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Variation in grain arsenic assessed in a diverse panel of rice (*Oryza sativa*) grown in multiple sites

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Summary

- Inorganic arsenic (As_i) in rice (*Oryza sativa*) grains is a possible threat to human health, with risk being strongly linked to total dietary rice consumption and consumed rice As_i content. This study aimed to identify the range and stability of genetic variation in grain arsenic (As) in rice.
- Six field trials were conducted (one each in Bangladesh and China, two in Arkansas, USA over 2 yr, and two in Texas, USA comparing flooded and nonflood treatments) on a large number of common rice cultivars (*c.* 300) representing genetic diversity among international rice cultivars.
- Within each field there was a 3–34 fold range in grain As concentration which varied between rice subpopulations. Importantly, As_i correlated strongly with total As among a subset of 40 cultivars harvested in Bangladesh and China.
- Genetic variation at all field sites was a large determining factor for grain As concentration, indicating that cultivars low in grain As could be developed through breeding. The temperate *japonica*s exhibited lower grain As compared with other subpopulations. Effects for year, location and flooding management were also statistically significant, suggesting that breeding strategies must take into account environmental factors.

Introduction

Arsenic (As) in rice (*Oryza sativa*) grains is present mainly as inorganic arsenic (As_i) (arsenite and arsenate) and the organic species dimethylarsinic acid (DMA), with lower concentrations of the organic species monomethylarsinic acid (MMA) and tetramethylarsonium (Williams *et al.*, 2005; Hansen *et al.*, 2011). With regard to accumulation of As in the grain, rice is more problematic than other cereals as it has much higher concentrations as a consequence of the method of rice cultivation in anaerobic soil, where As is more available (Williams *et al.*, 2007; Xu *et al.*, 2008).

While dietary As is generally not a concern with the highly varied diets typical of Western cultures, it can be of serious concern for those on a rice subsistence diet, especially if they are restricted to rice that was produced in As-contaminated soil or irrigation water. The US Environment Protection Agency considers a 1/10 000 cancer risk to be the threshold for establishing legally enforceable thresholds on toxins, and the As_i ingested through consuming rice contributes to an elevated cancer risk above 1/10 000 in all countries where this has been assessed, with risks being proportional to the quantity of rice consumed (Meharg *et al.*, 2009). Elevated As in cultivated land can arise from a number of sources. In the Bengal Delta, it has been established that the groundwater has high concentrations of As (Fendorf *et al.*, 2010) and that shallow tube wells have been used to tap into this water for crop irrigation (Williams *et al.*, 2006), leading to increased concentrations of As within agricultural soils (Saha & Ali, 2007), and consequently elevated concentrations of As within the edible parts of crops (Williams *et al.*, 2006). Other areas where there is increased As within the soil include regions with a history of mining and mining-associated industries (Zhu *et al.*, 2008a,b), and the use of arsenical pesticides and wood preservatives (Meharg & Hartley-Whitaker, 2002; Zhao *et al.*, 2009). In addition to anthropogenic contaminants being a source

of As, it has recently been established that in some areas of Bangladesh baseline soil As concentrations (i.e. not raised by human pollutants) are high enough to cause substantial accumulation of As within rice grains grown there (Lu *et al.*, 2009).

A number of approaches have been proposed to reduce the accumulation of As in rice grains (Daum *et al.*, 2002; Imamul Huq *et al.*, 2006; Xu *et al.*, 2008; Brammer, 2009; Li *et al.*, 2009; Zhao *et al.*, 2010a). One of the simplest and most cost-effective approaches would be to select rice cultivars that biologically restrict the accumulation of As in their grains (Carbonell-Barrachina *et al.*, 2009; Zhao *et al.*, 2010a). This could be achieved by selecting cultivars that have low As uptake from soil, and/or cultivars that restrict the transfer of As from roots to shoots and/or from shoots to the grain. These cultivars could immediately be used in As-contaminated regions, as well as being suitable genetic stock for breeding programs introducing low grain As into agronomically improved varieties suitable for modern commercial rice production. A field study looking at grain As variation in Bangladesh determined that the variation observed was largely attributable to genetics (Norton *et al.*, 2009a); however, when a subgroup of the cultivars were tested in a wider environmental context in three countries (Bangladesh, India and China) there was a large environmental effect on grain As concentration (Norton *et al.*, 2009b). Grain As variation of cultivars (90 in total) grown in West Bengal was found to be very large, with the grain As concentration ranging from 79 to 2700 μg kg^{-1}, with 69 of the cultivars tested identified as unsafe for people on a rice subsistence diet (Tuli *et al.*, 2010). A study performed in Arkansas, USA, measuring the performance of *c.* 20 cultivars across multiple years, has identified the major factor affecting grain As, under their moderate As field conditions, to be genotypic variation; however, there were effects of both year and year-by-cultivar interactions that determined grain As concentration (Pillai *et al.*, 2010). Recently, a study examining the effects of cultivar, environment, and cultivar-by-environment interaction on grain As was conducted in Bangladesh across 10 locations with varying concentrations of soil and water As; the findings suggested that a large proportion of the variation in grain As was attributable to the environment that plants were grown in (Ahmed *et al.*, 2011). However, low grain As cultivars could be identified; in the dry (boro) season, cultivar BR3 was identified to have the lowest grain As (Ahmed *et al.*, 2010), which is in agreement with Norton *et al.* (2009a), but for other cultivars the rankings were not in agreement. This suggests that a complex control of grain As is operating, and is affected not only by genetics but also by the location where the rice is grown and the environmental fluctuations that occur between years.

In the present study, genetic variation in grain As was investigated using the same set of *c.* 300 cultivars grown in six field trials, one each in Bangladesh and China, two in Arkansas, USA over 2 yr, and two in Texas, USA comparing flooded and non-flood treatments. The comparison of flooded and nonflooded treatments was performed to determine if the genetic variation in grain As is the same under flooded and nonflooded conditions. Daum *et al.* (2002) showed that even short, intermittent periods of soil aeration can alter soil chemistry significantly enough to reduce grain As concentration. Therefore, the Texas study employed an unconventional extended flood as described in the Materials and Methods section, with the aim of maximizing As accumulation in those grains. Variation in total As was investigated by genotype and by rice subpopulation group (*aus*, *indica*, and tropical and temperate *japonica*s). We compared grain As concentrations with important traits such as yield and flowering time to determine if strategies to reduce grain As will affect agronomic characteristics. Additionally, year-to-year variation in grain As in the Arkansas site was assessed. Furthermore, the inadvertent effect of breeding on As content among the US rice cultivars was explored. Inorganic forms of As are more toxic to animals than organic As species, yet analysis of total As is significantly less complex and more affordable than analysis of As_i. To investigate relationships between total As and the different As species in rice grains, a subset of 40 cultivars grown in Bangladesh and China were analysed for As speciation in the grain. Finally, using this As speciation data and current As intake guidelines, the number of cultivars grown at the Bangladesh and China sites that were found to contain concentrations of As_i high enough to be of concern for people on a rice subsistence diet is reported.

Materials and Methods

Field sites

The field trials were conducted in four locations. Two of the four sites were selected for unusually high soil As content (Bangladesh and China), while two US field locations (Arkansas and Texas) allowed observation of the same genotypes when grown under low soil-As conditions. Each site was planted and managed according to methods common to commercial rice production in that region, except for the Texas site where both the flooded and unflooded fields were under atypical water management, described further below. Plants were transplanted into flooded paddies at the two Asian sites, and mechanically drill-seeded into dry soil at the US sites, then irrigated to initiate germination.

The field site in Bangladesh was located at Faridpur (latitude 23°35.1′; longitude 89°47.1′). Rice seeds were germinated in December 2008, transplanted to the field in January 2009, and harvested in May 2009. The field site at Faridpur was maintained under continuous flood. The field site was fertilized with 70 kg ha^{-1} nitrogen (N) (split over three equal applications), 20 kg ha^{-1} phosphorus (P), 50 kg ha^{-1} potassium (K), 15 kg ha^{-1} sulphur (S) and 2 kg ha^{-1} zinc (Zn). Once a majority of the cultivars had flowered, the irrigation was stopped and the field allowed to dry before harvest of the grains.

The second field site was located in China near Qiyang city, Hunan province (latitude 26°45′; longitude 111°52′). Rice seeds were germinated in April 2009 and harvested in July/August 2009. The field site in Qiyang was under continually flooded conditions, and fertilizer was applied three times during the field trial. On the day of transplanting the seedlings, 200 kg ha^{-1} of compound fertilizer (N, P, K at a ratio of 5 : 2 : 1) was applied. Eight days after transplanting, 60 kg ha^{-1} of urea was applied, and 30 d after transplanting, 100 kg ha^{-1} of the same compound

fertilizer was applied. This provided a seasonal total of 100 kg ha^{-1} N, 30 kg ha^{-1} P, and 15 kg ha^{-1} K. Flooding was performed as described for the Faridpur field site.

