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Population differentiation of two Thai fragrant rice landraces revealed by SSR marker analysis

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ABSTRACT

This study aimed to survey the *badh2* allele, an 8-bp deletion in exon 7 of the *fgr* gene, in a representative rice collection of two traditional fragrant rice landraces, Hohm Nangnuan and Hohm Thung, grown in the northeastern region of Thailand. The study also determined the population genetic structure of these two traditional rice landraces by using 13 SSR markers surrounding the aroma loci mapped on chromosome 8. It was found that rice accessions of Hohm Nangnuan from Mahasarakham, Ubon Ratchathani (type 2), Yasothon, and Sakon Nakhon provinces showed the fragrant genotype (*badh2/ badh2)*, while Hohm Nangnuan type 1 from Ubon Ratchathani showed the non-fragrant genotype (*Badh2/Badh2*). For the Hohm Thung variety from Ubon Ratchathani province, all rice accessions with the rice straw husk used in this experiment carried the fragrant genotype, whereas rice accessions with the rice brown husk carried the non-fragrant genotype. Based on the data of SSR genotype over the 13 SSR loci, the STRUCTURE simulation illustrates a spatial pattern that corresponds to their name.

Keywords: rice; betaine aldehyde dehydrogenase gene (*badh2)*; microsatellite marker

INTRODUCTION

Rice genome sequence information on the identity and characteristic of genes associated with both qualitative and quantitative phenotypic variation is readily available to the public. Information pertaining to fragrance or aroma (Shao *et al*., 2010); eating and cooking qualities (Yi *et al*., 2009); lodging resistance and panicle characters (Matsushita *et al*., 2014) is of particular interest to rice breeders. They require data on genetic diversity and population structure of rice cultivars to help them better understand their germplasm, guide their breeding plans, and better exploit genetic variation (Lu *et al*., 2005). A better understanding of the population structure is important to avoid identifying spurious associations between phenotype and genotype.

In Thailand, traditional rice landraces grown by local farmers have been passed down from generation to generation. Varieties such as Jek Chuey, a local rice variety in Saraburi province in central Thailand, and Sung Yod, an outstanding high quality variety from Patthalung province in southern Thailand, are good examples. Fragrance is considered one of the most important factors in determining market price and is related to both local and national identity (Fitzgerald *et al*., 2009). The characteristic of fragrance in aromatic rice has been shown to be associated with the presence of 2-acetyl-1-pyrroline (Buttery *et al*., 1983; Chen *et al*., 2008; Nui *et al*., 2008). Genetic investigations have revealed that the fragrance trait of rice is controlled by a single recessive locus, which is located on chromosome number 8 (Ahn *et al*., 1992). Furthermore, several PCR-based markers for the single locus of the fragrance gene (*fgr*) have been developed and utilized for differentiation between fragrant and non-fragrant rice varieties (Bradbury *et al*., 2005; Chen *et al*., 2006; Prathepha, 2009; Shao *et al*., 2010).

Two glutinous fragrant rice landraces, *Hohm Thung* and *Hohm Nangnuan* are Thai traditional rice landraces grown by local farmers in the northeastern region of Thailand. These two rice landraces have been recognized as aromatic rice by farmers. *Hohm Thung* has been selected by local farmers for growing in the flood areas of the Mun River basin of northeastern Thailand. Farmers grow this rice early in the rainy season (May or June) by using a transplanting method. Mature seeds are harvested in September. *Hohm Thung* is photoperiod insensitive, which is a unique characteristic and distinct from other traditional rice landraces that are photoperiod sensitive. This rice landrace can grow as a major rice in the monsoon season (May to October) and a minor rice in the dry season (January to April) in Thailand. A preliminary survey revealed that *Hohm Thung* may be classified into two populations based on seed morphology (seed size, seed shape and seed color). In addition, there is no available genetic information that shows the genetic differentiation of these two groups of this rice landrace.

Hohm Nangnuan is grown only in northeastern Thailand. This rice landrace is a photoperiod sensitive variety that has flowering and harvesting time around September and October of the year (harvesting about 30 days after flowering). This rice landrace is adapted to lowland paddy cultivation and is grown as a major rice in the monsoon season of Thailand (May to October). Since ancient times, this landrace has been recognized as glutinous aromatic rice by northeastern farmers.

