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Genetic Analysis of Atypical U.S. Red Rice Phenotypes: Indications of Prior Gene Flow in Rice Fields?

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Weedy red rice is a troublesome weed problem in rice fields of the southern United States. Typically, red rice plants are much taller than rice cultivars, and most biotypes are either awnless with straw-colored hulls (strawhull) or have long awns with black-colored hulls (blackhull). Outcrossing between rice and red rice occurs at low rates, resulting in a broad array of plant types. Simple sequence repeat (SSR) markers were used to evaluate the genetic backgrounds of atypical red rice types obtained from rice farms in Arkansas, Louisiana, Missouri, and Mississippi, in comparison to standard red rice types and rice cultivars. Principal coordinates analysis (PCoA) and population structure analysis of atypical red rice accessions suggested that short-stature awnless (LhtsA−) and awned (LhtsA+) types, each representing a total of about 5% of a 460-accession collection, usually were closely genetically related to their normal-sized counterparts, and not with cultivated rice. A short-awned, intermediate height type, 'Sawn', representing about 4% of the accessions was genetically distinct from all of the other types. Key alleles in Sawn types appeared to be shared by both standard awnless (StdRRA−) and awned (StdRRA+) red rice, suggesting that Sawn types could have arisen from gene flow between awned and awnless red rice types.
Nomenclature: Weedy red rice, *Oryza sativa* L.; Rice, *Oryza sativa* L.
Key words: Weedy red rice, *Oryza sativa* L., strawhull red rice, blackhull red rice, rice (*Oryza sativa* L.), rice-red rice hybrid, tropical japonica rice, simple sequence repeat (SSR), genetic diversity.

Weedy red rice is among the most troublesome and costly weed species in southern U.S. rice fields, and has been estimated to cost farmers \$274 ha^{-1} in losses annually (Burgos et al. 2008). Typically, the red rice plant biotypes in the southern United States are much taller than rice cultivars, and most are either awnless with straw-colored hulls (strawhull) or have prominent awns and black-colored hulls (blackhull) (Delouche et al. 2007; Shivrain et al. 2010b). Important progress into the genetic identities and evolution of U.S. red rice types has been achieved in recent years. The most prevalent types were shown to be descended from *Oryza sativa* ancestors of Asian origin (Londo and Schaal 2007). Strawhull awnless types were estimated to have diverged from cultivated *Oryza sativa* L. subsp. *indica* S. Kato (indica) rice concurrent with its establishment in the United States, up to 400 yr ago or within the last 1,000 yr, whereas blackhull awned types probably diverged from cultivated indica var. Aus rice near the time of rice domestication at least several thousand years ago (Reagon et al. 2010).

Key traits of red rice can contribute to its negative impact as a weed. Among these are weed–crop outcrossing potential, seed shattering, and red seed pericarp color. Rice and red rice are normally self-pollinated, but are conspecific and capable of intercrossing to produce highly diverse progeny (Gealy et al. 2003, 2006). The extent to which progeny of such crosses have introgressed permanently into red rice in U.S. rice fields remains largely undetermined. Seed shattering, which facilitates natural reseeding, is greater in U.S. red rice than in cultivated rice (Thurber et al. 2010), and the responsible gene has been linked to the domestication of rice (Li et al. 2006). The red pericarp color can reduce significantly the market values of rice grain contaminated with red rice seeds (Delouche et al. 2007).

In the United States, the predominant red rice types are ~30 to 60 cm taller than most modern rice cultivars (D. Gealy, unpublished data). These height differences help create a competitive advantage for red rice and might have increased in modern times with the introduction of shorter rice cultivars. It is notable, however, that some red rice plant types in commercial fields have been identified by farmers as possible hybrid (or hybrid progeny) red rice plants with heights as short as ~85 to 125 cm.

Preliminary analysis of allele sizes and phenotypic traits (Estorninos et al. 2006; Gealy et al. 2005, 2007) indicated that certain red rice types might be progeny of recent crosses between long grain rice and red rice, and that some of these produce atypical phenotypes with unusually short plant height or awn length (Gealy et al. 2006; D. Gealy, unpublished data). Potentially, such crosses could affect the management and production of rice, particularly if short red rice plant types, which are difficult to distinguish from rice plants in the field, were to result in stealthy proliferation of herbicide-resistant weed types derived from gene flow with herbicide-resistant rice cultivars. Recent reports have indicated that early-generation hybrids or hybrid progeny from imidazolinone-resistant rice–red rice crosses have been found in farm fields and research fields (Gealy et al. 2005; Shivrain et al. 2007, 2008, 2009a; Zhang et al. 2006, 2008). However, genetic evidence regarding the potential presence and level of longer-term introgression between rice and red rice predating the herbicide-resistant rice production era has been limited, speculative, or unsettled (Delouche et al. 2007; Gealy et al. 2003; Reagon et al. 2010; Shivrain et al. 2010a). Thus, we screened the USDA–ARS red rice collection, which predates the herbicide-resistant rice production period, for plants of unusually short height or awn length to ascertain whether these atypical accessions were genetically linked to the rice cultivars or predominant red rice types of the region, or were genetically distinguishable as independent populations.

The specific objectives were: (1) to determine the genetic backgrounds of three atypical red rice types in relation to standard red rice types and rice cultivars; and (2) to determine the degree of shared genetic background among these atypical and standard red rice types, and rice.

