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Sequence Variation and Haplotype Structure in the *Lox3* Gene of *Oryza sativa* L.

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ABSTRACT

For 24 rice (*Oryza sativa* L.) varieties, genetic variation was studied of the *Lox3* gene in samples of the two subspecies, japonica and indica. The genomic DNA was amplified, followed by DNA sequencing to detect sequence variation. A total of 13 single nucleotide polymorphisms (SNPs) was detected in the exon 4 containing lipoxygenase domain. The nucleotide diversity in the *Lox3* region of *O. sativa* was 0.00112, which could be classified into ten haplotypes. Phylogenetic relationships among varieties were inferred using neighbor-joining methods. Cluster analysis showed that all populations could be clustered into five groups. The results also revealed that the haplotype of *Oryza sativa* ssp.japonica rice is distinctively separated from that of *Oryza sativa* ssp.indica rice.

Keywords: genetic variation, nucleotide diversity, haplotypes, lipoxygenase, *Oryza sativa*

INTRODUCTION

Rice is a major food crop for almost half the world's population. All rice is milled before consumption, producing hull, bran, germ and white rice. Rice bran is a useful by-product from rice polishing and is normally used as food for human consumption and as animal feed. Embryos and aleurone layers are the main components in bran, which are major tissues where lipids are deposited (Juliano, 1977; Fujino, 1978). Rice bran contains valuable nutritional constituents, which have great potential as a supplementary source to a normal dietary intake. Rice bran oil (RBO) lowered plasma cholesterol more effectively than other commonly used vegetable oils rich in linoleic acid (Rukmini and Raghuram, 1991); this effect can be attributed to the occurrence of specific components in RBO, γ- oryzanol (and its constituents, triterpene alcohols) and tocopherols (Nicolosi *et al*., 1991; Rukmini and Raghuram, 1991; Sugano and Tsuji, 1997). These properties make rice bran oil ideal as a health-promoting product.

The quality of RBO depends on the quality of the rice bran. The physical and chemical natures of bran depend on the rice variety, treatment of the grain before milling, type of milling system and degree of milling. The utilization of rice bran as a food and feedstock is confined, due to its instability caused by the development of hydrolytic and oxidative rancidity.

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Important factors are enzymes, such as lipoxygenase, which is generally activated when tissue is disrupted or injured. Lipoxygenases (LOX, linoleate;oxygen oxidoreductase, EC 1.13.11.12) are associated with the oxidation of polyunsaturated fatty acids having a cis, cis-1,4-pentadiene structure (such as linoleic and linolenic acids), to conjugated hydroperoxy fatty acid. Hydroperoxides are further transformed into various volatile compounds, causing changes in or adding flavor and deteriorated color to flavor and nutritive properties.

LOX activity in rice grain is present in a bran-milling fraction (Shastry and Rao, 1975; Yamamoto *et al*., 1980). Rice embryos contain three isozymes, Lox-1, Lox-2 and Lox-3, with lipoxygenase activity and with the Lox-3 isozyme as the major component (Ida *et al*., 1983). In previous studies, the absence of the Lox-3 protein in ungerminated seeds of the Thai variety, Daw Dam, was determined by immunoblot analysis and enzyme assay (Suzuki *et al*., 1993; Suzuki and Matsukura, 1997). Genetic analysis showed that the absence of Lox-3 was inherited as a single recessive gene (Suzuki, 1995; Suzuki *et al*., 1996a). Suzuki *et al*. (1996b) reported that the peroxidation products of unsaturated fatty acids were lower in the Daw Dam bran fraction during storage than in rice varieties with Lox-3 in their seeds. These results suggest that the absence of Lox enzymes in rice grains alleviates oxidative deterioration.

