragrant when grown at Chiang Mai University and rated by a sensory test, KH, NDLP and PLD were found to be strongly aromatic in the same range as KDML105. Those found to be moderately aromatic similar to the elite variety PTT1 were BNM4, BNM-CMU and SKH. BH62, BH96, HS, JPD and PS were slightly aromatic, with no aroma detected in the remaining 43% of the germplasm (Table 4 and Table S4).

Gelatinization temperature assessed by alkali spreading assay was found to be low in four accessions of the landraces (BNM1, BNM2, EL and HB), comparable to KDML105 and PTT1, which remained soft when the cooked rice was allowed to cool to room temperature. Nine accessions (BNM3, BNM4, BNM5, BNM6, BNM7, BNM8, BNM9, JPD, and NU) had intermediate gelatinization temperatures similar to the advanced breeding line BNM-CMU, and the remaining 43% of the germplasm had high gelatinization temperatures similar to SPR1, a standard firm textured rice (Table S3).

Table 4. Nucleotide sequences of *Badh2/badh2* allele on exon 7, sensory test and alkaline test of 22 landrace fragrant rice varieties, 2 breeding line varieties, and 3 check varieties.

Haplotype	**Variety**	**Nucleotide Position**								**Sensory Test** [#]	**Gelatinization Temperature**
		2415	**2901**	**2903**	**2905-2912**	**2913**	**3233**	**3386**	**3482**		
Haplotype 1	BNM4	Insertion G	T	T	8 deletion	T	G	C	T	Moderately aromatic	Medium
	KH	Insertion G	T	T	8 deletion	T	G	C	T	Strongly aromatic	High
	NDLP	Insertion G	T	T	8 deletion	T	G	C	T	Strongly aromatic	High
	PLD	Insertion G	T	T	8 deletion	T	G	C	T	Strongly aromatic	High
	BNM-CMU	Insertion G	T	T	8 deletion	T	G	C	T	Moderately aromatic	Medium
	KDML105	Insertion G	T	T	8 deletion	T	G	C	T	Strongly aromatic	Low
	PTT1	Insertion G	T	T	8 deletion	T	G	C	T	Moderately aromatic	Low
	SuYuNuo[δ]	insertion G	T	T	8 deletion	T	G	C	T	*ND*	*ND*
Haplotype 2	BH62	Insertion G	A	A	No deletion	C	G	C	T	Slightly aromatic	High
	BH96	Insertion G	A	A	No deletion	C	G	C	T	Slightly aromatic	High
	HS	Insertion G	A	A	No deletion	C	G	C	T	Slightly aromatic	High
	JPD	Insertion G	A	A	No deletion	C	G	C	T	Slightly aromatic	Medium
	PS	Insertion G	A	A	No deletion	C	G	C	T	Slightly aromatic	High
	SKH	Insertion G	A	A	No deletion	C	G	C	T	Moderately aromatic	High
Haplotype 3	EL	Insertion G	A	A	No deletion	C	A	G	C	Non-aromatic	Low
	HBK	Insertion G	A	A	No deletion	C	A	G	C	Non-aromatic	High
	HNK	Insertion G	A	A	No deletion	C	A	G	C	Non-aromatic	High
	NU	Insertion G	A	A	No deletion	C	A	G	C	Non-aromatic	Medium
Haplotype 4	BNM1	-	A	A	No deletion	C	A	G	C	Non-aromatic	Low
	BNM2	-	A	A	No deletion	C	A	G	C	Non-aromatic	Low
	BNM3	-	A	A	No deletion	C	A	G	C	Non-aromatic	Medium
	BNM5	-	A	A	No deletion	C	A	G	C	Non-aromatic	Medium
	BNM6	-	A	A	No deletion	C	A	G	C	Non-aromatic	Medium
	BNM7	-	A	A	No deletion	C	A	G	C	Non-aromatic	Medium
	BNM8	-	A	A	No deletion	C	A	G	C	Non-aromatic	Medium
	BNM9	-	A	A	No deletion	C	A	G	C	Non-aromatic	Medium
	HB	-	A	A	No deletion	C	A	G	C	Non-aromatic	Low
	SPR1	-	A	A	No deletion	C	A	G	C	Non-aromatic	High
	Nanjing11 [δδ]	-	A	A	No deletion	C	A	G	C	*ND*	*ND*

[#] Sensory test using 10 testers, [δ] fragrant and [δδ] non-fragrant varieties obtained from NCBI accession numbers EU770320.1 and EU710319.1 respectively, *ND* = not determined.