Materials and Methods

Acquisition of Plant Samples. Weedy red rice accessions analyzed in this study were selected from a USDA-ARS

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Table 1. Phenotypic descriptors for atypical and typical red rice accession groups, and rice cultivars analyzed for this study.

Group name	Group description	Number of entries[a]	Plant height[b]		Typical awn length	Emergence to heading[c]	
			Range	Average		Range	Average
			cm			d	
LhtsA−	Short plants without awns	8	~101–128	111	0	70–97	90
LhtsA+	Short plants with awns,	7	~112–127	118	≫ 1	102–105	103
Sawn	Plants with uncharacteristically short awns and an intermediate height	17	115–146	130	< 1	80–89	85
StdRRA−	Typical awnless strawhull red rice types (included as standards)	13	132–154	144	0	80–92	87
StdRRA+	Typical awned red rice types (included as standards)	15	130–167	155	≫ 1	89–105	95
StdHyb	Standard F_1 hybrids of imidazolinone-resistant rice × strawhull awnless red rice	2	NA	NA	0	NA	NA
StdRice	Rice cultivars	6 (57)	104–125	116	0	90–96	92

[a] Number of entries for StdRice from present analysis = 6 (all tropical japonica) (Table 2); total number with inclusion of core collection = 57 (45 tropical japonica, 2 temperate japonica [plus 5 admixtures], and 4 indicas [plus 1 admixture]) (Table 2 and Supplemental Table 1).

[b] Plant height range and average values for StdRice were obtained from three representative cultivars: Cypress (104 cm), Kaybonnet (119 cm), and Starbonnet (125 cm).

[c] Emergence to heading range and average for StdRice were obtained from three representative cultivars: Cypress (90 d), Kaybonnet (91 d), and Starbonnet (96 d).

collection of approximately 460 red rice accessions (Estorninos et al. 2006), representing the largest area of rice production in the southern United States as described previously (Gealy et al. 2009). Seeds of most accessions were sampled at commercial dryers from ''rough rice'' grain that had been delivered from farm fields in 2000 and 2001. Accessions were then propagated from single seeds and placed into the Dale Bumpers National Rice Research Center (DBNRRC) collection in 2001 and 2002. U.S. red rice accessions were coded using the convention described by Gealy et al. (2009) (i.e., state of acquisition–year placed into collection–permanent identification code). Five additional accessions (Gealy et al. 2009) were obtained from different sources as early as 1994, and included with the other standards.

The 460-accession USDA–ARS collection was grown to maturity in a preliminary field study in 2004 (D. Gealy, unpublished data) to obtain basic phenotypic data and serve as a source of DNA. Healthy green leaves from individual plants were sampled and stored in plastic bags in freezers for future DNA extraction and SSR analysis.

A small fraction of the red rice accessions in the collection exhibited unusual phenotypic traits compared to those of the typical awnless and ''long''-awned red rice accessions of the region. These traits included short height (7% were < 120 cm and 5% were < 115 cm), and very short awns (4%) (data not shown). The unusual phenotypic traits were consistent with progeny from known hybrids and hybrid progeny (Gealy et al. 2006). Thus, subgroups of atypical phenotypes (uncharacteristically short plants with and without awns, and plants with uncharacteristically short awns) consisting of 7 to 17 individuals each were chosen for SSR analysis and genetic comparison to standard red rice types and rice (Table 1) (Gealy et al. 2005). Typical awnless strawhull and awned red rice types were included as standards. Accessions in each group were chosen at random from the collection master list. In order to assure a broad geographic representation of LhtsA−, LhtsA+, and Sawn accessions within the region sampled, we attempted to include at least one individual matching the desired trait from each county in which the traits were identified, and sometimes omitted individuals belonging to one of these three trait groups when multiple accessions with the same trait were present in the same county. The areas sampled were primarily from Arkansas, but also included counties from Missouri, Mississippi, and Louisiana. Collectively, these areas represented approximately 70% of the southern U.S. rice area. Examples of the plant types and panicles from each group are shown in Supplemental Figure 1.

The LhtsA− accessions were mostly from northeast Arkansas counties (Clay, Independence, Lawrence, Jackson, Poinsett, Randolph, and Woodruff; Desha County in southeast Arkansas was an exception) (Table 2; Supplemental Figure 2). The LhtsA+ accessions were mostly located in central or east central Arkansas counties (Arkansas, Faulkner, Jackson, Lincoln, Lee, and Lonoke). Sawn accessions were found throughout eastern Arkansas (especially central and south; Chicot, Desha, Drew, Jefferson, Lincoln, Phillips, Prairie, St Francis, White, and Woodruff counties), and were present in northeast Louisiana (Morehouse), southeast Missouri (Dunklin), and western Mississippi (Coahoma).

The semidwarf rice cultivars, 'Cypress', 'CL121', and 'CL161', and taller cultivars, 'Kaybonnet', 'Starbonnet', and 'Wells', were included as standards and grown in the same field plots with the red rice. The height of Cypress was 104 cm, and Kaybonnet and Starbonnet averaged 122 cm. DNA was obtained from leaves of two F_1 hybrids of imidazolinone-resistant rice x strawhull awnless red rice (StdHyb) that had been developed from manual crossing (Shivrain et al. 2008) and maintained continuously in a greenhouse. In order to supplement the data from the rice standards above, SSR data from 48 additional U.S. rice entries (StdRice) and two indica cultivars ('Guichao2', and 'Teqing') in the USDA–ARS Core collection (Agrama et al. 2010; Yan et al. 2007) and IR 64 from the USDA Mini Core Collection (Agrama et al. 2009) were included in our genetic analyses (Supplemental Table 1; GRIN 2011).