Several studies have reported that genetic variability occurs among rice landraces, but few studies have assessed the genetic diversity within a local rice landraces (though see Kobayashi *et al.* (2006) and Pusadee *et al.* (2009)). Within a landrace, polymorphism also accounts for the fact that the same local variety name can be used in reference to populations that do not have exactly the same phenotypic and genotypic composition (Barry *et al*., 2007). *Hohm Thung* and *Hohm Nangnuan* are two local rice landraces that have a fragrant characteristic and are recognized as a great important resource for further rice genetic improvement because they have adapted to specific local conditions and can be used for human consumption within a region. The genetic diversity of the two rice landraces is therefore the most useful and economically valuable component. Thus, efficient conservation and use of these germplasm resources, and an understanding of the genetic differentiation of the fragrance gene related to morphological variation of these local rice landraces, are required. In addition, the population genetic structure of traditional rice landraces is significantly affected by natural processes such as selection, genetic drift, seed selection, and exchange among local farmers (Parzies *et al*., 2004). Based on this idea, when analyzing the genetic data obtained from the experiment, the useful information could explain the dynamics of these rice landraces either increasing or decreasing in genetic diversity. In this study the population of the two local rice landraces was examined to address the question: is the genetic character (i.e. fragrance) associated with rice landrace name, and what are the patterns of genetic differentiation among populations?

MATERIALS AND METHODS

Sample collections

Two hundred and forty-five rice genotypes of two rice landraces (*Hohm Nangnuan* type 1 and 2 and *Hohm Thung* type 1 and 2), were randomly collected from a set of seed samples from 14 farmers in 4 villages in Ubol Ratchthani, Yasothon, Maha Sarakham and Sakon Nakhon provinces of northeastern Thailand (Table 1). Seeds were germinated in Petri dishes for 5 days then transferred to 30-cm diameter pots. Genomic DNA was extracted from leaf samples of each individual 1 month-old plants using a modified cetyltrimethyl ammonium bromide extraction (CTAB) method (Doyle and Doyle, 1987). For *Hohm Nangnuan* landrace some morphological traits were observed and recorded as shown in Table 1.

Genotype determination of fragrance gene

Samples of two traditional rice landraces were examined for the allele *badh2* of the *fgr* gene, which is responsible for the fragrance, by using a polymerase chain reaction (PCR) assay following a previous reported by Prathepha (2009). The nucleotide sequences of PCR primers was as follows: Os2AP-exon7.1F: 5′-TGCTCCTTTGTCATCACACC-3′ and Os2AP-exon7.1R: 5′-TTTCCACCAAGTTCCAGTGA-3′). The PCR reaction was performed in a 20 µl reaction mixture containing 10 ng DNA template, 50 pmole each of the primer pairs, 2.0 mM $MgCl_2$, 2 units *Taq* polymerase (Promega, USA), 0.1 mM dNTPs. Cycling conditions were 94°C (5 min); then 40 cycles of 94°C (1 min), 60°C (1 min), 72°C (1.5 min), and a final extension of 72°C (5 min). A fragrant rice, KDML 105 (*O. sativa* L. subspecies *indica*) and a non-fragrant rice, Chainart 1, were used as positive and negative controls, respectively. The PCR products were separated in 4.5% denaturing polyacrylamide gels of 200 × 125 × 1 mm (length × width × thickness). After electrophoresis, the bands were visualized using silver-staining (Song *et al*. 2003). The PCR product of 396 bp obtained from Thai jasmine rice (KDML 105) was present in every sample with the recessive allele (the 8 bp deletion). As a result, the bands were recorded as a homozygous genotype (*badh2/badh2 or Badh2/Badh2)* or a heterozygous genotype (*Badh2/badh2)* of each rice plant.

Table 1 Number of accessions examined (n=245) and locations of two aromatic traditional rice landraces in farmer’s fields of four provinces of north-eastern Thailand.