3.3. *Badh2* Sequence Analysis

Sequences of the *Badh2* gene in exon 7 of 27 rice varieties were compared with the sequences of the two rice varieties, SuYuNuo and Nanjing1, obtained from the NCBI database, accession numbers EU7703020.1 and EU710319.1, respectively. The 1323 base pair segments of *Badh2* gene, covering from intron 4 to intron 8, revealed 8 base pair deletion in exon 7 and three single nucleotide polymorphisms (SNPs), similar to those found in the *badh2*-E7 allele of the check genotype SuYuNuo. Deletion of *badh2*-E7 was found in the strongly aromatic KH, NDLP, PLD and KDML105 and moderately aromatic BNM4, BNM-CMU, and PTT1.

Four haplotypes were identified in the analysis of the *Badh2* gene in exon 7 sequence (Figure 2). The strongly aromatic KH, NDLP, PLD and moderately aromatic BNM4, together with the advanced breeding line BNM-CMU and the elite varieties KDML105 and PTT1 were identified as haplotype H1. Haplotype H2 comprised the moderately aromatic SKH, and the slightly aromatic BH62, BH96, HS, PS, and JPD, containing non-8 bp deletion and 3 SNP at 2901, 2903 and 2913. Haplotype H3 comprised EL, HBK, HNK and NU, displaying similar SNP to haplotype H2, except at the 3233 sites, which showed the G/A transition and the 3482 sites showing the T/C transition. The last haplotype, H4, contained BNM1, BNM2, BNM3, BNM5, BNM6, BNM7, BNM8, BNM9 and HB, which had no aroma in the sensory test, along with the non-aromatic check, SPR1, containing similar sequences to non-aromatic Nanjing1 (Figure 3).

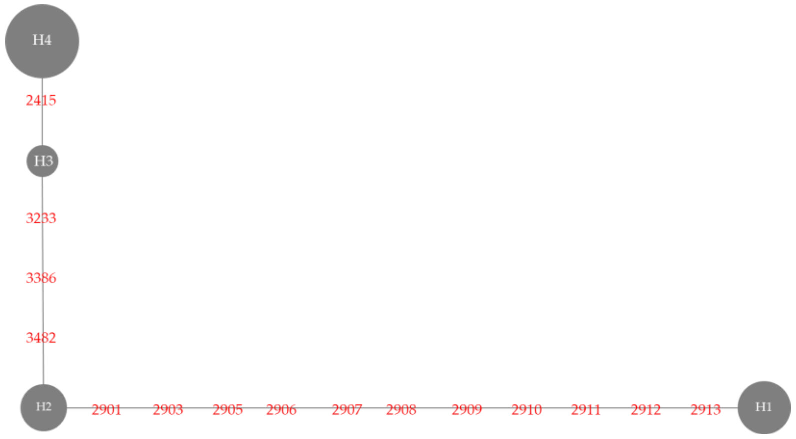

Figure 2. The haplotype network of the *Badh2* gene. Letters inside the circle correspond to the haplotype name. The numbers on the lines indicate the position of nucleotide substitution polymorphisms. The size of each circle is proportional to the number of individuals varieties possessing that haplotype.