The two field sites in the USA were 550 km apart, with one being located at Stuttgart, Arkansas (latitude 34°4.6′; longitude – 91°40.6′) and the other at Beaumont, Texas (latitude 30°4.3′; longitude – 94°17.3′). The Arkansas field site was located near the United States Department of Agriculture – Agricultural Research Service (USDA-ARS) Dale Bumpers National Rice Research Center outside of Stuttgart. Fields were fertilized before planting with 8.4 kg ha^{-1} P and 46.3 kg ha^{-1} K, which was incorporated during seedbed preparation. N was applied as urea at the rate of 56 kg ha^{-1} N just before a permanent flood was applied to the field in both years. The flood was applied when plants were at the five-leaf stage, and drained 15–20 d after almost all the cultivars had flowered to allow fields to dry before harvest.

For the Texas site, fields were fertilized before planting with 14.4 kg ha^{-1} P, which was incorporated during seedbed preparation. Urea was applied at a rate of 73 kg ha^{-1} N at planting. The fields were flush-irrigated until plants reached an average 18 cm height, at which time a 10-cm permanent flood was applied to the flooded paddy, with the nonflooded paddy receiving continued flush irrigations to keep the root zone damp but not saturated. Unique to this site compared with the other flooded fields in the study is that a minimum flood depth of 10 cm was maintained on the flooded field until all seeds were hand-harvested. This allowed all plots, from early to late flowering, to produce seeds under equally saturated soil conditions, avoiding concerns that even short, intermittent periods of soil aeration can measurably reduce grain As concentration (Daum *et al.*, 2002).

Soil properties

For the Bangladesh and China soils, As was determined using the method described in Stroud *et al.* (2011), which involves a strong digestion using perchloric acid and yields near-total As for measurement. For the US soils, the As was determined using the method described in Adomako *et al.* (2009), which involves overnight digestion in nitric acid followed by microwave heating (to 55°C; hold 10 min; to 75°C; hold 10 min; to 95°C for 30 min). The nitric acid digestions are less stringent than the perchloric acid digestions, rendering *c.* 70% of the total soil As for measurement. For both laboratory analyses, soil samples comprising the top *c.* 10 cm of the field surface were air-dried and then passed through a 2-mm (10 mesh) sieve.

The soil at the Faridpur, Bangladesh field site had a pH of 8.2, and 1.81% total carbon (C). The field site had a history of boro irrigation with As-contaminated water from tubewells. The Faridpur field site had an average soil As content of 14 ± 0.3 mg kg^{-1}. At the Qiyang field site, the soil had a pH of 6.3, and 2.20% C. The As contamination at this site was derived from geologically elevated concentrations of As. The average soil As content was 65 ± 2 mg kg^{-1}.

The soil at the field site near Stuttgart, Arkansas is classified as a Dewitt silt loam (fine, smectitic, thermic Typic Albaqualf (Soil Conservation Service, 1995)). When measured before planting in 2007, the soil had a pH of 5.5. Fields at this site are managed in a 2-yr rotation of rice and soybean (*Glycine max*) and, based on historical records, the soil is presumed to never have been exposed to As-containing pesticides that would artificially increase the soil As content. The As content was determined in early 2011 by collecting soil samples from the locations where the field studies were conducted in 2006 and 2007. The soil As content was 4 ± 2 and 5 ± 1 mg kg^{-1} in the 2006 and 2007 locations, respectively, with 0.68% total C. The soil at the field site in Texas is classified as a League clay (fine, smectitic, hypothermic Oxyaquic Dystrudert (Soil Conservation Service, 1995)). The soil had a pH of 5.5, 1.06% total C, and a soil As content of 3 ± 1 mg kg^{-1} for the flooded field and 2 ± 0.2 mg kg^{-1} for the nonflooded field. The field site has been used solely for rice field-plot research since 1948, and, as such, is presumed to have never been exposed to any As-containing pesticides that would artificially increase the soil As content. Soil samples were collected just before planting, after the seedbed had been cultivated and prepared for planting.

Plant material

The cultivars used in this study are from an established rice diversity panel (Ali *et al.*, 2011; Tung *et al.*, 2010; Zhao *et al.*, 2010b; http://www.ricediversity.org). The seed source for the population is described in Ali *et al.* (2011). For the US field experiments, seeds were obtained from the Genetic Stocks-Oryza (GSOR) collection directly. For the Bangladesh and China field experiments, seeds were first sent to Aberdeen, UK, where the seeds were bulked before being sent out to the field sites in Bangladesh and China. A total of 312 *Oryza sativa* L. cultivars were grown at the Faridpur field site, 295 cultivars at the Qiyang field site, 346 at the Arkansas field site in 2006 and 352 cultivars in 2007, and 377 cultivars at the Texas field site. The cultivars embody a wide range of the geographical and genetic diversity of *O. sativa*, originating in 79 different countries and representing all five of the major subpopulations of rice. Subpopulation identity was determined based on simple sequence repeat (SSR) and single nucleotide polymorphic (SNP) markers (Ali *et al.*, 2011; Zhao *et al.*, 2010b). The *aus*, *indica*, and tropical and temperate *japonicas* were well represented (at least 55 cultivars from each subpopulation) at each field site.

Field layout

At the field sites in Bangladesh and China, the plants were transplanted in a randomized complete block design (RCBD) with four replicates. In each replicate, each genotype was planted by hand in a single row of 2 m with 10 hills, each hill (one seedling) 20 cm apart and each row 20 cm apart. The central six plants were harvested and pooled together for As analysis; the yield is also expressed as the combined yield from these six plants. To separate the test genotypes, two hills of a check cultivar were planted at each end of the 10-hill test rows. Between each row of test genotypes, one row of check cultivar was planted. Flowering time was recorded in only one of the four replicates at each of the

Asian field sites. The six representative plants were harvested and threshed by hand, and a subsample of the grain was dehusked for grain As determination.

For the field site in Arkansas, the field layout in both years was a RCBD with two replications. Seeds of each cultivar were planted with a Hege Model 90 Plot Drill seeder *c.* 2 cm deep in a single row 5 m long with spacing of 25 cm between the plants and 50 cm between the rows. Seedlings emerged *c.* 1 wk after planting. Fields were flush-irrigated twice before a permanent flood was applied to the fields *c.* 2–3 wk after seedling emergence. Flowering time was recorded for three representative plants per row per each of the two replications. The same representative plants were harvested by hand and threshed with an Almaco small bundle thresher to obtain the seeds for the grain As determination.

For the field site in Texas, the *c.* 300 genetic lines were grown in adjacent fields (flooded and nonflooded) using an RCBD with three replications. Plots were planted using similar machinery and methods to those used in Arkansas. Five seeds per cultivar were drill-seeded *c.* 2 cm deep into 13-cm length lines, hereafter called hillplots. Five hillplots were planted per row with 61 cm between hillplots within each field row, and 25 cm between rows. Genotypes were represented by one hillplot per replication. Flowering time was recorded per hillplot. Twenty fully mature seeds per hillplot were dehulled, from which three seeds were randomly selected for analysis of grain As.

As analysis

The following method was used for the analysis of the Faridpur and Qiyang samples. Trace-element grade reagents were used for all digests, and for quality control replicates of certified reference material (CRM; rice flour (NIST 1568a) or Oriental tobacco (*Nicotiana tabacum* leaves (CTA-OTL-1)) were used; spikes and blanks were included. Rice grain samples were dehusked and oven-dried (80°C), and 0.2 g was weighed into 50-ml polyethylene centrifuge tubes. The rice total shoot biomass (except panicles) was harvested 10 cm above the ground (at the time of harvest), chopped into small pieces, subsampled, oven-dried and finely chopped, and 0.2 g was weighed into 50-ml polyethylene centrifuge tubes. Samples were microwave-digested with concentrated HNO_3 and H_2O_2 as described in Sun *et al.* (2009). Total As analysis was performed by inductively coupled plasma mass spectrometry (ICP-MS) (Agilent Technologies 7500, Santa Clara, CA, USA). Rhodium (10 μg l^{-1}) was run on an external line as the internal standard. The analysis was performed as described in Sun *et al.* (2009).

