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Is there a second fragrance gene in rice?

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Summary

Aromatic rice is highly prized by most rice consumers, and many countries cultivate traditional and improved aromatic varieties. 2-Acetyl-1-pyrroline (2AP) is the major aromatic compound in rice, and is believed to accumulate because of an eight-base-pair (8-bp) deletion in an allele at the fragrance locus. In this study, 2AP was quantified and the presence or absence of the fragrance allele (*fgr*) was determined in 464 samples of traditional varieties of rice from the T.T. Chang Genetic Resources Centre at the International Rice Research Institute. It was shown that a number of aromatic varieties, primarily from South and South-East Asia, do not carry the 8-bp deletion, but 2AP was identified in both raw and cooked rice of these varieties. We suggest that the 8-bp deletion in *fgr* is not the only cause of aroma, and at least one other mutation drives the accumulation of 2AP. The amount of 2AP in most uniform *fgr* genotypes was not significantly different from that in aromatic n*fgr* genotypes, but several *fgr* genotypes, primarily from South Asia, reproducibly accumulated exceptionally large amounts of 2AP. We suggest that the mutation leading to 2AP in aromatic n*fgr* varieties possibly originated several times and, through either domestication or evolution, the *fgr* gene and other alleles leading to 2AP have combined in South Asia, leading to several highly aromatic traditional varieties. The identification of multiple mutations for 2AP will enable rice breeding programmes to select actively for multiple genetic sources of 2AP, leading to the development of highly aromatic and, consequently, high-quality varieties of rice.

Keywords: 2-acetyl-1-pyrroline, aroma, fragrance, fragrance allele, rice.

Introduction

Aromatic rice is highly valued in many countries of the world and commands premium prices at all levels of the global rice trade. India, Pakistan and Thailand are the major exporters of aromatic rice, but it is cultivated and prized in many other countries of the world. Most people recognise two major types of aromatic rice: basmati from India and Pakistan; and jasmine from Thailand. However, the T.T. Chang Genetic Resources Centre (GRC) at the International Rice Research Institute (IRRI) contains both glutinous and non-glutinous accessions of traditional varieties of aromatic rice collected from many rice-growing countries.

Many volatile compounds have been detected that are unique to aromatic rice, but the major compound of aroma is always cited as 2-acetyl-1-pyrroline (2AP) (Buttery *et al*., 1983; Widjaja *et al*., 1996b; Grimm *et al*., 2001; Mahatheeranont *et al*., 2001; Jezussek *et al*., 2002). 2AP occurs in many plants (Yoshihashi *et al*., 2002), and also occurs in cooked foods as the product of the Maillard reaction between 1-pyrroline, a degradation product of proline, and a two-carbon sugar fragment (Adams and De Kimpe, 2006). However, several lines of inquiry have suggested that 2AP in cooked rice grains is not the product of the Maillard reaction, but is synthesised within the plant during its growth: (i) the detection of a gene locus that associates with aroma (Bradbury *et al*., 2005a); (ii) histochemical studies that have localised 2AP in uncooked rice grains (Nadaf *et al*., 2006); (iii) the formation of 2AP at room temperature in plants labelled with precursors of 2AP (Yoshihashi *et al*., 2002); and (iv) the extraction and detection

of 2AP from rice grains without using heat (Sriseadka *et al*., 2006).

It has been reported that 2AP is produced through the agency of a single recessive allele at a locus on chromosome 8 (Lorieux *et al*., 1996; Jin *et al*., 2003; Chen *et al*., 2006). With this allele (*fgr*), the gene has an eight-base-pair (8-bp) deletion on exon 7, introducing a premature stop codon upstream of key coding regions (Bradbury *et al*., 2005a). The functional allele, n*fgr*, contains the 8 bp missing in *fgr*, and its transcription is proposed to lead to the metabolism of 2AP into its proline and acetyl groups, whereas any product of the *fgr* allele is considered to be unable to metabolise 2AP, leading to its accumulation in aromatic varieties (Bradbury *et al*., 2005b). Bradbury *et al*. (2005a), after screening 14 aromatic and 64 non-aromatic varieties, suggested that all aromatic rice shares a common allele by descent from a single ancestral fragrant genotype. The validity of this suggestion depends largely on the effective diversity of the aromatic varieties used in the study in relation to the full diversity of aromatic rice. If breeders used the same aromatic variety to introduce aroma into the 14 varieties studied, the effective diversity of the sample would be much less than if there were 14 unrelated varieties.