DNA sequencing is a powerful tool for studying genetic variation and DNA sequences to collect information that can be important in assisting the understanding of the evolutionary dynamics of a species. Nucleotide diversity reveals an abundant history of selection, migration, recombination and mating systems (Buckler and Thornsberry, 2002). The conserved DNA sequences, which differ at single positions, are the most frequent kind of genetic variation revealed in natural populations of a species. In addition, nucleotide polymorphism across a genome is the origin of most of the phenotypic variation, because of changes in the conservation of amino acid correlated with functional effect (Buckler and Thornsberry, 2002).

This study was designed to reveal the level and pattern of sequence variation of *Lox3* rice, never previously investigated in a natural population of Thai rice. Therefore, the study aimed to characterize a subset of rice varieties with different oxidative stability in their rice bran oils due to nucleotide diversity, using sequencing data and analysis of peroxide values among molecularly characterized haplotypes. The sequence variation was investigated in the coding region of the *Lox3* (E03480) consisting of four exons that covered 2.83 kb on chromosome3 within the rice species.

MATERIALS AND METHODS

Plant materials

Eighteen accessions of Daw Dam and two accessions of Payaluemkang all having a glutionous endosperm, which originated from different provinces in the North and Northeast were obtained from the Pathum Thani Rice Research Center, Thailand. Two additional cultivars with non-glutinous endosperm, namely, the economic rice of Thailand (KDML105) and Jao Hom Nin, showing high rancidity in their rice bran, and two Japanese cultivars, namely, Nipponbare and Koshihikari, each having normal lox3 activity (Table 1), were added to the current study. Seeds of these accessions were germinated in the greenhouse for DNA isolation.

Genomic DNA extraction, polymerase chain reaction (PCR) amplification and sequencing

Total genomic DNA was isolated from fresh leaves of three-week old seedlings using a DNA Trapping Kit (DNA Technology Laboratory, Thailand). Seven specific PCR primers for the lipoxygenase-3 gene identified from four exons

Table 1 List of rice varieties used.

Accessions	Cultivars' name	Species/ subspecies	Locality/ province
1430	Daw Dam	*O. sativa*/ Indica	Northern Thai/ Lampang
3354	Daw Dam	*O. sativa*/ Indica	Northeastern Thai/Si Sa Ket
5614	Daw Dam	*O. sativa*/ Indica	Northeastern Thai/ Yasothon
5645	Daw Dam	*O. sativa*/ Indica	Northeastern Thai/ Yasothon
5647	Daw Dam	*O. sativa*/ Indica	Northeastern Thai/ Yasothon
5978	Daw Dam	*O. sativa*/ Indica	Northern Thai/ Chiang Rai
6702	Daw Dam	*O. sativa*/ Indica	Northern Thai/ Phrae
6710	Daw Dam	*O. sativa*/ Indica	Northern Thai/ Phrae
7734	Daw Dam	*O. sativa*/ Indica	Northern Thai/ Phayao
7751	Daw Dam	*O. sativa*/ Indica	Northern Thai/ Phayao
10748	Daw Dam	*O. sativa*/ Indica	Northern Thai/ Phayao
12127	Daw Dam	*O. sativa*/ Indica	Northeastern Thai/Nakhon Phanom
13852	Daw Dam	*O. sativa*/ Indica	Northern Thai/ Lampang
14013	Daw Dam	*O. sativa*/ Indica	Northern Thai/ Lampang
19004	Daw Dam	*O. sativa*/ Indica	Northern Thai/ Phrae
19086	Daw Dam	*O. sativa*/ Indica	Northern Thai/ Phrae
19557	Daw Dam	*O. sativa*/ Indica	Northern Thai/ Phrae
20904	Daw Dam	*O. sativa*/ Indica	Northern Thai/ Uttaradit
7620	Payaluemkang	*O. sativa*/ Indica	Northeastern Thai/Nakhon Phanom
14549	Payaluemkang	*O. sativa*/ Indica	Northeastern Thai/Nakhon Phanom
KDML105	-	*O. sativa*/ Indica	Northeastern Thai/ Surin
JHN	-	*O. sativa*/ Indica	Central Thai/ Nakhon Pathom
Koshihikari	-	*O. sativa*/ Japonica	Japan
Nipponbare	-	*O. sativa*/ Japonica	Japan