The following method was used for the analysis of the grains from the Texas and Arkansas sites. Three whole grains of dehusked rice (*c.* 0.05 g) were digested with 1.0 ml of concentrated HNO_3 in 16 × 100 mm Pyrex tubes, at temperatures stepped from ambient to 110°C over a period of 12 h. Indium (EM Science, Gibbstown, NJ, USA) was added to the acid to a final concentration of 20 μg l^{-1} as an internal standard. Samples were diluted to 10.0 ml and analysed on a PerkinElmer (Waltham, MA, USA) Elan DRCe ICP-MS for As. Portions of the samples were combined and used as a matrix-matched standard for drift correction, measured after every nine samples. Samples were normalized to the averaged signals of the best-measured elements and weights of seven samples per run.

The methods for As analysis were different, as the optimized standard procedures at the institutes where the samples were analysed differed (the Bangladesh and China samples were analysed at Aberdeen, UK, and the Texas and Arkansas samples were analysed at Purdue, IN, USA).

There is no direct comparison between the grain As concentrations determined using the two different digestion methods and ICP-MS instruments used in this study, but both labs use internal controls (CRM at Aberdeen and matrix-matched standard at Purdue). Although the variable methodology employed precludes comparison of absolute concentrations between the USA and Asian sites, the data are sufficient for the primary purpose of the study, to identify genotypes high or low in As concentration across multiple sites.

As speciation

A subset (n = 40) of the cultivars from the field sites in Bangladesh and China were selected for As speciation. This subgroup comprised 10 randomly selected cultivars from each of the four well-represented subpopulations (*indica*, *aus*, temperate *japonica* and tropical *japonica*). The samples were dehusked and powderized, and 0.2 g was weighed into 50-ml polyethylene centrifuge tubes. Samples were microwave-extracted with 1% HNO_3 as described by Sun *et al.* (2009). The As speciation was performed by anion-exchange HPLC-ICP-MS. Details of the protocol for As speciation analysis are given by Sun *et al.* (2009).

Statistical analysis

Analysis of variance (ANOVA) and a general linear model (GLM) analysis were performed per individual field site using MINITAB 15 Statistical Software (Minitab Inc, State College, PA, USA). All trait data were tested for normality (only the grain As data from the Arkansas 2007 field site were not normal and were corrected using a $\log_{10}$ transformation). Replication effects proved minor (< 2% of total variance in each site) compared with genetic effects (ranging from 40.1 to 63.4%). One-way ANOVA was used to test for statistically significant differences between traits for the cultivars at individual field sites. For the cultivars in common across the two Asian field sites, GLM was used with cultivar and field site as the main effects. Correlations between grain As and other traits were obtained using the least squared means for the cultivars at each site. Analysis of the flowering time data by linear regression (covariance) analysis was not appropriate because the error variances proved to be non-normally distributed (variances were correlated with means). Therefore, to determine if there was an interaction between flowering time and grain As, the cultivars were grouped into flowering time intervals (5 or 10 d per bin based on division into six to eight bins per location) allowing a GLM analysis to be conducted in MINITAB.

Results

Genetic variation in grain As

The mean, median and range of brown rice As concentrations in all the cultivars per location are presented in Table 1. Additionally, for the flooded sites the mean, median and range of brown rice As concentrations in only the cultivars grown at all locations are presented. The range in grain As for all the cultivars for each site is presented in Fig. 1(a–f). Mean total grain As was the highest at the Qiyang (drained after heading) and Texas (flooded until grain maturity) field sites. There was a 14-fold difference in mean grain As between the nonflooded and flooded cultivars at the Texas site. When descriptive values of variation obtained for all cultivars at each site were compared with those obtained only for cultivars common across all sites, little difference was found between the means, medians and ranges (Table 1).

In all field trials there were significant differences in grain As across the four main rice subpopulations (Table 1, Fig. 2a–f). The subpopulation groups with the highest and lowest grain As varied among field sites. At the Faridpur site and three flooded field sites in the USA, the temperate *japonica* subpopulation had the lowest grain As, while at the Qiyang field site varieties from the tropical *japonica* subpopulation had the lowest concentration of grain As. The ratio of grain As in flooded/nonflooded conditions at the Texas field site showed significant subpopulation differences ($P < 0.001$, $F = 16.53$, df = 3), with the *aus* subpopulation having the largest ratio (Supporting Information Fig. S1), showing that it had the largest proportional increase in As uptake in response to flooding. Flooding is known to change the form of As in soil solution (reduces As(V) to As(III)), making it more available for plant uptake. In addition, at the Texas and Arkansas field sites (under flooded conditions), the six modern US genotypes that are currently cultivated had lower grain As compared with the 22 US cultivars that are no longer in common cultivation (Tables 2, S1). Although the mean As contents were slightly lower for the modern US cultivars compared with the historical ones, they were not significantly different when grown under nonflooded conditions at Texas nor when grown at the field sites in Bangladesh and China (Table 2). When flowering times were compared between the modern and historical US cultivars, no significant difference was found at the Texas field site under flooded conditions, nor at the Arkansas 2007 field site. At the Arkansas field site in 2006, the modern cultivars flowered on average 9 d earlier than the historical US cultivars ($P = 0.014$, $F = 7.1$, df = 1).

Although the method for As analysis differed, when the cultivars common among the different field sites and years were compared, a number of significant correlations were found across sites for grain As (presented in Table 3). There were significant positive correlations ($P < 0.001$) between the three flooded US field sites (Arkansas 2006 vs Arkansas 2007 ($r = 0.509$, df = 322); Texas vs Arkansas 2006 ($r = 0.506$, df = 328); Texas vs Arkansas 2007 ($r = 0.460$, df = 344)). Also, there was a significant correlation between the flooded and nonflooded cultivars at the Texas field site ($P < 0.001$, $r = 0.416$, df = 368) (Table 3). The US sites had weakly significant correlations with the two Asian sites (r values ranged from −0.149 to 0.299), which were not significantly correlated with each other.

As the Arkansas and Texas experiments were designed differently from the Qiyang and Faridpur experiments, with grains analysed by different methods, it is not possible to estimate the partitioning of variance for grain As between all field trials. However, it is possible to determine the partitioning of variance for the three experiments separately. Comparing the field sites in Bangladesh and China, there were significant ($P < 0.001$) cultivar differences ($F = 4.1$, df = 288), site effects ($F = 1675$, df = 1) and cultivar-by-site interactions ($F = 4.1$, df = 288); these factors respectively explained 40.3, 13.7, and 36.5% of the variance. For the Arkansas field site there were significant ($P < 0.001$) effects of cultivar ($F = 10.91$, df = 325), year ($F = 1167$, df = 1) and cultivar-by-year interactions ($F = 3.94$, df = 325); these factors respectively explained 31.0,

Table 1 Mean and range of grain arsenic (As) concentration for the six field trials

Variable	Faridpur	Qiyang	Arkansas 2006	Arkansas 2007	Texas flooded	Texas nonflooded
No. of cultivars	312	295	346	352	377	374
Mean grain As (μg kg^{-1})	443 (443)	676 (681)	376 (389)	253 (266)	632 (653)	45
Median grain As (μg kg^{-1})	437 (441)	662 (666)	360 (372)	205 (217)	620 (646)	42
Min. grain As (μg kg^{-1})	192 (192)	363 (363)	104 (123)	30 (30)	172 (172)	9
Max. grain As (μg kg^{-1})	899 (820)	1266 (1266)	988 (988)	1035 (951)	1682 (1682)	126
First quartile value (μg kg^{-1})	361 (361)	553 (555)	283 (303)	159 (166)	478 (505)	32
Third quartile value (μg kg^{-1})	509 (506)	780 (784)	456 (465)	276 (297)	742 (756)	56
F-value for significant difference among cultivars	7.77***	3.58***	4.25***	9.09***	4.8***	3.16***
Proportion of the variation explained by cultivar (%)	63.4	40.1	41.2	59.7	56.2	42.1
F-value for significant difference among subpopulations	14.4***	40.7***	17.3***	25.9***	18.2***	10.1***

Analyses of cultivar differences and subpopulation differences are presented as the *F* value from one-way ANOVA. The numbers in brackets are the values calculated when using only the 263 cultivars common across all the flooded field sites. The Arkansas and Texas studies used different methodologies for field management and grain analyses. Therefore, direct comparison of As values between the US sites and the two Asian sites should not be made.
***, $P < 0.001$.