Population Abbreviation/no. accessions	Location (District and province name)	Landrace’s name	Genotype
SKN (25)	Phananikhom District, Sakon Nakhon Province	Hohm Nangnuan (HNN)	*badh2/badh2*
MSK (36)	Muang District, Maha Sarakham Province	Hohm Nangnuan (HNN)	*badh2/badh2*
YST (36)	Kudchum District, Yasothon Province	Hohm Nangnuan (HNN)	*badh2/badh2*
UBT1 (66)	Sawangweeravong District, Ubon Ratchathani Province	Hohm Nangnuan (HNN) type1 (straw hull, green primary leaf, light purple leaf sheath)	*BADH2/BADH2*
UBT2 (36)	Sawangweeravong District, Ubon Ratchathani	Hohm Nangnuan (HNN) type 2 (straw hull, purple leaf, dark purple leaf sheath)	*badh2/badh2*
UBT3 (62)	Sawangweeravong District, Ubon Ratchathani	Hohm Thung type 1 (straw hull) (HT)	*badh2/badh2*
UBT4 (9)	Sawangweeravong District, Ubon Ratchathani	Hohm Thung type 2-1 (brown hull) (HT)	*BADH2/BADH2*

Microsatellite analysis

Thirteen SSR markers surrounding the aroma loci mapped on chromosome number 8, as reported by Jain *et al.* (2006), were used to characterize and analyze the genetic diversity of the two rice landraces that carried the fragrant genotype determined by a PCR assay, as mentioned above, and a fragrant rice of Thailand, cv. KDML 105. A total of 13 microsatellite primer pairs were chosen for this study as follows: RM42, RM44, RM137, RM152, RM195, RM223, RM256, RM284, RM310, RM325A, RM331, RM339 and RM342A (Jain *et al*. 2006). Microsatellite polymorphism was analyzed by PCR. Amplification of DNA was performed in 20 µL reactions consisting of 20–50 ng DNA, 0.25 mM dNTP, 0.2 µM each primers, and 0.5 unit of *Taq* DNA polymerase (Invitrogen, USA). Cycling conditions were 94°C (5 min); then 40 cycles of 94°C (1 min), 55°C (1 min), 72°C (2 min), and a final extension of 72°C (7 min). Amplified products were visualized by using silver-staining.

Allele scoring and cluster analysis

For each SSR locus, the most intensely amplified band was scored based on its migration relative to a molecular-weight size marker ((PUC 19 DNA digested by *Msp*I) (Figure 1). According to the results from fragrance gene determination in rice samples, with the expectation that microsatellite variation may be associated with the characteristic of fragrance, SSR profiles of all 13 SSR markers were recorded. Each sample set (i.e., population), the frequency of microsatellite polymorphism, and the polymorphism information content (PIC) value of each locus were calculated using PowerMarker version 2.3.2 (Liu and Muse, 2004).

The genetic structure of the samples (n=142) of two local rice landraces collected from four provinces of northeastern Thailand, which have fragrant genotype, were determined the true K (number of populations) by using the model-based approach of Pritchard *et al.* (2000) implemented in the software package STRUCTURE version 2.2