in chromosome 3 (BAC AC117988) were designed using the Primer3 program. The PCR reaction mixture contained 25 ng of DNA template, 0.1mM dNTPs, 2.0 mM $MgCl_2$, 0.25 mM of each forward and reverse primer, 0.25 U Taq polymerase and 1x thermophilic DNA Poly buffer (Promega) in a total volume of 10 uL. The DNA template was initially denatured at 94°C for 3 min. followed by 35 cycles of PCR amplification with the following temperature profile: 94°C for 30 sec, 55°C for 30 sec and 72°C extension for 2 min. The final extension was set at 72°C for 5 min. The amplified products were examined by electrophoresis on 1% agarose gel in a 0.5 × TBE buffer, stained with ethidium bromide and photographed under UV light. The PCR products were sequenced with an ABI PRISM 377 XL, using a BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) in both directions, for all individuals.

Measurement of the peroxide value

The peroxide value (PV) in the total lipids has been used to analyze the flavor quality and stability of rice bran oil. The standard method of IUPAC 2.501 was used for determination of PV. This involved weighing a sample of 5 g of oil into a clean 250mL flask and adding 25 ml of acetic acid-chloroform (3:2 v/v) mixture, followed by the addition of 1 mL of saturated KI solution and swirling for 1 min. The mixture was kept in the dark for 5 min and then 75 ml of deionized water was added and the mixture shaken. After that, the

contents were titrated with 0.01 N $Na_2S_2O_3$ solution in a 0.5-mL starch indicator solution (5%), until the blue color had just disappeared (end point) and the amount of $Na_2S_2O_3$ used was recorded. A blank was also run for all the solutions, but without the oil sample added. The peroxide value was evaluated by measuring the amount of iodine released from the potassium iodide titrated with sodium thiosulphate solution and recorded as the average of the triplicate measurements. The PV was expressed as milliequivalents of hydroperoxide per kg of oil.

Data analysis

Sequencing data were aligned manually with the CLUSTALW program (available from: http://align.genome.jp). The DNA haplotypes were assessed to determine nucleotide substitutions. The analyses were performed using the DnaSP software version 4.0 package (Rozas and Rozas, 1999). Levels of polymorphism were estimated by θ (Watterson, 1975) and π (Nei, 1987)(no-edit from cropscience). This program was also used to perform Tajima's D test (Tajima, 1989), Fu and Li's test (Fu and Li, 1993) and to estimate haplotype diversity. The phylogenetic tree was inferred by the neighbor-joining (ClustalX) method. Bootstrap analysis, using 1,000 replicates, was calculated in ClustalX. The programs NJPlot and TreeView (distributed with the ClustalX package) were used for tree construction. Analysis of variance (ANOVA) was performed using CropStat 7.2 software (IRRI, 2007).

RESULTS

Sequence variation

The genetic variation of the *Lox3* gene-coding region was analyzed. DNA sequences were determined for all 24 samples. There were 13 sites polymorphic for nucleotide substitutions (2 transveÇsion and 11 transition substitutions) and no insertion/deletion variation. Sequences of the *Lox3* gene were located on chromosome3 of the rice. Within the coding regions, five changes were synonymous and eight changes were nonsynonymous with the positions in the Lox3 domain (Figure 1). The genomic sequences of Daw