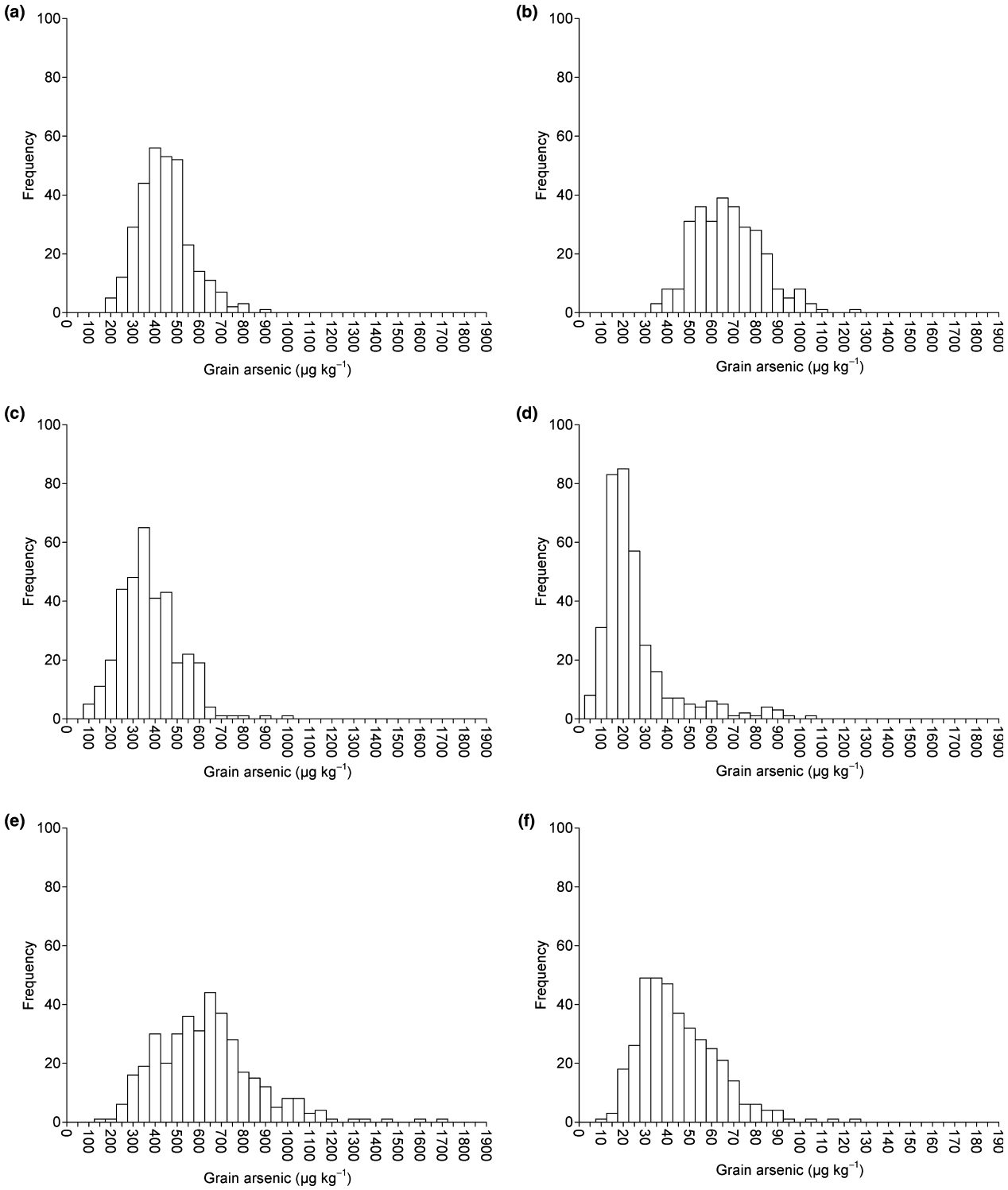

Fig. 1 Variation in arsenic (As) concentration in brown grain for the cultivars grown at the Faridpur (a), Qiyang (b), Arkansas 2006 (c), Arkansas 2007 (d), Texas (flooded) (e), and Texas (nonflooded) (f) field sites. Note that for (f) the *x*-axis scale is 10-fold lower.

22.6, and 17.1% of the variance. Comparing the effect of flooding in Texas, there were significant ($P < 0.001$) effects of cultivar ($F = 4.89$, df = 370), flooding ($F = 11169$, df = 1) and cultivar-by-flooding interactions ($F = 4.28$, df = 370). Even though the partitioned variance is dominated by the flooding treatment (99.9%), the cultivar effects were also found to be highly significant ($P < 0.001$) along with the cultivar-by-flooding interaction ($P < 0.001$).

For each flooded trial, those cultivars common across all trials were ranked by mean grain As. Comparing across the rankings it

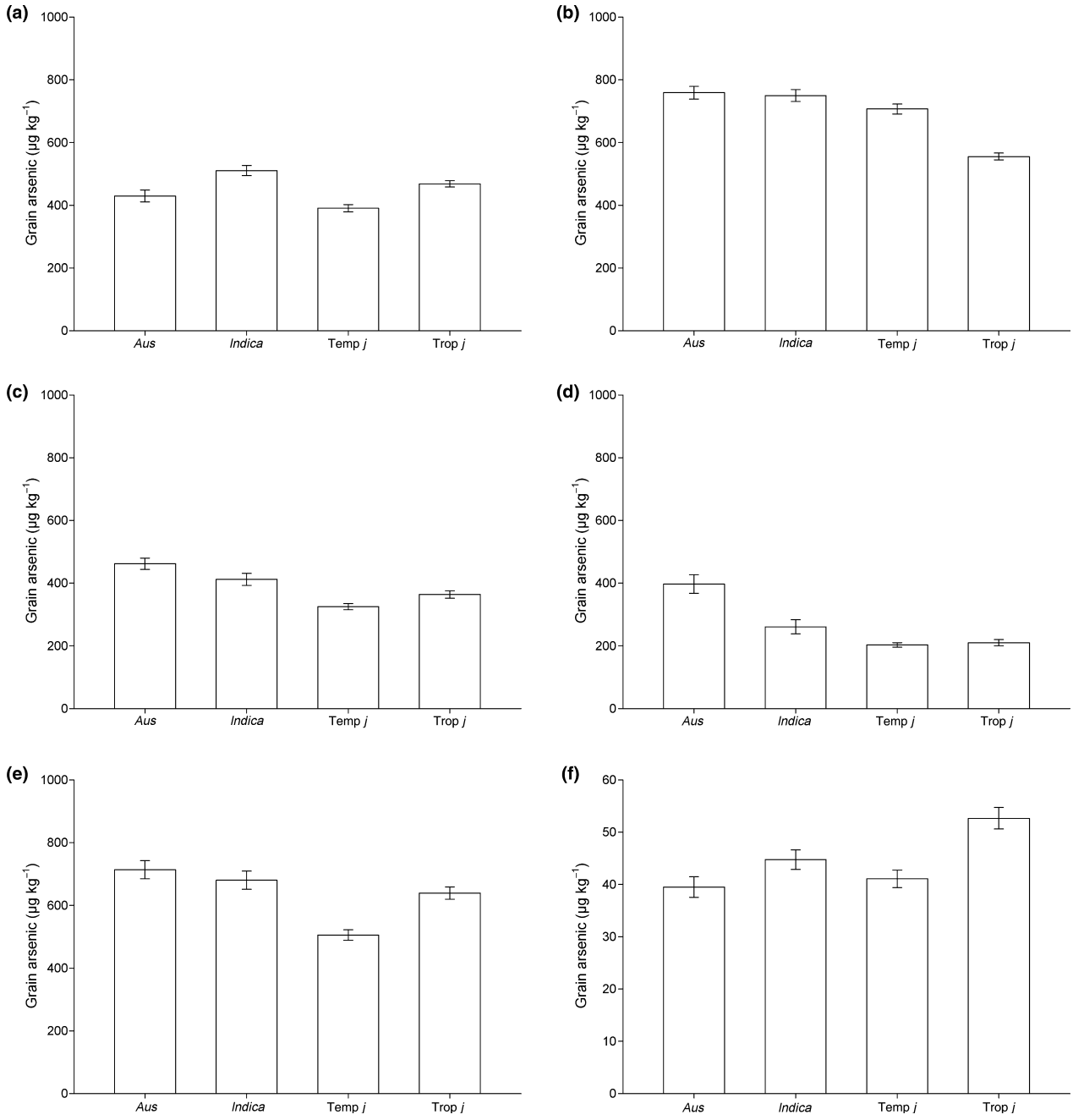

Fig. 2 Mean brown grain arsenic (As) concentration in subpopulations (the error bar is the SEM for the subpopulation) at the Faridpur field site (a), Qiyang field site (b), Arkansas 2006 field site (c), Arkansas 2007 field site (d), Texas flooded field site (e), and Texas nonflooded field site (f). Temp *j*, temperate *japonica*; Trop *j*, tropical *japonica*.

was possible to identify two cultivars that were in the highest 20% for grain As in all five field trials; these cultivars were Dawebyan and Sabharaj (both *indica*s) (Table 4). Fifteen cultivars were ranked in the upper 20% in four out of the five trials; 13 of these cultivars were either from the *aus* or *indica* subpopulations or were admixtures of these. No cultivars were identified that were consistently present in the bottom 20% for grain As across all sites. However, three temperate *japonica*s, Azerbaidjanica, Kon Suito, and Norin 20, were identified as having low grain As in four of the five field trials.