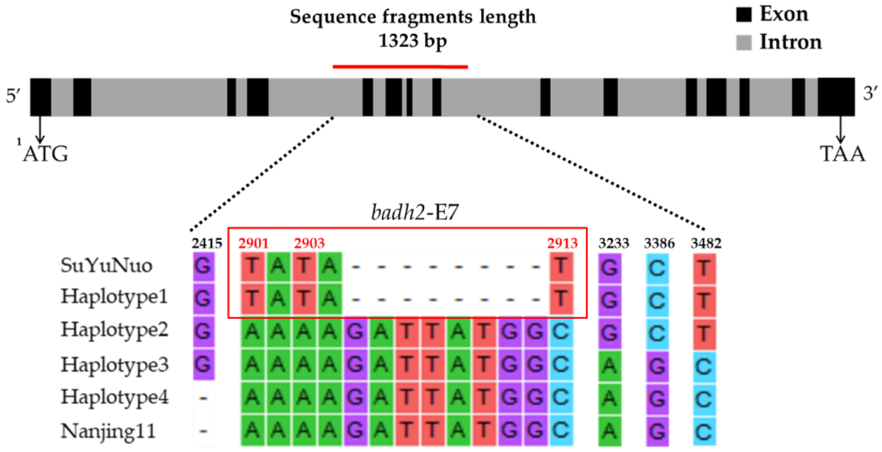

Figure 3. Extended haplotypes. Haplotype analysis of the sequence covering in the vicinity of *badh2*-E7 regions. 2415 site was in intron 4; 3233, 3386 and 3482 sites were in intron 8 of the *Badh2* gene.

Statistical T-test analysis identified significant differences between the sensory test score of the varieties in each haplotype pool versus the score of the high check line (Figure 4a) and low check line (Figure 4b).

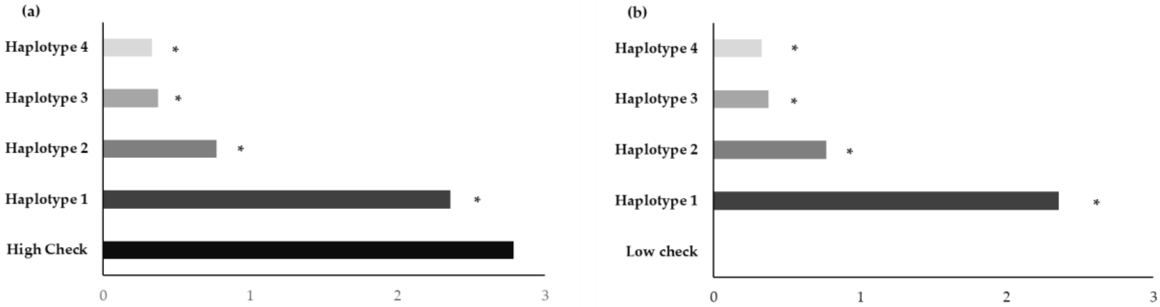

Figure 4. Statistical T-test analysis between the mean sensory test score of the varieties in each haplotype pool versus the score of high check line (**a**) and low check line (**b**); *, significant at the 5% level. Aroma was scored as 0 for non-aromatic, 1 for slightly aromatic, 2 for moderately aromatic, and 3 for strongly aromatic.

4. Discussion

The 22 rice landraces from northern, northeastern, and southern Thailand and two advanced breeding lines recognized locally as fragrant varieties have been found to vary considerably in their grain morphology, cooking quality, aroma and allelic variation of the fragrant gene *Badh2*. The landraces were classified mainly as large grain type, especially those from the highland of the north and northeast, where large grain type rice is preferred, as in the neighboring Lao PDR [58–60]. Diversity of local taste was also indicated in gelatinization temperature variation, ranging from low, in the same range as KDML105 and PTT1; intermediate, similar to the advanced breeding line BNM-CMU; to high as in the elite high yielding, non-aromatic variety SPR1. The premier Thai rice variety KDML105, with its long slender grain, low amylose content and low gelatinization temperature, has become the international standard for aromatic jasmine rice, just as it has long been in Thailand [61]. The grain quality features of KDML105 provide the benchmark for the selection of jasmine type grain quality in Thai breeding programs [31,36], and other areas [62–64], which commonly utilize KDML105 or other genotypes with similar grain quality characteristics as the parent. The rice landraces in this study clearly differentiate from KDML105 in their grain quality. The majority of the accessions belong to the large grain type. The few slender grain accessions either had high gelatinization temperatures or were non-aromatic when grown in Chiang Mai. Similar to the local Indian aromatic rice varieties of small and medium grain that are classed as non-Basmati [2], the local landraces in this study may be classed as non-jasmine type aromatic rice. It should be recognized as a distinctive and valuable set of rice genetic resources utilized and preserved on-farm. Unique among the landraces studied was NDLP, which is a glutinous rice with pigmented pericarp, sold as a premium priced, special quality rice [30]. It has potential health and pharmaceutical applications due to its anthocyanin content, phenolics and flavonoids, and high anti-oxidative properties [65].