In the present study, the validity of the suggestion that all aromatic rice shares a common allele was tested by assessing the diversity of the sources of aroma studied by Bradbury *et al*. (2005a), and by determining the fragrance genotype and phenotype in a wide range of traditional varieties from 23 countries spanning North, South, South-East, and Central Asia, Africa and the Americas. It was found that many aromatic varieties carried the deletion reported by Bradbury *et al*. (2005a), but a significant proportion did not. Thus, it is suggested that the deletion in the *fgr* allele is not the only cause of 2AP synthesis and accumulation in rice, and that there is at least one other mutation at a second gene locus leading to 2AP. This mutation would enable breeding programmes to select actively for both pathways, leading to the development of highly aromatic varieties.

Results and discussion

Is the 8-bp deletion in the *fgr* allele the only cause of 2AP?

The 8-bp deletion in exon 7 of the fragrance gene is thought to lead to the accumulation of 2AP instead of the breakdown of 2AP (Bradbury *et al*., 2005a). The aroma in the 14 varieties studied by Bradbury *et al*. (2005a) comes from only four traditional varieties and an old USA variety Delitus (Table 1).

Table 1 The five sources of aroma in the varieties used by Bradbury *et al*. (2005a)

Variety/breeding line	Origin	Aromatic parent
Kyeema	Australian	Delitus*
YRF203	Australian breeding line	Basmati 370*
00210-0-15	Australian breeding line	Basmati 370*
YRF207/1202	Australian breeding line	Delitus*
Yasmin	Jasmine 85 (IR841)	Khao Dawk Mali 105†
Amber	Iraq	(Traditional variety)†
Dumsorkh	Iran	(Traditional variety)‡
Dellmont	USA	Delitus†
YRF207/1202	Australian breeding line	Delitus*
YRF204	Australian breeding line	Delitus*
00210-33	Australian breeding line	Basmati 370*
Basmati 370	Pakistan	(Traditional variety)†
Goolarah	Australia	Delitus*
Khao Dawk Mali 105	Thailand	(Traditional variety)†

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Three of the traditional varieties come from adjacent countries (Pakistan, Iran, Iraq), and the fourth is from Thailand. Delitus was released in the USA in 1918 as a selection from the Italian variety Bertone (Jodon and Sonnier, 1973), which had been introduced into Italy in 1872 from an unknown country (Mantegazza *et al*., 2004). It was determined that Delitus carries the fragrance allele (IRGC1697 and IRGC1698; IRGC, International Rice Germplasm Collection) and contains 2AP; thus almost one-half of the 14 samples in Table 1 obtained the *fgr* allele from Delitus. Such a narrow genetic base provides little support for the suggestion of Bradbury *et al*. (2005a) that all aromatic varieties could have inherited their fragrance gene from a single aromatic ancestor during evolution.

Of the 464 traditional varieties genotyped and phenotyped, 313 varieties from 17 countries were classified as aromatic by the presence of 2AP. Most of these, 279, carried the *fgr* allele, but 15 varieties from nine countries did not, and 19 were mixtures (Table 2). Conversely, seven varieties with a uniform fragrant genotype did not contain 2AP, and five samples containing mixtures of both alleles of the fragrance gene also did not contain 2AP (Table 2). The amount of 2AP in the samples containing mixtures is likely to be a feature of the proportion of *fgr* and n*fgr* alleles in each, explaining why 2AP in the mixtures is significantly lower than for the other aromatic genotypes, or below the limit of detection (Table 2). Environmental and post-harvest conditions influence the amount of 2AP in grains (Widjaja *et al*., 1996a; Suzuki *et al*., 1999; Champagne *et al*., 2004; Itani *et al*., 2004), which might explain why 2AP could

Table 2 Number of accessions showing aromatic or non-aromatic phenotypes in varieties that are uniform *fgr*, uniform n*fgr* or mixtures of the two, and the average amount of 2-acetyl-1-pyrroline (2AP) (μg/g) for each group of aromatic accessions

Gene	Aromatic	2AP (μg/g)	Non-aromatic	Non-aromatic (%)
fgr	279	0.69a	7	2.5
n*fgr*	15	0.56b	139	89.7
fgr/n*fgr*	19	0.47b	5	20.8

Total number of accessions is 464. $LSD_{0.05}$ = 0.11. Values followed by the same letter are not significantly different.