DNA Extraction and Marker Analyses. DNA was extracted from healthy, green leaf tissue obtained from plants in the preliminary 2004 field study (described above) at the reproductive stage following the procedures described in Lu et al. (2005). Thirty-seven polymorphic SSRs varying in polymorphism information content (PIC) values were selected in a process similar to that used previously for genetic diversity analysis in *O. sativa* (Gealy et al. 2009; Thomson et al. 2007)

Table 2. Phenotypic identification of the atypical red rice accessions obtained from Arkansas and adjoining states compared to red rice standards and selected rice standards.

Abbreviated name[a]	Analysis grouping	Hull color[b]	Plant height (cm)[c]	County in Arkansas[d]	Numeric code for corresponding entry on PCoA graph[e]	Principal coordinates for marker-based genetic distances between individual entries on PCoA graph[e]	
						Principal coordinate 1 (x-axis)	Principal coordinate 2 (y-axis)
AR01_1013	LhtsA−	S	104	Jackson	1	0.934	0.911
AR01_1064	LhtsA−	S	114	Independence	2	1.140	1.229
AR01_1092	LhtsA−	S	116	Randolph	3	1.381	1.199
AR01_1151	LhtsA−	S	107	Poinsett	4	1.308	1.268
AR01_1156	LhtsA−	S	107	Woodruff	5	1.262	1.153
AR02_1226	LhtsA−	S	108	Clay	6	1.340	1.190
AR02_1290	LhtsA−	S	101	Randolph	7	1.295	1.375
AR02_1111	LhtsA−	S	128	Desha	8	1.046	0.933
AR01_1167	LhtsA+	B	115	Faulkner	9	0.780	−1.459
AR01_1176	LhtsA+	B	116	Faulkner	10	0.757	−1.341
AR02_1287	LhtsA+	B	112	Lee	11	0.777	−1.461
AR01_1078	LhtsA+	B	114	Jackson	12	0.784	−1.479
AR01_1096 (PI653430)	LhtsA+	B	118	Arkansas	13	0.694	−1.368
AR01_1142	LhtsA+	B	122	Lonoke	14	0.888	−1.393
AR01_1194	LhtsA+	B	127	Faulkner	15	0.726	−1.347
AR02_1099	Sawn	Br	121	Phillips	16	1.527	0.780
LA02_1312	Sawn	S	133	Morehouse, LA	17	1.476	0.522
AR01_1111	Sawn	S	130	Woodruff	18	1.612	−0.166
AR02_1147	Sawn	S	132	Prairie	19	1.541	−0.077
AR01_1183	Sawn	S	130	Chicot	20	1.526	−0.022
AR02_1218	Sawn	S	131	St. Francis	21	1.608	−0.075
AR01_1043	Sawn	S	124	Lincoln	22	1.564	−0.447
AR01_1124	Sawn	S	132	White	23	1.607	−0.393
MO02_1300	Sawn	Br	132	Dunklin, MO	24	1.607	−0.393
AR02_1148	Sawn	S	126	Prairie	25	1.608	−0.350
AR02_1157	Sawn	S	127	Desha	26	1.607	−0.393
AR01_1186	Sawn	S	136	Drew	27	1.607	−0.393
AR02_1271	Sawn	Br	115	Lincoln	28	1.363	−0.228
AR02_1406	Sawn	S	125	Woodruff	29	1.660	−0.336
MS02_1430	Sawn	S	126	Coahoma, MS	30	1.607	−0.393
AR02_1202	Sawn	Br	146	Jefferson	31	1.438	−0.484
AR02_1254	Sawn	S	146	Jefferson	32	1.438	−0.545
AR02_1039	StdRRA−	S	141	St. Francis	33	1.272	1.271
AR02_1046	StdRRA−	S	139	Phillips	34	1.336	1.189
LA02_1105	StdRRA−	S	148	East Carroll, LA	35	1.420	1.162
AR01_1132	StdRRA−	S	146	Yell	36	1.338	1.208
MO01_1187	StdRRA−	S	145	Ripley, MO	37	1.361	1.362
MO02_1263	StdRRA−	S	132	Stoddard, MO	38	1.368	1.160
AR02_1288	StdRRA−	S	140	White	39	1.393	1.331
AR02_1358	StdRRA−	S	154	Craighead	40	1.248	1.101
AR94_StgS (PI653423)	StdRRA−	S	144	Arkansas	41	1.254	1.047
AR01_1131	StdRRA−	S	143	Cross	42	1.272	1.138
LA01_1160 (PI653437)	StdRRA−	S	142	Morehouse, LA	43	1.401	1.259
LA02_1243	StdRRA−	S	142	Morehouse, LA	44	1.401	1.259
AR02_1418	StdRRA−	S	150	Jefferson	45	1.267	1.219
AR01_1154	StdRRA+	B	152	Desha	46	1.438	−0.272
LA02_1431	StdRRA+	Br	151	East Carroll, LA	47	1.238	−0.596
LA95_LA3 (PI653420)	StdRRA+	S	158	LA	48	1.114	−0.569
AR94_StgB (PI653422)	StdRRA+	B	165	Arkansas	49	0.984	−0.756
AR02_1022	StdRRA+	B	141	White	50	0.963	−0.908
MO01_1061	StdRRA+	B	152	Butler, MO	51	0.937	−1.442
AR94_11D (PI653417)	StdRRA+	S	—[f]	Arkansas	52	1.047	−1.419
AR94_8 (PI653425)	StdRRA+	B	—	Prairie	53	1.013	−1.398
AR02_1052	StdRRA+	B	160	Lee	54	0.973	−1.388
AR01_1034	StdRRA+	B	167	St Francis	55	0.995	−1.477
AR01_1042	StdRRA+	S	153	Jefferson	56	0.520	−0.910
AR02_1043	StdRRA+	B	159	Monroe	57	0.939	−1.378
AR01_1060	StdRRA+	B	161	Craighead	58	0.995	−1.187
AR01_1115	StdRRA+	B	160	Lawrence	59	0.961	−1.432
AR02_1349	StdRRA+	B	135	Arkansas	60	0.976	−1.407
CL161×RR	StdHyb	S	—	—	61	0.223	0.529
RR×CL121	StdHyb	S	—	—	62	0.111	0.477
CL121	StdRice	S	—	—	63	−0.862	−0.028
CL161	StdRice	S	—	—	64	−0.823	−0.047
Kaybonnet	StdRice	S	119	—	65	−0.808	0.178
Wells	StdRice	S	—	—	66	−0.799	−0.012
Starbonnet	StdRice	S	125	—	67	−0.857	−0.058