Genetic variation in shoot As and shoot-to-grain transfer

The shoot As concentration was measured only at the Faridpur field site, where the 312 genotypes grown there had shoot As concentrations ranging from 4 to 23 mg kg^{-1}, with an average concentration of 9 mg kg^{-1}. There were significant genotypic differences ($P < 0.001$, $F = 1.89$, df = 311) which explained 19.4% of the variation. For the four main rice subpopulations there was a significant difference in shoot As concentration ($P < 0.001$, $F = 9.15$, df = 3). Cultivars from the temperate

Table 2 Mean grain arsenic (As) concentrations for the modern and historical US cultivars at all field sites

	Grain As (μg kg^{-1})*		One-way ANOVA	
Field site/treatment	Modern US cultivars (n = 6)	Historical US cultivars (n = 22)	F-value	P-value
Texas flooded	429 ± 35	705 ± 38	28.7	< 0.001
Texas nonflooded	50 ± 5	52 ± 7	–	NS
Arkansas 2006	278 ± 17	408 ± 28	13.1	0.002
Arkansas 2007	149 ± 15	231 ± 25	7.11	0.014
Faridpur	441 ± 23	487 ± 28	–	NS
Qiyang	549 ± 33	562 ± 45	–	NS

The Arkansas and Texas studies used different methodologies for field management and grain analyses. Therefore, direct comparison of As values between the various field sites should not be made.
*, mean ± SE; NS, not significant.

Table 3 Correlations between mean grain arsenic (As) concentrations for the cultivars common to all field trials (n = 263)

	Faridpur 2009	Qiyang 2009	Arkansas 2006	Arkansas 2007	Texas 2009 flooded
Qiyang	NS	–	–	–	–
Arkansas 2006	0.239***	0.241***	–	–	–
Arkansas 2007	NS	0.264***	0.509***	–	–
Texas 2009 flooded	0.299***	0.134*	0.506***	0.460***	–
Texas 2009 nonflooded	0.187***	–0.149*	0.166**	0.222***	0.416***

*, P < 0.05; **, P < 0.01; ***, P < 0.001; NS, not significant.

Table 4 Cultivars that were in either the upper (high grain arsenic) or lower (low grain arsenic) 20% in the five flooded field trials

Cultivar	Country of origin	Subpopulations based on SSRs	Grain arsenic	Faridpur	Qiyang	Arkansas 2006	Arkansas 2007	Texas flooded
Kon Suito	Mongolia	Temperate *japonica*	Low	X		X	X	X
Norin 20	Japan	Temperate *japonica*	Low	X		X	X	X
Azerbaidjanica	Azerbaijan	Temperate *japonica*	Low	X		X	X	X
Dawebyan	Myanmar	*indica*	High	X	X	X	X	X
Sabharaj	Bangladesh	*indica*	High	X	X	X	X	X
Bala	India	*indica*	High	X	X	X		X
DM 43	Bangladesh	*aus*	High		X	X	X	X
ARC 10177	India	*aus*	High	X		X	X	X
Shim Balte	Iraq	*aus*	High		X	X	X	X
Manzano	Zaire	Tropical *japonica*	High	X		X	X	X
519	Uruguay	*indica*	High		X	X	X	X
P 737	Pakistan	*aus*	High		X	X	X	X
Khao Pahk Maw	Thailand	*aus*	High		X	X	X	X
Pao-Tou-Hung	China	*indica*	High	X		X	X	X
BJ 1	India	*aus*	High		X	X	X	X
TOg 7178	Senegal	Admix (*indica*, *aus*)	High		X	X	X	X
DA16	Bangladesh	Admix (*indica*, *aus*)	High	X	X	X		X
Kalubalavee	Sri Lanka	*aus*	High		X	X	X	X
LD 24	Sri Lanka	*indica*	High	X		X	X	X
Padi Pagalong	Malaysia	Tropical *japonica*	High	X		X	X	X

The value is based on the mean grain arsenic value in the cultivars that are common across the five trials. 'X' denotes that that cultivar was in the upper or lower 20% at that field site.

japonica subpopulation had a higher concentration of shoot As compared with the other three subpopulations, with the *aus* subpopulation having the lowest shoot As (Fig. S2a) at the Faridpur field site. There was a significant positive correlation between grain As and shoot As for the cultivars (P < 0.001, r = 0.341, df = 309).

The ratio of grain:shoot As, termed the transfer factor, ranged from 0.023 to 0.134, with an average of 0.057. There were significant genotypic differences (P < 0.001, F = 2.5, df = 310), which explained 29.4% of the variance. For the four main rice subpopulations there was a significant difference in grain:shoot As (P < 0.001, F = 22.79, df = 3). Cultivars from the temperate

japonica subpopulation had a lower transfer factor compared with the other three subpopulations (Fig. S2b).

Genetic variation in grain yield and flowering time

Data on yield were analysed only for the Faridpur and Qiyang sites. Genotypes were significantly different for grain yield (from six plants) at both sites (Faridpur, $F = 7.76$, df = 311; Qiyang, $F = 8.37$, df = 300; $P < 0.001$), with yield for the cultivars at the Faridpur field site ranging from 18.5 to 205.8 g, with an average yield of 79.1 g, while at the Qiyang field site grain yield ranged from 5.4 to 352.4 g, with an average of 84.1 g. There was a significant ($P < 0.001$) difference in grain yield (Faridpur, $F = 70.6$, df = 3; Qiyang, $F = 48.85$, df = 3) between the four main rice subpopulations. At the Faridpur site cultivars from the *indica* subpopulation had the highest grain yield (Fig. S3a), while at the Qiyang site *aus* cultivars had the highest yield (Fig. S3b). At both sites there was a significant ($P < 0.001$) positive correlation between grain yield and grain As (Faridpur, $r = 0.214$, df = 309; Qiyang, $r = 0.404$, df = 299). There was also a significant positive correlation ($P < 0.001$, $r = 0.461$, df = 292) between the grain yields of the cultivars at the Faridpur and Qiyang field sites.

At the Faridpur field site, the time from transplanting to flowering ranged from 78 to 149 d, with an average of 111 d. At Qiyang it ranged from 57 to 114 d, with an average of 96 d. At the Arkansas site, in 2006 it ranged from 55 to 122 d, with an average of 89 d, and in 2007 it ranged from 52 to 127 d, with an average of 87 d. At the Texas site, it averaged 92 d and ranged from 61 to129 for the flooded field, and averaged 97 d and ranged from 52 to 129 for the nonflooded field site. There was a significant ($P < 0.001$) difference in days to flowering between the rice subpopulations at all sites (Faridpur, $F = 18.6$, df = 3; Qiyang, $F = 36.2$, df = 3; Arkansas 2006, $F = 24.8$, df = 3; Arkansas 2007, $F = 29.4$, df = 3; Texas flooded, $F = 21.3$, df = 3; Texas nonflooded, $F = 28.1$, df = 3), where the temperate *japonica*s flowered, on average, earlier than the other subpopulations (Fig. S4a–f). At all of the flooded sites, grain As was significantly ($P < 0.001$) different between flowering time groups (Faridpur, $F = 22.2$, df = 5; Qiyang, $F = 4.23$, df = 5; Arkansas 2006, $F = 8.7$, df = 6; Arkansas 2007, $F = 30.1$, df = 6; Texas (flooded), $F = 9.37$, df = 6), with the earliest cultivars having lower grain As at Faridpur (Fig. 3a) and at the Arkansas site in 2007 (Fig. 3d). At the Qiyang, Arkansas 2006 and flooded Texas field sites, the earliest and latest cultivars had lower grain As (Fig. 3b,c and e, respectively), and at the nonflooded field site there was no significant effect of flowering time group on grain As (Fig. 3f). These data suggest a potential relationship between grain As concentration and flowering time that appears to differ between locations, and warrants consideration and further investigation in future studies.