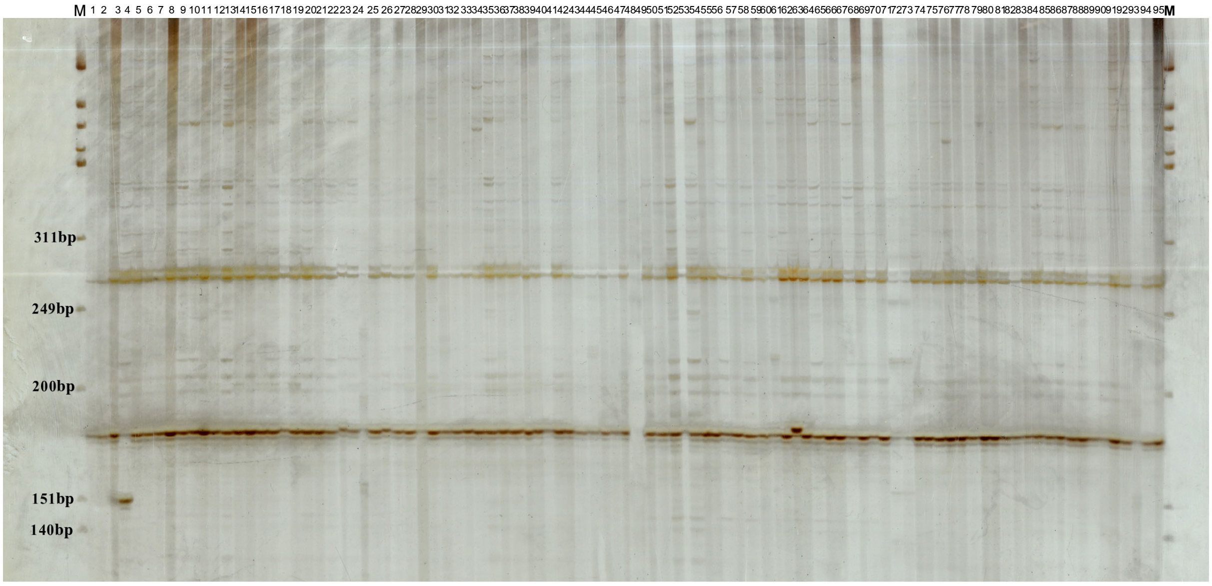

Figure 1 The PCR products using microsatellite primer RM331 of rice accessions of two fragrant rice landraces were separated in 4.5% denaturing polyacrylamide gel. M, marker. Lane 1-24, HNN-MSK; 25-48, HT-UBT2; 49-72, HNN-SKN; 73-95, HNN-YST.

(http://www.pritch.bsd.uchicago.edu) and the program Structure Harvester (http://taylor0.biology.ucla.edu) used with STRUCTURE. In this method, a number of populations (*K*) are assumed to be present and contribute to the genotypes of the examined rice sample. A series of tests was performed using a different number of population (*K*=1 to 6, each with five independent runs) to guide an empirical estimate of a number of identifiable populations without consideration of sampling origins. In each run, the admixture model, without prior population information, was applied with a 10,000 burn-in of 10,000 followed by sampling from 5,000 step in the Markov Chain Monte Carlo (MCMC). The approach of Evanno *et al*. (2005) was used to decide on the number of clusters (K-value). Briefly, in their simulation of populations, an *ad hoc* statistic ΔK based on the rate of change in the log probability of data between successive *K*-values could accurately detect true *K*. The suggested $\Delta k=m(|L(k+1)-2L(k)+L(k-1)|)/s[L(k)]$, where $L(k)$ represents the k^{th} LnP(D), m is to the mean of five runs and their standard deviation. A graphical display of the STRUCTURE results was generated and illustrated that the genotype of each individual is a junction of the allele frequencies in these *K* populations and a proportion of its genotype drawn from each of the *K* populations. Individuals were assigned probabilistically to a population if their genotype profile indicated admixture (Weckworth *et al.*, 2005).

RESULTS

Fragrant and non-fragrant genotypes of rice samples

The accessions of *Hohm Nangnuan* landrace from Mahasarakham (36 acc.), Ubon Ratchathani (type 2, 36 acc.), Yasothon (36 acc.) and Sakon Nakhon (25 acc.) provinces, revealed the fragrant genotype (*badh2/badh2*, 8-bp deletion mutation*)* (Figure 2), while the *Hohm Nangnuan* type 1 from Ubon Ratchathani showed the non-fragrant genotype (*Badh2/Badh2)*, meaning that this genotype carried is the dominant allele of the *fgr* gene. For *Hohm Thung* from Ubon Ratchathani province, all rice accessions with the rice straw husk used in this experiment carried the fragrant genotype, whereas rice accessions with the rice brown husk carried the non-fragrant genotype.