Table 2. Continued.

Abbreviated name[a]	Analysis grouping	Hull color[b]	Plant height (cm)[c]	County in Arkansas[d]	Numeric code for corresponding entry on PCoA graph[e]	Principal coordinates for marker-based genetic distances between individual entries on PCoA graph[e]	
						Principal coordinate 1 (x-axis)	Principal coordinate 2 (y-axis)
Cypress	StdRice	S	104	—	68	−0.796	−0.072
LSD (0.05)			14.9				

[a] Complete names for the Abbreviated names of red rice accessions in table are listed in Supplemental Table 2. Names in parentheses are codes for the red rice accessions that are available from USDA-ARS GRIN at http://www.ars-grin/npgs/. Descriptions for analysis groupings are presented in Table 1.

[b] Hull Color: S, strawhull; B, blackhull; Br, brownhull.

[c] Plant height: distance from ground to tip of tallest panicle. Heights of red rice accessions and rice cultivars in 2004 field nursery are means over three replications. LSD (0.05) applies to 445 entries analyzed; only a subset of these is shown in table. Leaves of all red rice accessions were pubescent except for LA02-1105, which was considered to have intermediate pubescence. Leaves of all rice entries were glabrous.

[d] County of origin is in state of Arkansas unless otherwise noted; state abbreviation is listed if county is unknown.

[e] Cross-references to data points on PCoA graph (Figure 1A).

[f] Indicates data not available or not applicable.

Table 3. Summary information for 37 single sequence repeat (SSR) marker loci and the alleles present among the U.S. red rice accessions and rice cultivars analyzed.[a,b,c]

SSR marker	Major allele frequency	Genotype number	Allele number	Gene diversity	Heterozygosity	Polymorphism information content (PIC)
RM283	0.46	9	7	0.64	0.025	0.58
RM5	0.31	9	8	0.77	0.017	0.74
RM488	0.48	6	4	0.58	0.074	0.50
RM1339	0.24	14	10	0.82	0.043	0.81
RM154	0.34	14	11	0.75	0.026	0.72
RM174	0.58	5	4	0.55	0.016	0.48
RM475	0.55	5	4	0.50	0.026	0.39
RM231	0.47	8	6	0.70	0.018	0.66
OSR13	0.95	3	3	0.10	0.000	0.10
RM232	0.26	10	9	0.82	0.017	0.80
RM55	0.56	8	6	0.60	0.026	0.55
RM551	0.37	16	12	0.74	0.044	0.71
RM317	0.50	8	6	0.64	0.029	0.60
RM124	0.59	5	3	0.56	0.026	0.50
RM507	0.54	3	2	0.49	0.018	0.37
RM413	0.45	10	7	0.66	0.042	0.60
RM146	0.51	4	3	0.54	0.030	0.45
RM133	0.53	3	2	0.49	0.017	0.37
RM190	0.38	11	8	0.76	0.028	0.73
RM253	0.43	11	6	0.72	0.074	0.69
RM454	0.89	3	2	0.19	0.030	0.17
RM162	0.31	10	8	0.79	0.017	0.76
RM234	0.74	9	7	0.44	0.044	0.43
RM118	0.53	3	2	0.49	0.018	0.37
RM408	0.39	7	5	0.66	0.017	0.59
RM44	0.31	11	7	0.77	0.042	0.74
RM210	0.51	12	9	0.66	0.045	0.64
RM316	0.42	6	5	0.69	0.017	0.64
RM215	0.40	9	6	0.71	0.034	0.67
RM271	0.44	6	4	0.65	0.017	0.59
RM484	0.69	4	3	0.46	0.021	0.41
RM536	0.32	11	7	0.77	0.034	0.74
RM206	0.58	7	4	0.56	0.061	0.50
RM224	0.22	13	9	0.83	0.034	0.82
RM512	0.62	4	3	0.52	0.030	0.45
Pi-ta[d]	0.61	3	2	0.47	0.017	0.36
RM277	0.54	4	3	0.50	0.017	0.39
Mean	0.49	7.7	5.6	0.61	0.029	0.56

[a] Chromosome and map locations, and the forward and reverse primer sequences for each marker can be obtained from the Gramene Version 29 database available (summarized at www.gramene.org/markers/microsat/). The map location data consulted for these markers were based on the Cornell 2001SSR genetic map; Gramene (2008).