Table 3 2-Acetyl-1-pyrroline (2AP) content of grains from each accession of Pandan Wangi, Paw San Hmwe and Kai Noi Leung grown in different years and different locations

Variety	Accession number	Place/year grown	2AP (μg/g)
Kai Noi Leung	LG6732	Laos 2005	0.36
Kai Noi Leung	LG6732	Laos 2006	0.71
Kai Noi Leung	IRGC91414	IRRI 1999	0.17
Kai Noi Leung	IRGC91415	IRRI 1999	0.10
Kai Noi Leung	IRGC95488	IRRI 2000	0.11
Kai Noi Leung	IRGC95490	IRRI 2000	0.28
Kai Noi Leung	IRGC111814	IRRI 2003	0.16
Kai Noi Leung	IRGC111815	IRRI 2003	0.31
Kai Noi Leung	IRGC111817	IRRI 2003	0.17
Paw Sam Hmwe 1		Burma 2007	0.49
Paw Sam Hmwe	IRGC97793	IRRI 2004	0.23
Pandan Wangi	IRGC18438	IRRI 2005	1.36
Pandan Wangi 1		Indonesia, Cianjur 2007	0.18
Pandan Wangi 2		Indonesia, Cianjur 2007	0.14
Pandan Wangi 3		Indonesia, Ciliway 2007	0.26

IRRI, International Rice Research Institute.

not be detected in some samples uniform for the deletion, but does not explain why some n*fgr* samples contained significant amounts of 2AP.

Given that only a small proportion of the aromatic varieties tested were found to be n*fgr*, several samples of three traditional aromatic varieties that did not carry the deletion were obtained from three countries in South-East Asia in order to confirm our findings (Set 2). All samples contained 2AP (Table 3) and none carried the *fgr* allele (Figure 1). This confirms our result, demonstrating that the *fgr* allele does not associate unequivocally with aroma. A number of options could explain this: (i) the compound is not actually 2AP but a co-eluting compound; (ii) 2AP is formed as the product of the Maillard reaction; (iii) there is a second mutation that leads to 2AP; (iv) there is a second mutation in the fragrance gene; or (v) the deletion on exon 7 of the *fgr* allele is not the genetic cause of fragrance.

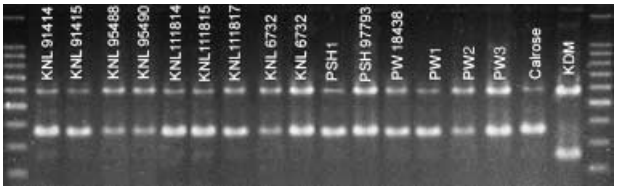

Figure 1 Gel of the varieties and accessions given in Table 3, showing that none have the 8-bp deletion that has been reported to cause the accumulation of 2-acetyl-1-pyrroline (2AP). Khao Dawk Mali 105 (KDM), which carries the deletion, and Calrose, which does not, are shown as controls. KNL, Kai Noi Leung; PSH, Paw San Hmwe; PW, Pandan Wangi.

Do aromatic n*fgr* varieties contain 2AP?

In most chromatography methods, the identification of a compound based on the elution time of its standard does not absolutely identify that compound, given the possibility of co-elution. Mass spectrometry (MS) measures the mass to charge ratio of fragments of a sample, and so is able to identify a peak definitively. The mass spectrum of the standard 2AP is identical to the mass spectrum of the peak from Khao Dawk Mali 105 that elutes at the same retention time as the 2AP standard (Figure 2). Khao Dawk Mali 105 is an aromatic *fgr* variety (Bradbury *et al*., 2005a). The mass spectrum of 2AP from Khao Dawk Mali 105 is identical to the mass spectrum of the same peak from Pandan Wangi (Figure 2), which is an aromatic n*fgr* variety (Table 3, Figure 1). The mass spectrum of the peak for all the n*fgr* aromatic varieties was identical to that of Khao Dawk Mali 105 (data not shown). This demonstrates unambiguously that the varieties without the *fgr* gene contain 2AP and, as 2AP is the most potent aromatic compound in rice (Yoshihashi *et al*., 2002), it can be concluded that the presence of 2AP in these varieties confers their aromatic quality.

Is 2AP in aromatic n*fgr* varieties the product of the Maillard reaction?

The method used to extract 2AP (Bergman *et al*., 2000) involves the incubation of rice at 85 °C. Therefore, it is highly plausible that 2AP in these varieties may be the result of the Maillard reaction caused by the acylation of 1-pyrroline by a two-carbon sugar fragment during heating. If this is the case, it could explain why these varieties do not carry the *fgr* allele, and could lead to other biological mechanisms worthy of exploration with regard to the induction of aroma during cooking. One of the problems of aromatic rice is that, on storage, the amount of 2AP decreases (Widjaja *et al*., 1996a; Archana and Pandey, 2007). The discovery of a process to form 2AP on demand could circumvent the problem of the loss of aroma on storage.