The presence of the aromatic *badh2*-E7 allele encoding the production of the compound 2AP [19] confirmed the strong aroma in KH, NDLP and PLD, as in KDML105. However, the presence of *badh2*-E7 in the moderately aromatic BNM-CMU, BNM4 and SKH, along with PTT1 that was previously shown to have a significantly lower 2AP concentration than KDML105 [34], suggests some yet to be identified genotype-specific attenuating factors. Variation in aroma and 2AP content of KDML105 by environment and management in the lowlands, on the other hand, is well established [66–69]. A strong G x E interaction effect on the concentration of 2AP, as well as the aroma by sensory test was found among accessions of Bue Nur Moo (BNM) grown at 330 and 800 m elevations, having no effect on the 2AP content of KDML105 [35]. The absence of aroma in the BNM accessions except for BMN4 in the present study, therefore, suggest a possible environmental effect on the aroma of these landraces from the highlands, and similarly, in the accessions from other regions. The absence of the *badh2*-E7 allele in many of the accessions, however, suggests that local perception of the aroma in rice may be complex and not determined simply by the compound 2AP.

The fragrance of rice is mainly controlled by the major gene, *Badh2*, but expression of the gene, and thus, the concentration of the aromatic compound 2AP is influenced by climatic conditions and crop management [70]. For instance, the soil conditions, nutrients, plant growth regulators, planting density, irrigation draining, harvesting, post-harvest practices and storage temperature all have an effect [71]. However, these rice landraces were all grown together under the same environment and management. The complete lack of aroma in the genotypes with haplotype 4 may either indicate a different local definition of aroma that does not involve the compound 2AP, or a case of misnaming as the haplotype also includes the non-aromatic elite variety SPR1. An as yet unknown regulatory mechanism controlling the expression of the *Badh2* gene and the strength of the aroma is suggested by the variation in the aroma among the genotypes of haplotype 1, including the well-established difference between KDML105 and PTT1 [34]. The occurrence of deletion/insertion at other locations of the *Badh2* gene or the presence of other genes related to aroma characteristics increasing or decreasing of 2AP formation in aromatic rice [10]. This appeared to have taken place with the difference in the nucleotide sequences between haplotype 1 and haplotype 2. However, it is yet to be verified if the absence of the aroma in the genotypes with haplotype 3 is location-specific or not.

In conclusion, the aromatic rice landraces from different regions of Thailand had grain qualities that differentiated them from the elite varieties of KDML105 and PTT1. The majority were large grain with medium to high gelatinization temperatures in contrast to the slender grain with a low gelatinization temperature of the typical jasmine varieties KDML105 and PTT1. Only four of the landraces shared the *badh2*-E7 haplotype with the elite varieties of KDML105 and PTT1, and the previously selected BNM-CMU. The absence of the *badh2*-E7 allele in most of the accessions suggests that the 2AP compound may not be the only determinant of aroma in rice as perceived by local consumers. These locally recognized aromatic rice landraces make up a distinctive set of rice genetic resources preserved on-farm. One with commercial potential is exemplified by the glutinous genotype with pigmented pericarp, NDLP. Therefore, the landraces found to belong to the H1 group will be considered for further evaluation and introduced as genetic resources for a fragrant rice breeding program to develop highly palatable cultivars by pure-line selection or improving variety.

Factors affecting 2AP biosynthesis in fragrant rice will be identified in further work. Not only the fragrant genes, but also the environmental factors and crop management practices [72,73] should be examined to identify genes and develop functional markers for fragrant rice landrace breeding. These *badh2* alleles and functional markers are important for the development and identification of new fragrant rice varieties using marker-assisted selection.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4395/10/6/779/s1, Table S1: Mean value and standard errors (3 replications) of the grain length (mm), grain width (mm), and grain thickness (mm) of paddy rice of 22 fragrant rice landrace accessions, 2 advanced breeding lines, and 3 elite variety checks; Table S2: Seed morphology of 22 landraces fragrant rice varieties, 2 breeding line varieties, and 3 check varieties (n=100); Table S3: Alkali spreading test (%) at six levels of 22 landraces fragrant rice varieties, 2 breeding line varieties, and 3 check varieties (n=100) Level; Table S4: Sensory scores by using 10 testers of 22 landraces fragrant rice varieties, 2 breeding line varieties, and 3 check varieties

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