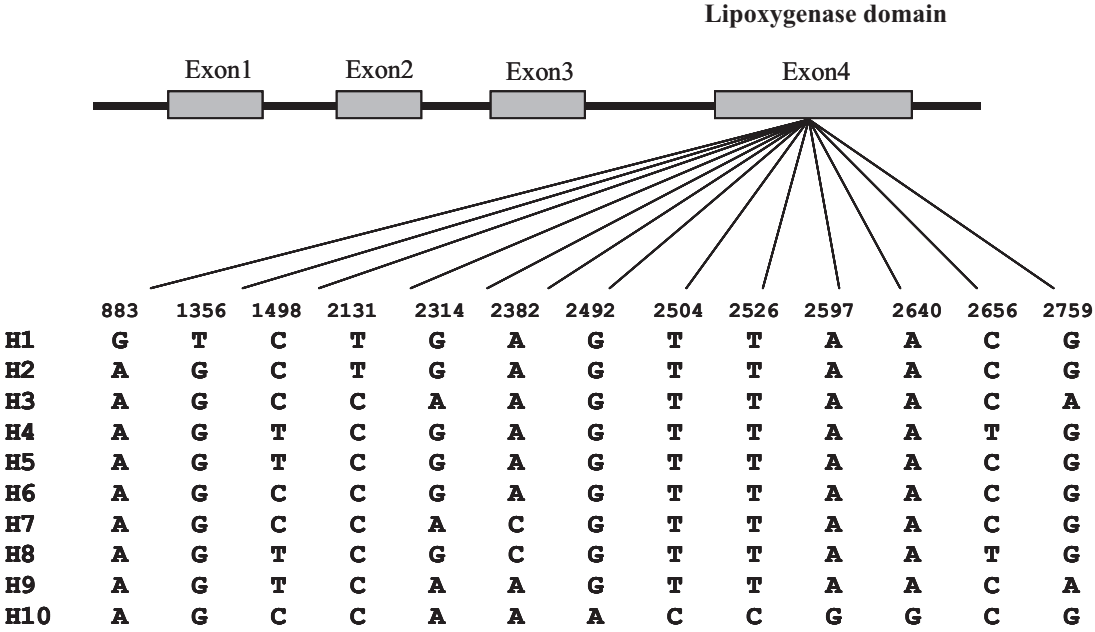

Figure 1 Haplotypes of *Lox3* gene on exon 4 in 24 accessions of *O. sativa*.

Dam, Payaluemkang, KDML105, JHN, Nipponbare and Koshihikari rice samples were compared and showed variation of the nucleotide sequence in the exon4, which was the lipoxygenase domain that results in rancidity. These were categorized by a phylogram (Figure 2).

Phylogenetic analysis and haplotype structure

The genetic relationships among rice genotypes can be revealed in a phylogram based on nucleotide sequences. A neighbour-joining tree was calculated using TreeView and bootstrap analysis was performed with 10,000 replicates to evaluate the accuracy for individual branches of the neighbor-joining tree (Figure 2). The phylogenetic result indicated that all genotypes could be grouped into five clusters in the phylogram; A, B, C, D and E. Cluster A included two haplotypes (H3 and H9) containing eight accessions of Daw Dam. Cluster B included two