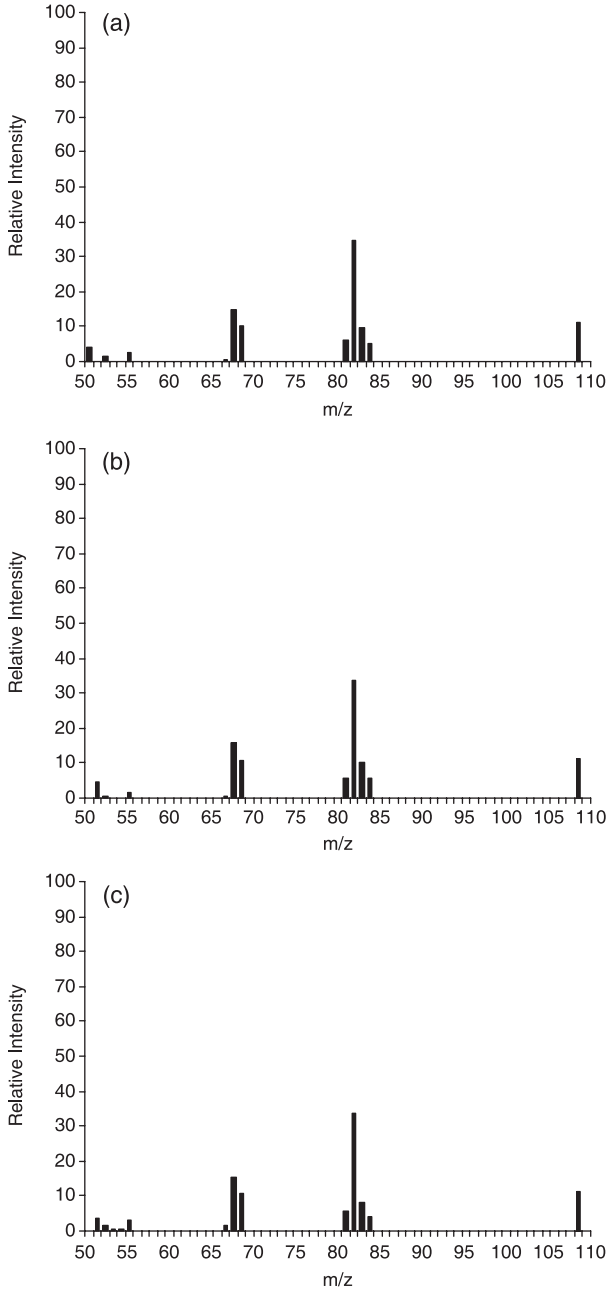

Figure 2 Mass spectra: (a) 2-acetyl-1-pyrroline (2AP) standard; (b) 2AP from Khao Dawk Mali 105; (c) same peak from one of the accessions of Pandan Wangi without the deletion. The National Institute of Standards and Technology (NIST) library identified the peak as being 2AP with 89% confidence.

Mass spectral analysis of the headspace from raw Kai Noi Leung rice (Figure 3), an aromatic n*fgr* variety (Table 3, Figure 1), shows that the spectral characteristics of the peak are similar to those of 2AP from cooked rice (Figure 2). A comparison between the mass spectra of 2AP in cooked rice (Figure 2) and raw rice (Figure 3) shows that the main difference lies in the absence of signals at masses of 50–55, 60–70 and 80–85.

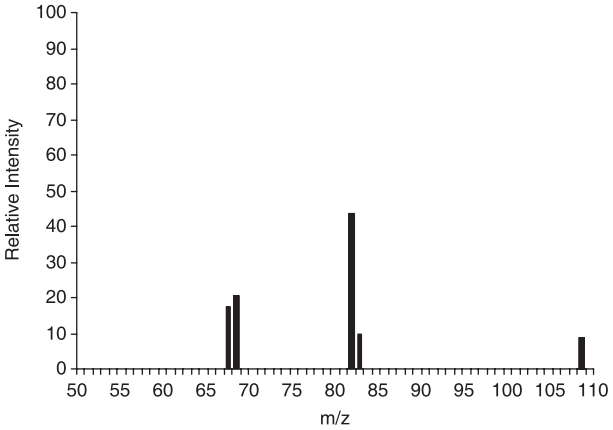

Figure 3 Mass spectrum of a compound that evaporated from flour of uncooked Kai Noi Leung with the same elution time as 2-acetyl-1-pyrroline (2AP). Using the National Institute of Standards and Technology (NIST) library, the compound at that elution time was identified as being 2AP with a likelihood of 92.5%.