On further inspection of the flowering time data in Arkansas, a highly noteworthy observation was made. In 2007, 41 cultivars, almost all of which were from the *aus* subpopulation, flowered much later than in 2006 (for the other cultivars there was a very strong correlation between 2006 and 2007 in flowering; $r = 0.936$, df = 276) (Fig. S6). In these later flowering cultivars alone grain As was 129% higher compared with the other cultivars in 2007 (in 2006 these cultivars had 31% higher grain As). In other words, a small group of cultivars were identified that flowered much later in 2007 and had much higher As in that year. This explains why in Fig. 3(d) there is such a large mean grain As in the two latest flowering groups. Other cultivars which flowered late were not high in As (hence large variation is apparent in these flowering time groups in Fig. 3d). This suggests that whatever environmental effect delayed flowering in these cultivars may also have caused their dramatically increased grain As. Also of note is that the cultivars that flowered before 100 d at the Arkansas field site in 2007 had lower grain As than the corresponding flowering time groups in 2006.

By contrast, there was no difference in grain yield between the flowering intervals at Faridpur (Fig. S5a), but at Qiyang yield varied with flowering time ($P < 0.001$, $F = 7.36$, df = 5) with the cultivars flowering between 91 and 100 d having the highest yield (Fig. S5b), as well as high grain As (Fig. 3).

Speciation of grain As

At the Faridpur field site, the As_i concentration in the grain for the cultivars ranged from 84 to 414 μg kg^{-1}, with an average of 196 μg kg^{-1}, which accounted for 65.5–93.2% of the total As. There was genotypic variation in As_i concentration and percentage As_i ($P < 0.001$, $F = 2.65$, df = 39 and $P = 0.003$, $F = 1.99$, df = 39, respectively). The DMA concentration in the grain for the cultivars ranged from 16 to 85 μg kg^{-1}, with an average of 36 μg kg^{-1}, which accounted for 6.8–34.5% of the total As. There was significant genotypic variation in DMA concentration ($P < 0.001$, $F = 2.50$, df = 39). MMA was detected in only five of the samples (and all the rice CRMs). For percentage total organic As (combined DMA and MMA), there was a significant genotypic difference ($P = 0.003$, $F = 1.98$, df = 39). Within the subset of cultivars tested for As speciation, variation in neither total As nor the percentage of inorganic or organic As was associated with genetic subgroup.

At the Qiyang field site, there was genotypic variation in As_i concentration in the grain ($P < 0.001$, $F = 6.53$, df = 37), which ranged from 106 to 572 μg kg^{-1}, with an average of 280 μg kg^{-1}. There was no genotypic variation in either grain DMA or MMA concentration, which had averages of 234 and 8 μg kg^{-1}, respectively. There was genotypic variation in both the percentage of As_i and the percentage of organic As ($P < 0.001$, $F = 3.71$, df = 37, and $P < 0.001$, $F = 3.91$, df = 37, respectively). The percentage of As_i ranged from 31.0 to 71.9%, with an average of 51.3%, while the percentage of organic As ranged from 26.7 to 68.0%, with an average of 46.8%. As for the Faridpur site, the variance seen for As_i was not associated with subgroup membership. In the Qiyang samples analysed for As speciation, 88% contained a cationic As species, which, in selected samples, was identified as tetramethylarsonium (identification of the As species can be found in Hansen *et al.*, 2010). There was significant variation ($P < 0.001$, $F = 5.4$, df = 37) in the percentage of tetramethylarsonium, ranging from 0.1 to 6.6% of total As.

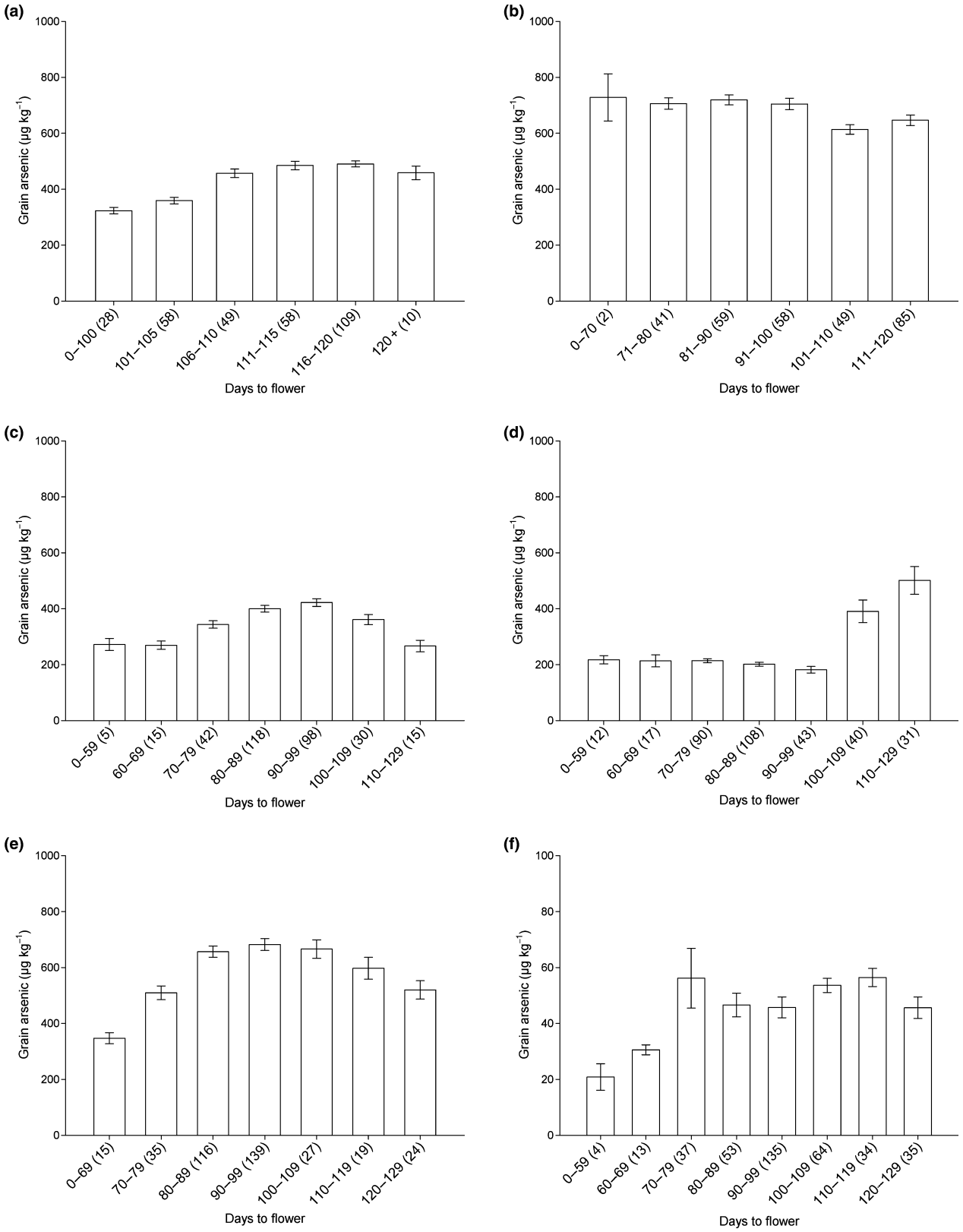

Fig. 3 Relationships between days to flowering and concentration of arsenic (As) in the brown rice grain. Grain As concentrations of the different cultivars are shown based on flowering time groupings at the Faridpur (a), Qiyang (b), Arkansas 2006 (c), Arkansas 2007 (d), Texas flooded (e) and Texas nonflooded (f) field sites. Numbers in brackets after the days to flower reflect the number of cultivars in each of the flowering groups.

When the results from the field sites in Bangladesh and China were compared, no correlation was found between sites for grain As_i (concentration or percentage) or organic As (concentration or percentage) in the 38 genotypes speciated at both field sites.

At the Faridpur field site, there were significant correlations of both As_i ($P < 0.001$, $r = 0.986$, df = 38) and organic As ($P = 0.002$, $r = 0.476$, df = 38) with total As (Fig. 4a). There were also significant correlations for both the percentage of As_i ($P = 0.018$, $r = 0.372$, df = 38) and the percentage of organic As ($P = 0.017$, $r = -0.376$, df = 38) with total As (Fig. 4c). At the Qiyang field site, there was a significant correlation between As_i and total As ($P < 0.001$, $r = 0.916$, df = 36; Fig. 4b), and significant correlations for both the percentage of As_i ($P < 0.001$, $r = 0.625$, df = 36) and the percentage of organic As ($P < 0.001$, $r = -0.621$, df = 36) with total As (Fig. 4d). There was no correlation between organic As (either total or percentage) and total grain As.