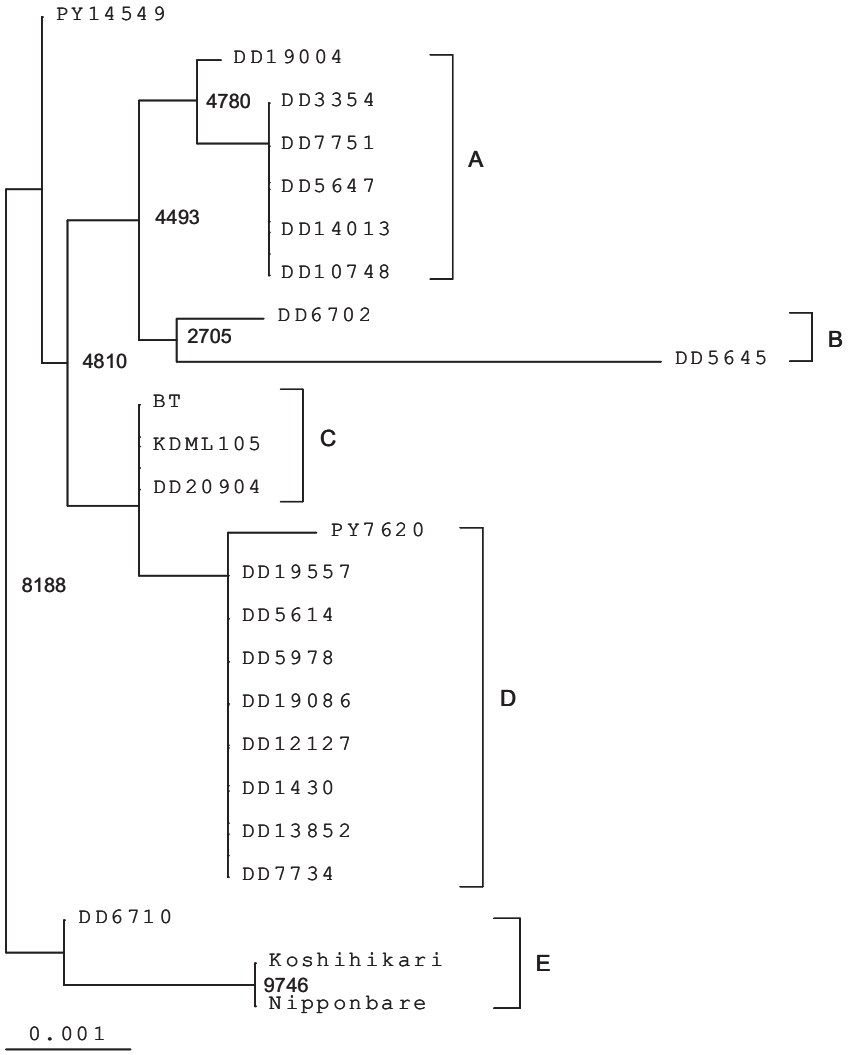

Figure 2 Phylogenetic tree based on the *Lox3* gene region sequences generated by neighbor-joining method (ClustalX) with Njplot. Maximum parsimony bootstrap tree, based on 10,000 bootstrap replicates.

haplotypes (H7 and H10) containing eight accessions of Daw Dam. Cluster C included one haplotype (H5) containing KDML105, JHN and one accession of Daw Dam. Cluster D included two haplotypes(H4 and H8) containing eight accessions of Daw Dam and one accession of Payaluemkang. Cluster E included two haplotypes (H1 and H2) containing Koshihikari and Nipponbare, which were japonica rice and one accession of Daw Dam. The genotype of PY14549 (H6) was not included in any cluster. Most samples of *O.sativa* indica were separated from *O.sativa* japonica. A total of ten different haplotypes within the lipoxygenase domain were constructed on the basis of the genotypic data from 13 SNPs in the sample of 24 accessions (Figure 1); the haplotype diversity was determined to be 0.848.

Phenotypic variation

The peroxide value was evaluated in all rice samples. A comparison of PV showed some differences among rice varieties (Table 2). The results of the oxidative stability analysis in rice bran oil indicated that Nipponbare, Koshihikari, KDML105 and JHN had high levels of PV, whereas most of the Daw Dam and Payaluemkang samples had lower PV values. One accession of Payaluemkang (PY 14549) presented the lowest PV (1.08 meq/kg). Moreover, the PV of the japonica rice samples was higher than for most of the indica rice samples, except for H5, which had the highest PV. There were significant differences among some haplotype groups. These groups, H3, H4, H7 and H10, gave approximately 2 meq/kg (Table 2).

Level of nucleotide variation and test of neutrality in the *Lox3* region of *O. sativa*

There were 13 segregation sites in the samples of the 24 sequences of the *Lox3* gene. The nucleotide variation found in the *Lox3* region of *O. sativa* is summarized in Table 3. The level of nucleotide polymorphism (p and q) in the coding region of the *Lox3* was 0.00112 and 0.00131, respectively. Tajima's D, and Fu and Li's tests were used to estimate the frequency spectrum of the alleles and deviated significantly from the neutral expectation, while in contrast, the genotypes of these rice samples did not show a significant deviation for Tajima's D test (D_T^* = -0.49403, P>0.1) and the Fu and Li's test statistic also gave significantly negative values (D*= -0.56020 F*= -0.63010) for the coding region of *Lox3*.