These masses are seen as the smallest peaks in the spectrum of 2AP extracted from cooked rice (Figure 2), indicating that they are amongst the weakest masses in the spectral signature of 2AP. The ubiquitous presence of these missing ions as background signals might have caused the deconvolution software, AMDIS (http://chemdata.nist.gov/mass-spc/amdis/), to exclude these signals from the extracted spectrum.

The amount of 2AP that volatilises from raw rice is likely to be less than the amount of 2AP recovered from heating the rice. Both amylopectin and amylose have been shown to bind aromatic compounds (Arvisenet *et al*., 2002; da Silva *et al*., 2002), although 2AP was not included in either of these studies. Nevertheless, it is commonly observed in quality evaluation programmes that aroma can best be tasted in a raw rice grain after the grain has been chewed several times and has reached a state of mastication and gelatinisation whereby amylases can act on the starch. This suggests that 2AP, like other aromatic compounds (Arvisenet *et al*., 2002; da Silva *et al*., 2002), is held within the starch matrix of the rice grain. Therefore, it is not unexpected for only low concentrations of 2AP to be evaporated from raw rice, which could easily explain why the weakest masses, at *m/z* values of 55, 66 and 80–85, were not detected in the spectral signature of 2AP from raw rice (Figure 3). Nonetheless, the presence of the large peaks in the signature indicates that the compound is 2AP, and was predicted, with confidence, to be 2AP by the National Institute of Standards and Technology (NIST) library. This shows that 2AP in aromatic n*fgr* varieties is not produced during cooking, and indicates that 2AP accumulates in aromatic n*fgr* varieties as a result of a genetic mutation other than the 8-bp deletion in the *fgr* allele.

There is at least one more allele leading to 2AP in rice

2AP is found in many plant species (Adams and De Kimpe, 2006), but its biochemical or metabolic role in these plants is unknown. 2AP is likely to be formed from a degradation product of proline (Yoshihashi *et al*., 2002), but its complete pathway of synthesis is unknown. Its presence in many species suggests that 2AP is a metabolic compound and is potentially a source of acetyl groups; thus, the synthesis of 2AP could easily be the result of multiple genetic perturbations that reach the same biochemical end-point.

Many studies have mapped aroma to the same locus on chromosome 8 (Pinson, 1994; Lorieux *et al*., 1996; Garland *et al*., 2000; Lanceras *et al*., 2000; Cordeiro *et al*., 2002; Jin *et al*., 2003; Jain *et al*., 2004; Bradbury *et al*., 2005a; Chen *et al*., 2006), providing a large body of evidence for the association between aroma and this region – the fragrance locus. Homology has been shown between the gene at this locus and the gene that encodes a betaine aldehyde dehydrogenase (*BADH2*) (Bradbury *et al*., 2005a; Chen *et al*., 2006), as well as a gene encoding two other enzymes (Chen *et al*., 2006). *BADH* genes are nonspecific aldehyde dehydrogenases (Trossat *et al*., 1997). Molecules of 2AP carry a ketone group, not an aldehyde group, and so 2AP is an atypical substrate for a *BADH* gene. However, a recent study has shown that post-transcriptional processing of transcripts of *BADH* genes in rice is unusual (Niu *et al*., 2007), perhaps leading to altered functionality. In the present study, it has been shown that the association between the 8-bp deletion and the presence of 2AP is strong (Table 2), regardless of the identity of the gene. However, by exploring the genetic diversity of the species, we have shown that the *fgr* allele cannot be the only genetic factor driving the accumulation of 2AP in rice.

The amount of 2AP in *fgr* varieties is significantly greater than the amount in aromatic n*fgr* varieties (Table 2); the reason for this is shown in Figure 4. The box and whisker plot (Figure 4) shows the concentration of 2AP in the aromatic varieties of each of the three aromatic genotypes: uniform *fgr*; uniform n*fgr*; and mixed. The boxes show that the 2AP values between the first and second quartiles span similar ranges for each genotype, and the median value of 2AP for each genotype is similar (Figure 4). Uniform *fgr* genotypes include outliers with extremely high concentrations of 2AP, extending above the third quartile, more than 1.5 times the interquartile range. The n*fgr* and mixtures include no such outliers (Figure 4). When the 2AP values for the outliers are separated from the 2AP values lying within the interquartile range, there is no significant difference between the 2AP content of *fgr* genotypes within the interquartile range and the 2AP value of aromatic n*fgr* genotypes (Table 4). This shows that both mutations lead to similar amounts of 2AP. It is tempting to speculate that the mutations occur in combination in the outliers, as they accumulate significantly more 2AP than the varieties falling within the interquartile range. If this is the case, then 2AP in the outliers must be driven by alleles of at least two different genes, not by different alleles of the same fragrance gene.