[b] The following markers were not used in analysis of rice standards from the Core collection: RM234, RM454, RM210, RM174, RM512, RM206, RM488, RM317, RM146, RM253 (Supplemental Table 1).

[c] Additional data not presented in table: Sample size was 119. Number of observations ranged from 109 to 119 for all markers except for RM484 (97) and the 10 markers omitted from the Core, which ranged from 61 to 68. Availability ranged from 0.92 to 1.0 for all markers except for RM484 (0.82) and the 10 markers omitted from the Mini Core collection, which ranged from 0.51 to 0.57. The inbreeding coefficient (F) values ranged from 0.84 to 1.0 and were below 0.94 in only RM253, RM488, RM206, RM210, RM234, and RM454.

[d] The Pi-ta marker is a SNLP marker that requires three primers; two forwards and one reverse (Jia et al. 2004). All other markers required one forward and one reverse primer.

Table 4. Genetic diversity of the weedy red rice and rice groups based on polymorphisms of the SSR markers used.[a]

Analysis group	No. of entries (N)	No. of different alleles (Na)	No. of effective alleles (Ne)	Shannon's information index (I)	Observed heterozygosity (Ho)	Expected heterozygosity (He)	Unbiased expected heterozygosity (UHe)	Fixation index (F)	Percentage of polymorphic loci (%P)
LhtsA−	7.4	1.6	1.3	0.293	0.004	0.184	0.198	0.979	51.4
LhtsA+	6.8	1.1	1.0	0.051	0.012	0.030	0.033	0.484	13.5
Sawn	16.6	1.8	1.2	0.232	0.028	0.132	0.136	0.691	64.9
StdRRA−	12.7	1.6	1.3	0.262	0.006	0.164	0.171	0.966	46.0
StdRRA+	14.2	2.1	1.5	0.455	0.023	0.275	0.286	0.845	75.7
StdHyb[b]	1.9	2.3	2.2	0.780	0.919	0.514	0.730	−0.806	94.6
StdRice	42.6	4.1	2.0	0.787	0.003	0.405	0.416	0.994	94.6
Grand mean over loci and groups.									
Total	14.6	2.1	1.5	0.409	0.142	0.244	0.281	0.517	62.9

[a] Values for each parameter are means over each group.
[b] The StdHyb group consisted of only two members. Thus, the values presented might not be indicative of those expected from a larger group.

(Table 3). These were selected from a subset of Garris et al. (2005) (i.e., approximately evenly spaced on chromosomes, and with a preference for markers that followed a stepwise mutation pattern), or were chosen based on previous results from weedy rice (Gealy et al. 2002, 2009). Polymerase chain reaction (PCR) was performed as described in Gealy et al. (2009). Samples were separated on a microcapillary DNA sequencer (ABI Prism 3730 DNA Analyzer, Applied Biosystems, Foster City, CA 94404) and the sizes of SSR fragments were determined and alleles binned using GeneMapper version 3.7 software (Applied Biosystems).

For the additional StdRice entries (Supplemental Table 1), SSR data were obtained from 27 markers that were common to the analysis of our red rice accessions (Table 3) and published rice analyses (Agrama et al. 2009, 2010).

Marker analyses were conducted using approaches similar to those of Gealy et al. (2009). SSR parameters associated with marker loci and plant groups were determined using PowerMarker software (Liu and Muse 2005). An analysis of molecular variance (AMOVA) was performed using Arlequin ver. 3.5 software (Excoffier and Lischer 2010). The computer program NTSYSpc 2.21 (Numerical Taxonomy System, Setauket, NY) was used to construct an Unweighted Pair Group Method with Algorithmic Mean (UPGMA) phylogenetic tree based on genetic distance (D_R) by Nei and Takezaki (1994). NTSYSpc 2.21 was also used to conduct Principal Coordinates Analysis (PCoA) for the red rice and rice entries and major groups using the marker data.

Population structure was determined via Bayesian clustering in the STRUCTURE software package (Falush et al. 2003; Pritchard et al. 2000). The program was run for an assumed number (K) of different subgroups ranging from 1 to 10, with 20 independent replications per K. For this we used the admixture model with uncorrelated allele frequencies setting a burn-in length of 20,000 iterations followed by 50,000 Markov Chain Monte Carlo iterations. For each value of K, STRUCTURE produces a Q-matrix (QST) that lists the estimated membership coefficients for each accession in each subgroup. An individual was assigned to a group if its inferred ancestry indicated the germplasm was 70% related to that (K) group or higher. Entries with inferred ancestry less than 70% were considered admixtures of two different populations. It was observed that the model choice criterion implemented in STRUCTURE, i.e., LnP(D), which is the estimate for the posterior probability of the data for a given K, frequently did not show a clear trend. To identify the most likely number K of subgroups, we additionally used an ad hoc statistic ΔK to determine the break in the slope of the LnP(D) probability function provided by STRUCTURE as described in Evanno et al. (2005).