DISCUSSION

Nucleotide variation in the *Lox3* gene has never been estimated for Thai rice. The current study characterized sequence variation across the *Lox3* locus. The DNA polymorphism in the *Lox3* region was analyzed for two subspecies of rice and 10 DNA haplotypes were distinguished, of which only one haplotype, H1, was found in the japonica rice, seven haplotypes were found in the DawDam accessions, two haplotypes were found in Payaluemkang and one haplotype was found in JHN and KDML105. The data indicated that the haplotype groups could be used to separate each cultivar. Futhermore, these haplotypes provided different sequence of indica and japonica rice.

The *Lox3* gene had a low level of DNA polymorphism (π=0.00112). This result indicated that the low level of variation may be caused by the lack of the power in the test, since the population size was small. Both the Tajima's D and the Fu and Li's tests gave significantly negative values for the coding region in the *Lox3*.

It has been previously reported that *Lox3* was associated with the level of PV in rice bran oil (Suzuki *et al*., 1996b). Therefore, the PV was compared among the haplotypes consisting of Daw Dam, Payaluemkang, JHN, KDML105, Koshihikari and Nipponbare. This information was used in the experiment to find the relationship between the phenotypic variability of economically important traits and the nucleotide

Table 2 Relationships between rice genotypes of *Lox3* region and peroxide value.

Cluster	Haplotype	Rice accessions	Peroxide value (meq/kg)	Mean PV of haplotype
A	H3	3354	1.75	2.072b
		7751	1.82	
		10748	1.88	
		14013	2.37	
		5647	2.54	
	H9	19004	2.89	2.89c
B	H7	6702	1.78	1.78b
	H10	5645	1.81	1.81b
C	H5	20904	2.02	3.07c
		JHN	3.54	
		KDML105	3.65	
D	H4	5978	1.34	2.012b
		7734	1.61	
		1430	1.75	
		12127	1.78	
		13852	2.01	
		19557	2.33	
		5614	2.41	
		19086	2.87	
	H8	7620	1.56	1.56a
E	H1	Kkoshihikari	2.95	3.01c
		Nnipponbare	3.07	
	H2	6710	1.42	1.42a
	H6	14549	1.08	1.08a
Mean			2.18	2.18
Min			1.08	1.08
Max			3.65	3.07
Probability			**	**
LSD 5%			0.06	0.47
CV			20.1	

** Significant at $p < 0.01$.

sequence. The haplotype H6 of PY14549 had the lowest PV compared with others and also showed a difference from other groups in its DNA sequence. In addition, the Payaluemkang cultivar did not have low bran rancidity reported previously. Payaluemkang, therefore, may be an interesting rice accession to study lipid oxidation in rice bran oil.

CONCLUSION

The data produced in the current study provide preliminary information from the association tests of the *Lox3* gene in a natural population and provide an opportunity for the consequent use of nucleotide diversity and some alleles for the improvement of the nutritional quality of rice.

Table 3 Statistics of nucleotide diversity in the *Lox3* gene of *Oryza sativa*.

	Coding region of *Lox3* gene
Data	
S	13
h	10
Population statistics	
p	0.00112
qw	0.00131
Hd	0.848
D_T*	-0.49403
D*	-0.5602
F*	-0.6301

S = Number of polymorphic sites.
H = Number of haplotypes.
π = The average number of nucleotide differences per site among all pairs of sequences (Nei, 1987).
θ = The number of segregating site is the estimate from Watterson (1975).
Hd = Haplotype diversity.
* = 0.10
D_T* = Tajima's D test.
D* = Fu and Li's D test.
F* = Fu and Li's F test.

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