Discussion

The genetic variations in grain As at the different field sites were large, and are comparable with those found in a number of other studies (Cheng *et al.*, 2006; Norton *et al.*, 2009a,b; Pillai *et al.*, 2010; Tuli *et al.*, 2010), while the larger range observed in the

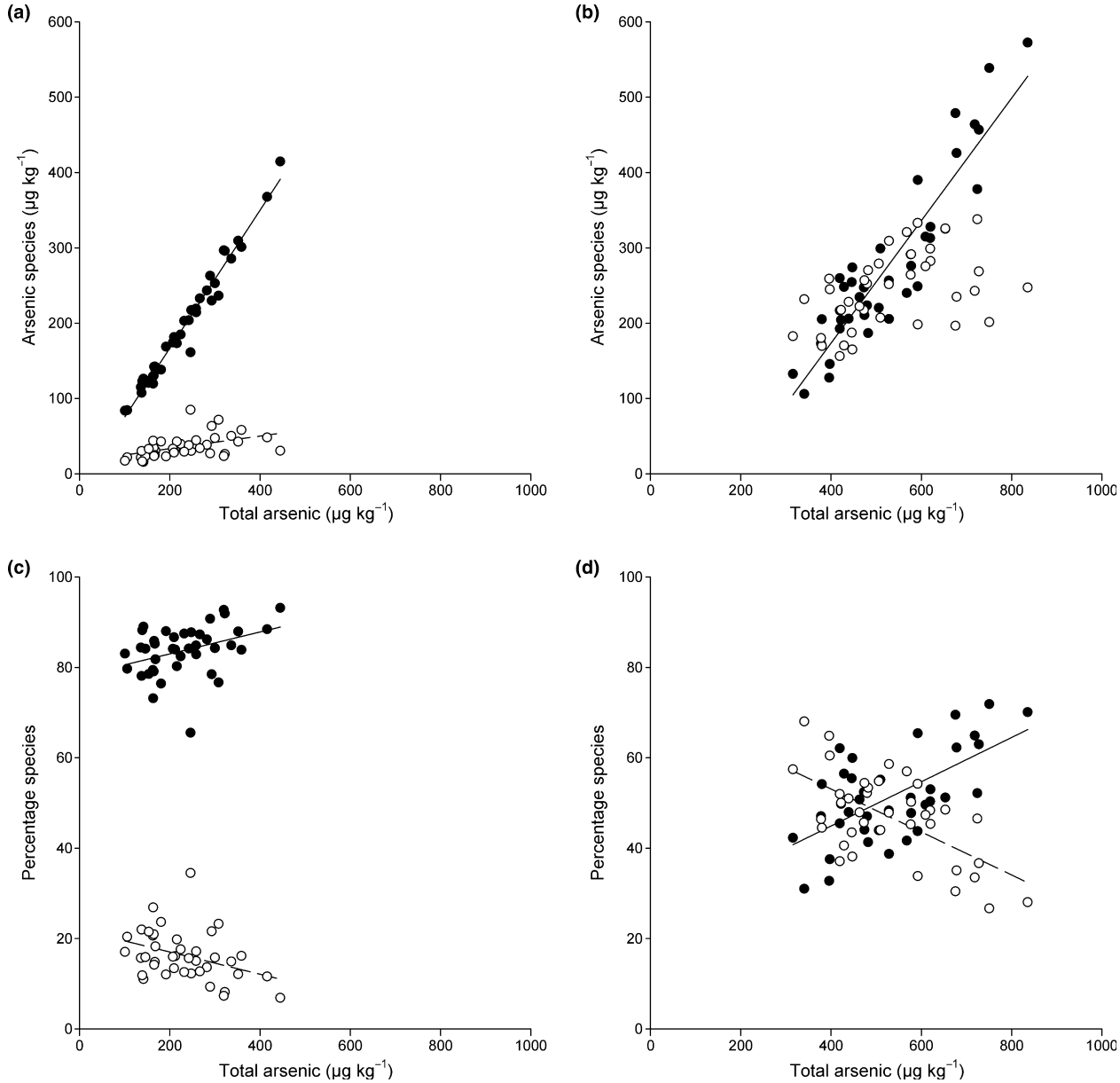

Fig. 4 Correlations between total grain arsenic (As) and grain concentrations of As species. (a, b) Correlations between total grain As and inorganic As (closed circles, solid line) and organic As (open circles, dashed line) for the Faridpur field site (a) and Qiyang field site (b). (c, d) Correlations between total grain As and the percentage of inorganic As (closed circles, solid line) and the percentage of organic As (open circles, dashed line) for the Faridpur field site (c) and Qiyang field site (d).

Arkansas field site in 2007 was primarily attributable to the very low As concentrations detected in some accessions, contrasted with the very high As observed in a subset of later flowering genotypes, as already mentioned. The range at the Texas nonflooded site was 14-fold, but the average and maximum grain As contents were the lowest of all sites. As well as genetic variation in grain As, it has been established that there is a significant genetic component to As variation in roots and shoots of rice plants (Zhang & Duan, 2008; Norton *et al.*, 2010a), and quantitative trait loci for root, shoot, and grain As concentrations have been reported (Zhang *et al.*, 2008; Norton *et al.*, 2010b).

The genetic variation in grain As accounted for 63.4 and 40.1% of the observed variance at the Faridpur and Qiyang sites, respectively, 41.2 and 63.6% of that at the Arkansas field sites in 2006 and 2007, respectively, and 56.2 and 42.1% of that under flooded and nonflooded conditions in the Texas field trials, respectively (Table 1). This suggests that the genetic variation in grain As is a major factor within sites. As in previous studies (Norton *et al.*, 2009b; Pillai *et al.*, 2010; Ahmed *et al.*, 2010), cultivar, environment (either location or year) and cultivar-by-environment interaction all had a significant effect on grain As accumulation. However, the strong genetic variation within field sites, along with the correlations between years (and weaker correlations between sites), show that there are opportunities to select for low grain As in breeding cultivars for particular field sites/environments. For the three flooded field trials in the USA (Arkansas 2006, Arkansas 2007 and Texas), there were positive correlations between sites in total grain As (Table 3), with all sites having a similar pattern in average grain As in the rice subpopulations (Fig. 2c–e). These correlations between years and sites suggest stability for the genetic variation within this relatively small geographic region.

The contrast in grain As concentrations between flooded and nonflooded conditions was very dramatic, with the average As concentration being 14 times greater in plants grown under flooded compared with nonflooded conditions (Table 1). Xu *et al.* (2008) found similar differences in grain As in pot-grown plants, comparing aerobic and anaerobic conditions. Under anaerobic conditions, As was mobilized into the soil pore water mainly as arsenite, whereas pore water As, predominantly arsenate, remained much lower under aerobic conditions (Xu *et al.*, 2008). Interestingly, the correlation in grain As between plants of a cultivar grown in flooded and nonflooded conditions only explained 17.2% of the variation, and the observation of a cultivar-by-treatment interaction suggests that different genetic regulation is involved in the accumulation of As within the grain under flooded and nonflooded conditions. The combination of markedly different As speciation and availability in soil pore water between flooded and nonflooded conditions (Xu *et al.*, 2008) and genetic variation in the activities of the different root uptake mechanisms for the various As species, with arsenate entering through the P-uptake transporters and arsenite being taken into and transported through the plants through silicon (Si)-uptake transporters (as discussed in Zhao *et al.*, 2009), provides a possible explanation for the cultivar-by-treatment interaction.

The identification of significant differences in grain As concentration between the flowering time bins or groupings suggests a possible relationship between flowering time and grain As concentration, but it may not be consistent over locations. At the Faridpur field site, mid- to late-flowering cultivars had significantly higher grain As compared with early-flowering cultivars (Fig. 3a). At the Qiyang field site, the relationship between grain As and flowering was much weaker and in the opposite direction. Interestingly, at the Texas site there was no effect of flowering time on grain As in the nonflooded field, while the flooded field site had lower grain As for the cultivars at the extremes of flowering; that is, both early- and late-flowering cultivars had less grain As compared with the cultivars with average flowering times.

This observation was also true at the Arkansas field site in 2006. However, in 2007 at the Arkansas site, there was a very pronounced effect of flowering time on grain As concentration (Fig. 3d), with cultivars displaying delayed flowering compared with 2006 having a much higher grain As compared with the cultivars that flowered at approximately the same time in both years. Even though flowering time seems to be related to variation in grain As, the relationship was not consistent across field sites (and years). Assuming that most initial plant translocation following uptake is via the xylem (Zhao *et al.*, 2009), then conditions that decrease translocation, such as suboptimal air or soil water temperatures or lengthy overcast periods, could decrease As uptake by late-flowering cultivars depending on the location and year. More research relating flowering time to grain As seems merited.