Results and Discussion

Genetic Diversity. SSR marker data from published research were included for additional rice cultivar standards (Supplemental Table 1). Ten fewer SSR markers were run on these rice standards than on the red rice accessions and rice standards analyzed in the present study (Table 2). Among the SSR markers, major allele frequency at each locus ranged from 0.22 to 0.95, allele number per locus ranged from 2 to 12, gene diversity ranged from 0.10 to 0.83, and PIC values ranged from 0.10 to 0.82 (Table 3). In comparing the *Oryza* groups, the number of different alleles per locus ranged from 1.1 in LhtsA+ to 2.1 in StdRRA+ among the five major red rice groups (StdHyb omitted), to 4.1 in StdRice (Table 4). Thus, rice exhibited greater genetic variation than the red rice groups in this study. This unexpectedly high variation in rice may have occurred because *Oryza sativa* L. subsp. *japonica* S. Kato (japonica), tropical japonica, and indica rice cultivars, each with distinctly different genetic backgrounds, were analyzed as part of the same "rice" group. Further, the diverse StdRice entries from the Core collection (Supplemental Table 1), which were analyzed using fewer markers than for red rice and the other rice cultivars, might also have added to the genetic variation observed in rice. Similarly, the number of effective alleles ranged from 1.0 in LhtsA+ to 1.5 in StdRRA+ among the major red rice groups, to 2.0 in StdRice. Shannon's information index (I) usually followed a pattern similar to that for effective alleles and gene diversity (not shown). With the exception of LhtsA+, the observed heterozygosity was inversely related to the fixation index (F) among the major red rice groups and rice. The StdRRA− standards had lower heterozygosity values than the StdRRA+ standards. The percentage of polymorphic loci among major red rice groups ranged from 13.5% in LhtsA+ to 75.7% in StdRRA+. Overall, the results showed that the StdRRA+ group was more genetically diverse than the other red rice groups. This is consistent with other findings from red rice collections in the southern United States in which awnless strawhull red rice types typically had less genetic variation than awned red rice (Gealy et al. 2009; Shivrain et al. 2010a). Awnless strawhull red rice comprises 70 to 80% of the red rice

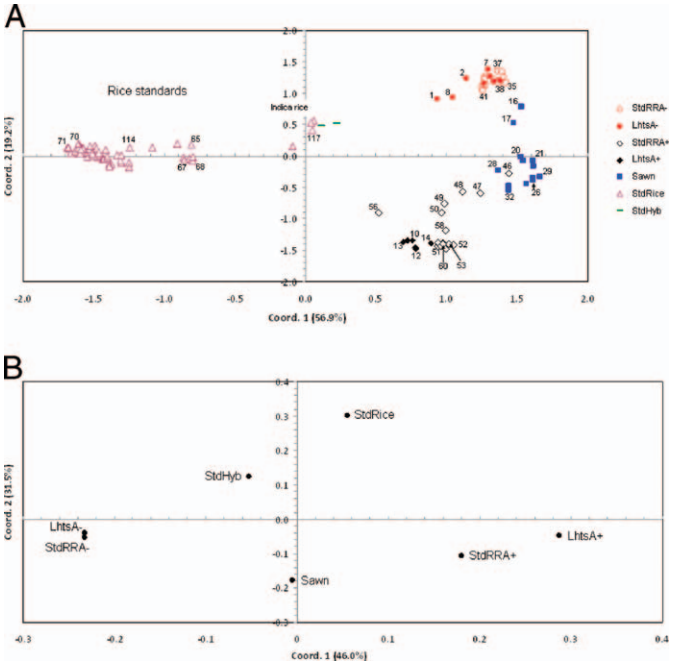

Figure 1. Principal coordinates analysis (PCoA) of red rice and rice based on marker-based genetic distances between individual entries (A) and groups (B).

in Arkansas (D. Gealy, unpublished data; Shivrain et al. 2010b) and is considered the most prevalent type in the southern United States. The genetic diversity in the LhtsA− group was greater than that for the other short-statured group, LhtsA+ (Table 4). For instance, the I values for these two groups were 0.293 and 0.051, respectively. This was in contrast to the results obtained for the StdRRA− and StdRRA+ red rice standards (I = 0.262 and 0.455, respectively), and might have been due in part to genetically divergent accessions (e.g., AR02-1111 [#8]; and possibly AR01-1013 [#1]) as indicated by PCoA (Figure 1A), and to the small sample sizes (Note: numeric codes [e.g., #8, above] are presented for each entry in Table 2 and Supplemental Table 1 to facilitate cross referencing between the entry names in the tables and the data points on the PCoA graph).

Analysis of Molecular Variance. More than 60% of the genetic variation occurred among groups (Table 5), which is consistent with the hypothesis that our initial phenotypic groupings were relatively well-defined and differed from each other genetically. With StdHyb plants omitted due to small sample size, AMOVA assigned the following genetic groups ($P < 0.001$). All LhtsA− accessions, except for AR01-1151, belonged to the StdRRA− group. Most of the StdRRA− accessions were assigned to their phenotypic group, but AR02-1039 (#33), AR01-1132 (#36), MO01-1187 (#37), AR02-1288 (#39), AR94-StgS (#41), and AR02-1418 (#45) were assigned to LhtsA−. Thus, LhtsA− and StdRRA− accessions were genetically very closely related. Sawn accessions belonged to a separate group, except for AR02-1099 (#16; assigned to StdRRA). Most Sawn accessions were genetically intermediate between the two red rice standards. The LhtsA+ and StdRRA+ accessions each belonged to separate, but closely related groups. Most indica rice entries were assigned to the StdRRA− group (detailed data not shown). Similar associations between U.S. red rice and indica rice have been shown previously (Gealy et al. 2009; Lee et al. 2011; Londo and Schaal 2007; Reagon et al. 2010; Vaughan et al. 2001). In a previous study, four of five U.S. *Oryza* collections differed from one another genetically, but blackhull awned and strawhull awned red rice types were similar (Gealy et al. 2009).