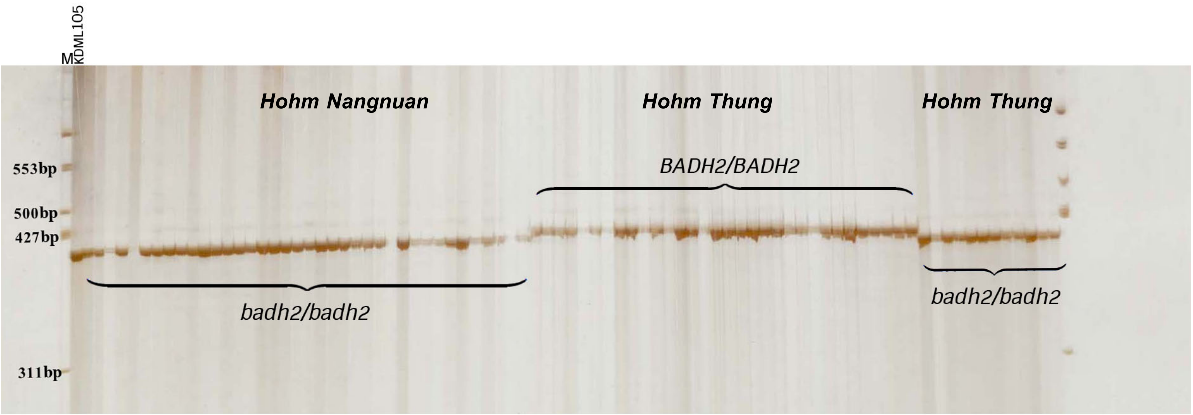

Figure 2 PCR products in a 4.5% polyacrylamide gel showing two genotypes (*BADH2/BADH2,* no deletion of the 8-bp fragment; and *badh2/badh2,* deletion of the 8-bp fragment) of the *fgr* gene in *Hohm Nangnuan* and *Hohm Thung*. Thai jasmine rice KDML105 showed genotype *badh2/badh2*. M, DNA molecular weight (bp, base pair).

Genetic variation within and among varieties

Rice accessions carrying the fragrant genotype were determined for genetic variation by detecting the 128 accessions (95 acc. for *Hohm Nangnuan*, 33 acc. for *Hohm Thung*) over 13 SSR loci located in chromosome 8. According to each SSR locus, an average allele number within *Hohm Nangnuan* (Na=2.428) was higher than *Hohm Thung* (Na=1.857). Heterozygosity ranged from 0 to 1 for both rice landraces with an average of 0.350 and 0.363 for *Hohm Nangnuan* and *Hohm Thung*, respectively (data not shown).

Population differentiation in Thai fragrant rice varieties

An analysis of the population structure of the 142 accessions of the two fragrant rice varieties (Table 1) and KDML 105 was included in this analysis by using STRUCTURE and STRUCTURE HARVESTER (Earl *et al.*, 2012). The magnitude change of LnP(D) relative to the standard deviation, called ΔK by Evanno *et al.* (2005), showed the highest peak at *k=2* (ΔK =20.058953), and there was one smaller peak at *k=3* (ΔK =8.115289). Therefore, *k=2* was selected for the true population number and showing the membership of rice accessions to the populations identified by STRUCTURE (Figure 3). Based on the data of SSR genotyping over the 13 SSR loci, the diagram illustrates a spatial pattern that corresponds to their name. It means that *Hohm Nangnuan* (95 acc.) and *Hohm Thung* (fragrant, 33 acc. and non-fragrant genotype, 10 acc.) are grouped into two different clusters.