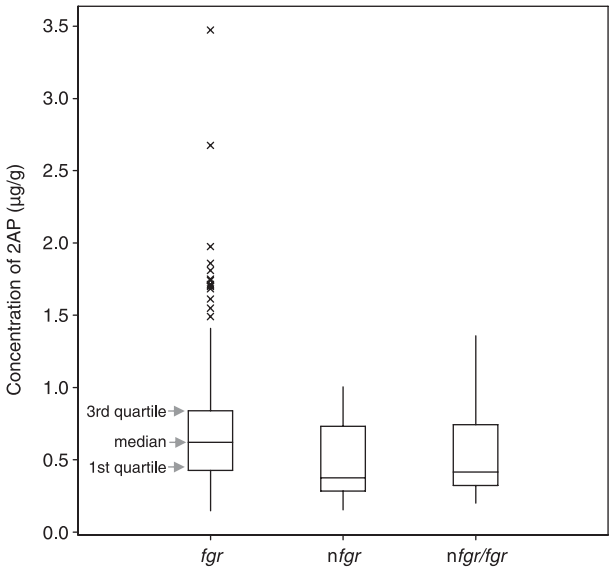

Figure 4 Box and whisker plot showing the median, first and third quartiles and range of values for the concentration of 2-acetyl-1-pyrroline (2AP), measured in aromatic accessions that carry (*fgr*) and do not carry (n*fgr*) the 8-bp deletion and mixtures (n*fgr*/*fgr*). Outliers (x) lying more than 1.5 times the interquartile range beyond the first and third quartiles are plotted individually.

Table 4 Average 2-acetyl-1-pyrroline (2AP) contents of *fgr* varieties beyond the interquartile range (IQR) in Figure 4, those within the IQR and of the n*fgr* genotypes, all of which are within the IQR in Figure 4

Genotype	Type	Mean 2AP content (µg/g)
fgr	Outlier	1.75a
fgr	Within IQR	0.62b
n*fgr*	Within IQR	0.56b

$LSD_{0.05}$ = 0.08. Values followed by the same letter are not significantly different.

The outliers in the uniform *fgr* group originate from a band of adjacent countries (Iran, Pakistan, India, Bangladesh, Burma and Thailand, as well as several from the Philippines), and belong to aromatic isozyme group 5 (Glaszmann, 1987). The aromatic n*fgr* varieties are more widely distributed than the *fgr* outliers (Figure 5), occurring throughout the centre of

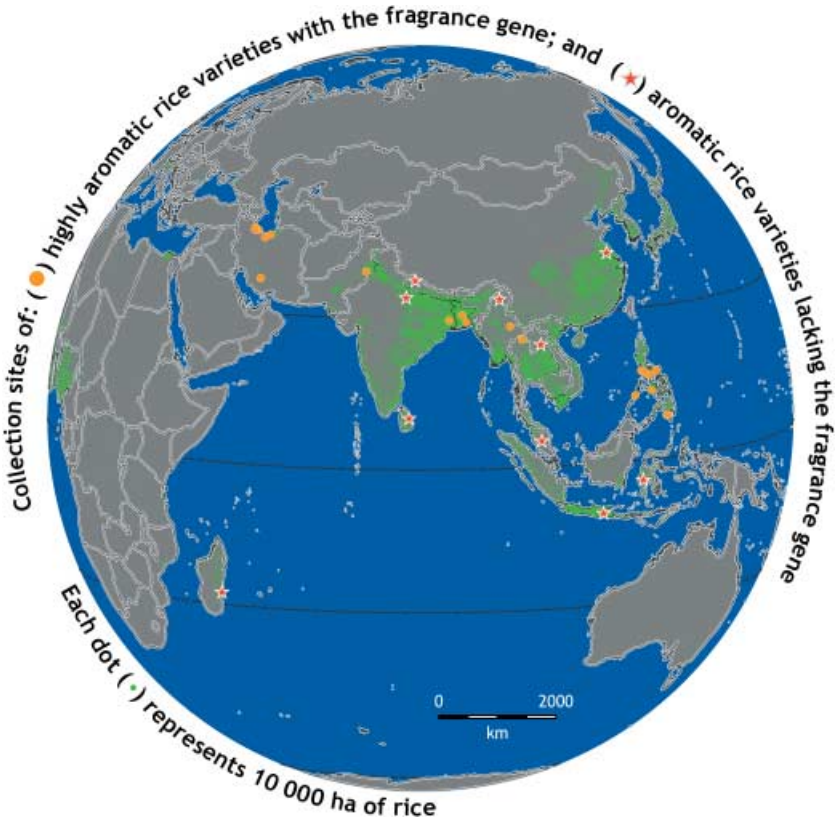

Figure 5 Country of origin, mapped as close to the provincial level as possible, for the aromatic accessions that contained 2-acetyl-1-pyrroline (2AP) but did not have the 8-bp deletion, and for the highly aromatic varieties that are outliers in Figure 4.