Principal Coordinate Analysis. The individual red rice and rice cluster groups identified in the AMOVA were also readily visualized on PCoA graphs (Figures 1A and 1B) which indicated large genetic distances between all rice cultivars and all red rice accessions, except for indica rice and rice × red rice hybrids, which were intermediate between tropical japonica rice and awnless red rice types. This relationship between U.S. red rice and indica rice was previously shown in the AMOVA (Table 5). The two awnless red rice groups clustered closely in the upper right quadrant of the graph, the LhtsA+ and StdRRA+ groups clustered less closely in the lower right, and the Sawn group clustered in a somewhat intermediate position between these awnless and awned groups, suggesting that this group might be derived from outcrossing between these two red rice types.

Based on the PCoA graph coordinates (Figure 1A) and individual allele size comparisons (Supplemental Table 2), other genetic similarities were identified among some red rice accessions and the different red rice or rice groups. The LhtsA− line, AR01-1013 (#1), could have been derived from rice or awned red rice because it shares an unusual 138-bp allele from RM317 with Starbonnet, as well as AR01-1060 (#58) and four other red rice lines. The StdRRA+ line, AR01-1042 (#56), also might be derived from rice or awnless red rice. With most rice entries, AR01-1042 shares the RM551-194-bp allele also found in LA95-LA3 (#48), and a rare RM316-214-bp allele also found in AR02-1111 [# 8] and AR01-1060 (Supplemental Table 2).

Some red rice accessions appeared to be hybrid progeny based on the presence of heterozygous alleles that were consistent with red rice × red rice or red rice × rice parentage.

Table 5. Analysis of molecular variance (AMOVA) for seven groups of red rice and rice based on data from 37 single sequence repeat (SSR) markers.[a]

Source	Degrees of freedom	Sums of squares	Mean square	Estimated variance	%
Among Groups	6	3193	532	36.0	61
Within Groups	112	2530	22.6	22.6	39
Total	118	5724		58.6	100
Statistic	Value	P			
PhiPT[c]	0.614	0.001			

[a] The groups consisted of three atypical red rice groups, two standard red rice groups, a known rice–red rice hybrid group, and commercial white rice cultivar standards. In total, 119 entries were included in the analysis.

[b] Abbreviations: P, probability; PhiPT, proportion of variance among groups relative to total variance.

[c] Probability, P, for PhiPT is based on permutation across the full data set. PhiPT = variance among groups/(variance within groups + variance among groups) = variance among groups/total variance.