One important observation made here is that the temperate *japonica*s at the Faridpur site accumulated less As in their grains compared with the other three subpopulations (Fig. 2a), despite accumulating the highest concentrations of As in the shoots (Fig. S2a), and thus had the lowest transfer factor for all the subpopulations (Fig. S2b). This may suggest that generally the temperate *japonica*s differ either in the extent of As competition between cellular compartmentation in vegetative tissue, and availability for translocation of As to other parts of the plant, or in the transporters of As from the shoots to the grain during grain filling. In the plant, the arsenite can be complexed with glutathione or phytochelatins, which predisposes it for sequestration in the vacuole (Liu *et al.*, 2010). This potential mechanism is compatible with the findings of Zheng *et al.* (2011) showing a restriction in As movement from lower to upper plant parts. If competition exists between sequestration within a tissue/part and availability for partitioning to other plant parts, then the expected outcome of such competition would be a gradient in As concentration along the path of net As movement. A similar phenomenon has been observed in internodal compartmentation of sucrose in culms of diverse sorghum (*Sorghum bicolor*) types (Tarpley *et al.*, 1994).

The very highly significant correlations seen between total grain As and grain As_i at the two Asian sites (Fig. 4a,b) suggest that breeding for reduced As_i could be accomplished using the less expensive As analysis rather than requiring the more complex As_i analysis. The relationships seen between total As and As_i differed for the two Asian sites, but can be used within those sites to

predict the grain As_i from the measured total As, as a means to explore dietary risk associated with the genotypes grown at these two Asian locations. It must be noted, however, that this risk assessment is presented for brown rice, not polished rice. Sun *et al.* (2008) have shown that on average just 40% of the As contained in brown rice was retained in milled rice, though the percentages ranged from 17 to 83% for the six *indica* cultivars investigated.

The maximum allowed for As_i in rice grain in China is 150 μg kg^{-1} (Zhu *et al.*, 2008a,b). Using the As to As_i model (Fig. 4b), 150 μg kg^{-1} As_i equates to 380 μg kg^{-1} As. Out of the 295 cultivars at the field site in China, all but two would be predicted to have > 150 μg kg^{-1} of As_i, based on the modelled As_i content. If milling were to remove 40% of the As_i, however, as roughly predicted by Sun *et al.* (2008), the threshold for total brown grain As becomes 633 μg kg^{-1}, which means that *c.* 50% of the cultivars were above the threshold. There is no specific As_i limit for rice grain in Bangladesh. Previously the World Health Organization (WHO) provisional tolerable weekly intake (PTWI) of 2.0 μg As_i kg^{-1} body weight per day from all sources (WHO, 1993) has been used to calculate the maximum intake of As_i from rice. Based on a 50-kg person consuming 450 g of rice per day (typical body mass and rice consumption rate for this region (Rahman *et al.*, 2009)), a concentration of 222 μg kg^{-1} As_i would be the maximum allowable threshold (Norton *et al.*, 2009b). Using the presently calculated model for rice grown at Faridpur, this equates to a total As concentration of *c.* 250 μg kg^{-1} As, which would have led to 298 of the 312 cultivars at the Bangladesh site exceeding the WHO guidance. However, the WHO has at present withdrawn the PWTI for As (WHO 2010), as it was observed that the As_i lower limit on the bench-mark lower dose for a 0.5% (BMDL0.5) increase in the incidence of lung cancer determined from epidemiological studies was 3.0 μg kg^{-1} d^{-1} (2–7 μg kg^{-1} d^{-1}) (WHO 2010). The previous PTWI of 15 μg kg^{-1} (equivalent to 2.1 μg kg^{-1} d^{-1}) is in the region of the BMDL0.5 and is therefore no longer appropriate (WHO 2010). This implies that probably > 298 of the 312 cultivars have As_i higher than acceptable risk levels if these quantities were consumed as brown rice. No As speciation was performed on the rice grain from the US field sites, so no risk assessment can be performed. However, as the average daily intake of rice in the USA is 24 g compared with 450 g d^{-1} in Bangladesh (Meharg *et al.*, 2009; Rahman *et al.*, 2009), the risk is much less for a person consuming the average US intake of rice compared with a person consuming the average Bangladeshi intake of rice. Furthermore, it has been shown that rice produced in the USA is similar to the observations made in the present study for rice grown in Qiyang, in that As_i makes up only *c.* 40% of the total grain As (Meharg *et al.*, 2009).

The strong correlations between total As and As_i at the two different Asian sites (where the percentage composition of the As_i species varies greatly; 65.5–93.2% at the Faridpur field site, and 31.0–71.9% at the Qiyang field site) are significant in terms of predicting the amount of As_i within rice grains based on total grain As. The data show similarly sloped linear relationships between As_i and total As in Qiyang and Faridpur (Fig. 4a,b), but at Qiyang the intercept is shifted by *c.* 250 μg kg^{-1} total As, which corresponds very well with the amount of organic As in grains at that site. This suggests that the higher organic As at Qiyang substantially elevates the total grain As at that site.

Recently it has been proposed that there are different controls for the unloading of As_i and DMA into rice grains, with DMA accumulating in the caryopsis before flowering and As_i being mainly transported into the caryopsis during grain filling (Zheng *et al.*, 2011). It has been established that DMA is poorly taken up by roots (Raab *et al.*, 2007a; Abbas & Meharg, 2008) yet can reach high concentrations in rice grains (Williams *et al.*, 2005; Norton *et al.*, 2009a,b). It has been demonstrated in As-fed excised panicles that the rate of shoot to grain translocation of As is drastically different for the two species, with DMA being translocated at an order of magnitude greater rate than As_i (Carey *et al.*, 2010). The As_i was primarily translocated via the phloem, whereas DMA was translocated via both phloem and xylem (Carey *et al.*, 2010). Efficient above-ground translocation of DMA may be attributable to its poor –SH (sulfhydryl) coordination, in contrast to inorganic arsenite (Raab *et al.*, 2007b). Finally, it is still unclear if the methylated organic species are directly taken up from the environment or if there is *in planta* biomethylation occurring. However, recent evidence does indicate that methylation of As can be performed by rhizosphere-associated bacteria (Arao *et al.*, 2011). With large variations in the percentage of DMA for the same cultivars across two different field sites, it seems likely that the variation is attributable primarily to environmental factors (Fig 4a,b).

To reduce the concentration of As in rice grains, breeding cultivars that produce grains with lower As is one of the simplest and most cost-effective approaches (Carbonell-Barrachina *et al.*, 2009; Zhao *et al.*, 2010a). This study shows that sufficient genetic variation exists to support such breeding goals. The currently cultivated US cultivars have lower grain As concentrations than the previously cultivated cultivars when grown under flooded conditions (Table 2), even though US rice breeders did not measure or consider grain As during the selection or breeding of these cultivars. These modern US cultivars showed decreased grain As across both US field sites and across both years at the Arkansas field site, which is important as environmental factors have previously been shown to have a large effect on grain As (Norton *et al.*, 2009b; Pillai *et al.*, 2010; Ahmed *et al.*, 2010). These current and previous US cultivars were not statistically significantly different in grain As at the two field sites in Asia, suggesting that this difference between these two groups of cultivars is related to adaptation to geographical regions. At present it is not clear why the current US cultivars have lower grain As compared with the previously cultivated US cultivars when grown on low As soils. Future work is needed to understand this observation.

The cultivars used in this study are part of a rice genome-wide association study based on 44 000 single nucleotide polymorphism markers (Tung *et al.*, 2010; Ali *et al.*, 2011); thus, these data will be further analysed for marker-trait associations related to grain As (Kang *et al.*, 2008). The data on the cultivar variation in grain As indicate that this is a good trait to use for genetic

mapping, because of the large percentage of the variation being partitioned to genetic background, and the high degree of variation within and between subpopulations. Knowing the influence of subpopulation structure on the genetic variation emphasizes the need to account for structure within any association study (Pritchard *et al.*, 2000; Kang *et al.*, 2008), which should also allow for a more precise association mapping. However, this study suggests that the genetic mechanisms regulating the concentration of As in the grain may not be simple. Previously, epistatic interactions rather than main effect loci have been shown to regulate grain As (Norton *et al.*, 2010b).

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Ratio of grain arsenic in different rice subpopulations grown under flooded and nonflooded conditions.

Fig. S2 Subpopulation variation in shoot arsenic concentration and arsenic transfer factor.

Fig. S3 Subpopulation variation in grain yield.

Fig. S4 Subpopulation variation in flowering time.

Fig. S5 Relationships between days to flowering and grain yield.

Fig. S6 Correlation between flowering times for the cultivars grown at the Arkansas field site in 2006 and 2007

Table S1 List of the six modern US rice cultivars and the 22 historical rice lines used to evaluate how breeding for modern cultivation has indirectly affected total arsenic (As) concentrations in brown grain

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