origin and diversity of rice, as well as further south in countries that border the Indian Ocean, from Madagascar to China. Mostly, the aromatic n*fgr* types belong to the tropical japonica group of varieties. Such a wide distribution of a relatively rare novel aromatic phenotype could have arisen either through long-distance trading or through repeated evolution of this phenotype. With the evidence available, it is not yet known whether the phenotype is a single genotype, arising by mutation one or more times, or whether different varieties have different mutations. In either case, it can be assumed that the outliers arose from hybridisation between the aromatic n*fgr* varieties and the aromatic *fgr* varieties of South Asia, capturing two or more alleles of aroma in these varieties. Thus, it can be concluded that aroma has evolved independently at least twice in rice, and perhaps the outliers in Figure 4 represent hybridisation events between domestication groups. Aroma is highly prized in almost every rice-growing country, but achieving highly aromatic varieties in a breeding programme is currently a result of fortuitous selection. The present study will eventually enable rice breeding programmes to select actively for multiple genetic sources of 2AP, leading to the development of highly aromatic and, consequently, high-quality varieties of rice.

Experimental procedures

Pedigree analysis for aroma

For the 14 samples used by Bradbury *et al*. (2005a), the biological origin of the fragrance allele was determined by analysis of the pedigree of the samples using the International Rice Information System (IRIS) (www.iris.irri.org) and from information provided by the Australian Rice Improvement Programme and the Iran Rice Research Institute.

Plant materials

Two sets of samples, 478 in total, were used to assess genetic diversity in the genepool of aromatic rice varieties. The first set was a group of accessions selected from the rice collection maintained by the GRC at IRRI, and the other set was used to validate our findings. The first set comprised 464 samples of traditional varieties of rice (*Oryza sativa*) which, on the basis of variety name, other passport data or preliminary characterization data, were considered to be putatively aromatic. The samples were harvested from a trial sown during the dry season of 2005 at IRRI's Experiment Station. Plants in the trial were protected against leaf-cutting insects but were not sprayed after flowering. Plants were grown with adequate moisture provided by intermittent irrigation. Complete fertiliser was applied to the field before transplanting at a ratio of 30 : 30 : 30 kg/ha N : P : K, and the plants were top-dressed with urea (46 : 0 : 0 kg/ha) at 30 days after transplanting at a rate of 30 : 0 : 0 kg/ha.

The second set, of 14 samples, was assembled from different sources as follows. Nine samples of the Lao variety Kai Noi Leung were obtained as paddy. Two of these were obtained directly from the Agricultural Research Centre Vientiane, Lao PDR, harvested from plots grown in lowland conditions in 2004 and in upland conditions in 2005. The other seven samples of Kai Noi Leung were obtained from the GRC as paddy produced in the dry seasons of 1999, 2000 or 2003. Three samples of the Indonesian variety Pandan Wangi were obtained as paddy directly from Indonesia to confirm the results obtained with Pandan Wangi (GRC accession IRGC18438) in the first set: two of these had been grown in 2007 in different areas of the Cianjur Province (mountains) and one in the Cilimaya Region (lowland). One sample (IRGC97793) of the Burmese variety Paw Sam Hmwe was obtained from the GRC as paddy, produced in the dry season of 2004. A second sample of the same variety was obtained directly from Burma, harvested in an upland region in 2007.

After harvest, all samples of paddy rice were dried to 14% moisture and then allowed to equilibrate at 25 °C for 3 months. After equilibration, 200 g of each large sample, harvested from trials, was dehulled (Otake FCY4 Dehusker, Oharu, Japan) and polished (Grainman, Miami, FL, USA). For the small samples obtained from the GRC, the rice was dehulled as above, and then polished using a home-made polisher. For the analysis of aroma, a subsample of polished grain (1 g) was ground to a coarse flour using a mortar and pestle. The remaining samples were sealed and stored at 4 °C to minimise the loss of volatile compounds.

Genotyping alleles of the fragrance gene

A sample of unpolished grains (5 g, approximately 100 grains) from each sample was ground to flour and mixed well; a subsample of this (100 mg) was used for DNA extraction. DNA was extracted exactly as described previously (Bergman *et al*., 2001).