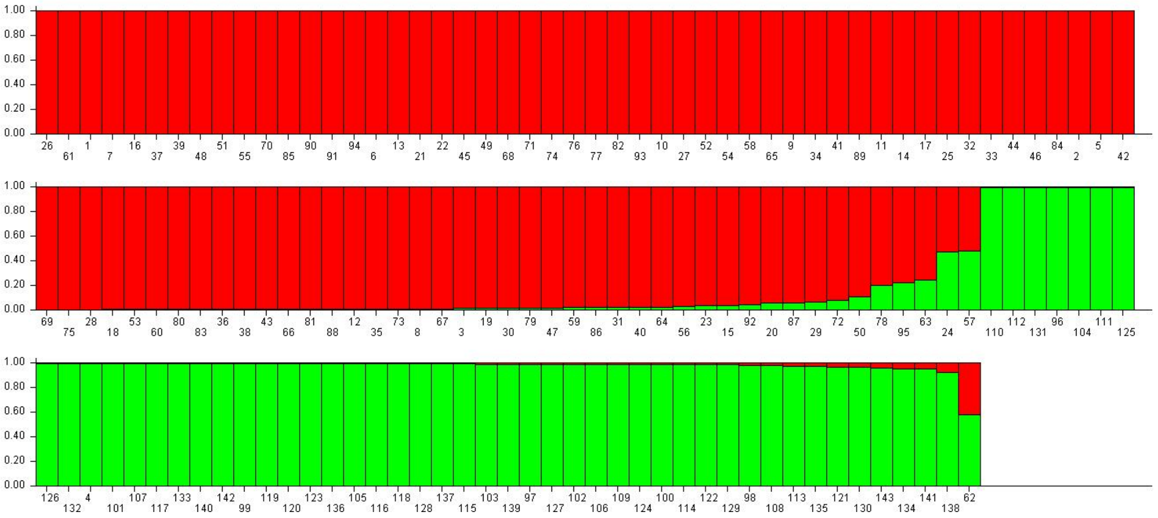

Figure 3 Histogram for K=2 using STRUCTURE. Every thin vertical bar corresponds to an accession of rice (no. 1-96, Hohm Nangnuan; 97-141, Hohm Thung; no. 142, 143, KDML105, RD 15) and individual code, which can be partitioned into K colored segments that represent the individual estimated membership to K cluster.

DISCUSSION

Recent findings suggest that the quality of rice grain is a multi-faceted trait. Quality traits encompass physical appearance, cooking and sensory properties and nutrition value. Among these valued traits, fragrance of the rice grain is one of the most valued grain quality traits. The dominant *Badh2* allele inhibits 2AP synthesis in non-fragrant rice, while the recessive *badh2* allele is responsible for 2AP accumulation (Bradbury *et al.,* 2005). The aroma characteristic of fragrant rice varieties that accumulates 2-acetyl-1-pyrroline (2AP), but does not carry the 7-bp deletion on exon 2 or the 8-bp deletion on exon 7 (*badh2-E7* allele) of *Badh2* (*fgr* gene) as reported by Fitzgerald *et al.* (2008), has been identified. Fragrant rice varieties which carry the 7-bp deletion on exon 2 (*badh2-E2* allele) were reported in japonica rice varieties (Shi *et al.,* 2008). There are two types of flavor and aroma of rice, the jasmine style and the Basmati style. The Thai jasmine rice, Khao Dawk Mali105, originated in Thailand and known as indica type rice, showed the 8-bp deletion on the exon 7 resulting in an accumulation of 2-acetyl-1-pyrroline (Prathepha, 2009). In this study, two traditional aromatic rice landrace from Thailand were examined. It was found that each named rice landrace (*Hohm Nangnuan* and *Hohm Thung*) carried the fragrant and the non-fragrant genotype. Results from this study provided information on locations of the population of the two rice landraces carrying the fragrant genotype. It would be beneficial for rice breeders to know the sources of fragrant rice, so they could use them in rice breeding programs because rice with the same landrace name and having similar phenotype such as *Hohm Nangnuan* exhibit different genetic background according to aroma characteristic.

Results from cluster analysis demonstrated that accessions of *Hohm Nangnuan* and *Hohm Thung* showed a spatial pattern that corresponded to their names. Accessions of *Hohm Nangnuan* were grouped into the same cluster. Likewise, accessions of *Hohm Thung* were grouped into the same cluster. These results suggested that there may be a differentiation of genetic structure between these two rice landraces because each has genetic changes in response to evolutionary forces such as selection and drift by the agricultural practices of local farmers. These processes interact to shape and change the population genetics of each landrace rice.

CONCLUSIONS

Traditional rice landraces, which have been passed down from generation to generation, are grown by local farmers in nearly every region. Farmers favor traditional rice landrace because they are better adapted to specific local conditions, and they are developed for regional uses of rice (Wood and Lenne, 1997). In the northeastern and northern regions of Thailand, local people eat glutinous rice as a staple food. Thus, some communities of farmers still use the local rice landrace that grows well in that particular region. The traditional rice landrace *Hohm Nangnuan* and *Hohm Thung* are the only two local glutinous rice landraces with fragrance characteristic grown in northeastern Thailand. Meanwhile, traditional rice landraces have been largely replaced by the improved variety named RD6, a popular glutinous variety and recommended by officials to grow in these areas. Nowadays, the RD6 variety is grown by all communities in the northeastern region of the country. The traditional rice landraces represent a unique and critical source of genetically variable traits that can serve as a resource for future rice improvement (Pusadee *et al.*, 2009). A challenge program aimed to improve the quality of the two glutinous fragrant rice landraces of northeastern Thailand is of interest, but several additional efforts are needed to constructively engage the Thai scientists in the development of new genotypes for local rice production, grain quality and food security.

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