The presence or absence of the 8-bp deletion was detected exactly as described previously (Bradbury *et al*., 2005b). In brief, polymerase chain reaction (PCR) was performed using 2 μL of DNA extract, 1 × Biomix (Bioline, MA, USA), 2.5 mM $MgCl_2$ and 0.2 mM each of allele-specific primers in a total volume of 12 μL. PCR was performed using a Palm Cycler (Corbett Life Science, Mortlake, NSW, Australia) with the following cycling conditions: 2 min at 94 °C, followed by 35 cycles of 94 °C for 30 s, 58 °C for 30 s and 72 °C for 35 s, with a final elongation step at 72 °C for 10 min, and then holding at 10 °C. PCR products were analysed by electrophoresis using an agarose gel (2%) stained with SybrSafe® nucleic acid stain (Invitrogen, Carlsbad, CA, USA), and visualized using a Non-UV Transilluminator (Dark Reader DR195M, Clare Chemicals, USA). When one allele was detected, all grains in the pooled sample carried either the *fgr* or n*fgr* allele and the sample was defined as uniform. Samples for which the association with 2AP did not hold were repeated twice more using completely new DNA extracts. When two alleles were detected, the samples were defined as mixtures, and DNA extraction and analysis were repeated.

Analysis of 2AP

2AP was extracted from the coarse flour of each sample with dichloromethane using a method described previously (Bergman *et al*., 2000) with some slight modifications. After extraction (once only), the extracts were heated in a water bath (85 °C) and then immediately injected into a gas–liquid chromatograph (model 6890N, Agilent, Santa Clara, CA, USA) equipped with a flame ionisation detector. An SPB-5 capillary column was used (length, 35 m; inside diameter, 0.25 mm; thickness, 0.25 μm; Supelco, Bellefonte, PA, USA). Samples were injected in splitless mode (2 μL) with a starting temperature of 35 °C for 1.8 min; the temperature was then increased at 50 °C/min to 70 °C, at 100 °C/min to 100 °C, and at 250 °C/min to 270 °C. Samples were held at 270 °C for 2 min. The injector and detector were set at 155 °C and 300 °C, respectively. Helium was the carrier gas, with a pressure of 210.5 kPa. Chemically synthesised 2AP, a gift from Dr T. Yoshihashi (Japan International Research Centre for Agricultural Sciences, Ibaraki, Japan), was used to quantify 2AP in the samples. For the set of 464 samples, 2AP analysis was replicated on 90 randomly selected samples. Furthermore, analysis of 2AP was carried out in triplicate for the outliers and for the aromatic n*fgr* samples.

For the analysis of extracted 2AP by gas chromatography-mass spectrometry (GC-MS), the gas chromatograph was equipped with a 5975 mass-selective detector, an HP-5 MS capillary column (length, 30 m; inside diameter, 0.25 mm; thickness, 0.25 μm; Agilent) was used, and the sample was introduced directly into the ion source. The electron impact mode was used. Injection parameters and heating steps were exactly as described above, but the interface temperature was 280 °C. Helium was the carrier gas at 1.0 mL/min. Mass spectra were acquired over 50–550 amu at one scan per second, with an ionizing electron energy of 70 eV, electron current of 0.3 mA, ion source at 230 °C and vacuum of 5–10 mmHg. The peaks were identified using NIST2005.

For the analysis of 2AP from raw rice, 1 g of polished rice was ground in liquid nitrogen and placed in a 2-mL GC vial. The vial was capped, held at room temperature for 24 h, and shaken constantly to maximise the accumulation of volatile components in the headspace. A polydimethylsiloxane/divinylbenzene (PDMS/DVB)-coated solid-phase microextraction (SPME) fibre (blue coded, Supelco) was exposed to the headspace of the rice for 30 min, and splitless desorbed at 250 °C in the injector port of the gas chromatograph-mass spectrometer. Separation was carried out by temperature programming: 80 °C for 2 min, heating at 8 °C/min to 250 °C, and then holding for 5 min at 250 °C. An HP5 column (length, 50 m; inside diameter, 0.32 mm; thickness, 1.025 μm) was used on a GC8000 chromatograph fitted with an MD800 mass spectrometer. Data were analysed using EXCALIBUR (Thermo Fisher, Waltham, MA, USA) and AMDIS, employing the NIST2005 mass spectral library for identification.

Statistical analysis

Statistical analysis to compare the amount of 2AP (Tables 2 and 4) was carried out using balanced analysis of variance (ANOVA) with a completely randomised design and partitioning the treatment sum of squares using CROPSTAT (version 6.1.2007.1). Pairwise comparison of means was performed using the least significant difference (LSD) at a 5% level of significance.

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