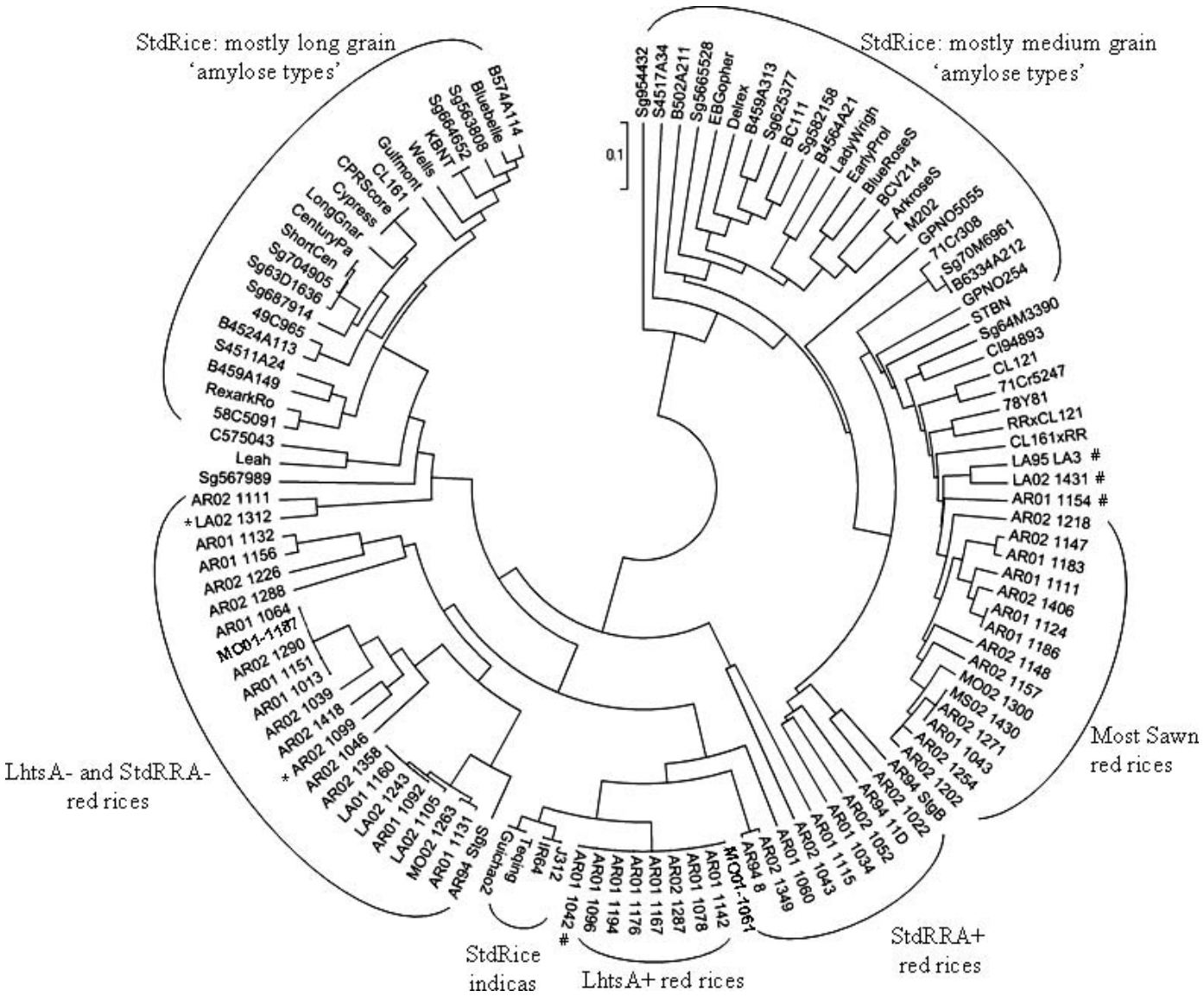

Figure 2. Phylogenetic tree showing genetic distance and clustering with Neighbor-joining between the U.S. red rice groups and rice standards. Symbols placed adjacent to particular entry names indicate that the entries are not aligned with other members of their groups on the phylogenetic tree: asterisk (*) indicates a Sawn type; pound sign (#) indicates a StdRRA+ type. Rice standards (StdRice) are mostly tropical japonica, consisting primarily of ''long grain amylose types'' (e.g., 20–22.3% amylose) and ''medium grain amylose types'' (e.g., 12.5–16.8% amylose); and a small number are indica and temperate japonica types (Supplemental Table 1).

The Sawn line, AR02-1099 (#16) has nine heterozygous alleles and clusters close to StdRRA− (Figure 1A). The Sawn line, LA02-1312 (#17), which clusters close to StdRRA−, and the StdRRA+ lines, AR01-1060 (#58) and AR02-1349 (#60), each have six heterozygous alleles. In AR01-1060, RM190 was heterozygous for alleles (105 and 118 bp) that were present in red rice but not rice, suggesting a red rice × red rice background. However, RM118 was heterozygous for a 156-bp allele nearly always present in rice and absent in red rice, and a 161-bp allele nearly always absent in rice and present in red rice, thus suggesting that AR01-1060 could have been derived from a rice × red rice cross.

Based on shared alleles, the LhtsA− line, AR02-1111 (#8) might be genetically related to Starbonnet and several other rice lines, or awned red rice. Starbonnet shares with it the 116-bp allele from RM5 and the 259-bp allele from RM507 (present in most rice lines). AR02-1111 has a unique 166-bp allele from RM551, and also shares the RM316-214-bp allele with the StdRRA+ line, AR01-1042 (#56). Population structure analysis also indicates that AR02-1111 might have a minor genetic relationship with Starbonnet-like rice, although it appears to have a much greater genetic connection with indica rice (see Figure 3 below).

Clustering of genetic distances between the U.S. weedy red rice groups and rice cultivars as shown in the phylogenetic tree (Figure 2) produced results consistent with those visualized in the PCoA graphs (Figures 1A and 1B), indicating a close genetic relationship between LhtsA− and StdRRA− groups, a close genetic relationship between LhtsA+ and StdRRA+ groups, an intermediate genetic relationship between the Sawn group and each of the other awned and awnless groups, and a distant relationship with most rice cultivars. Indica cultivars were genetically intermediate between tropical japonica rice and awnless red rice.

Some red rice accessions were genetically indistinguishable, even though their source locations were geographically diverse. The accessions AR02-1157 (Desha; #26), AR01-1186 (Drew; #27), and MS02-1430 (Coahoma, MS; #30) had identical

PCoA coordinates, and AR02-1148 (Prairie; #25) had coordinates nearly identical to these (Figure 1A; Table 2). Additional subsets from diverse locations were shown to be nearly genetically identical on the phylogenetic tree (Figure 2). One of these was concentrated in Faulkner County, AR (three accessions), broadly distributed in east and central Arkansas, and found as far north as Ripley County, MO. It consisted of the seven LhtsA+ accessions plus MO01-1061 (#51) and AR01-1042 (#56). Another subset, found only in northern Arkansas and northern Louisiana, included the StdRRA− types, LA01-1160 (#43), LA02-1243 (#44), and AR02-1358 (#40), plus AR01-1092 (#3). A third subset from north eastern Arkansas and southern Missouri included the LhtsA− types, AR01-1064 (#2), AR02-1290 (#7), AR01-1151 (#4), and AR01-1013 (#1), plus MO01-1187 (#37). Clearly, genetically closely-related accessions of red rice reside in geographically diverse areas, and vice versa (Table 2; Figures 1A and 2). In previous studies, generally low levels of correlation were found between geographic and genetic distances among U.S. red rice types (Gealy et al. 2009; Shivrain et al. 2010a). The current results as well as those from previous studies appear to be consistent with a scenario in which relatively few populations of red rice were introduced initially into the region, have remained relatively intact genetically, and have been reintroduced or moved with rice grain into different areas.

Population Structure Analysis. The analysis of population structure revealed that the greatest possible number of ancestral lineages (*K*) of these accessions is five (Figure 3; see also Supplemental Table 3). This is the number of subgroupings that optimizes by ΔK (Evanno et al. 2005). The genetically defined groups from the population structure analysis (Figure 3) generally coincided closely with the clusters visualized in the PCoA graphs (Figures 1A and 1B) as well as with the major genetic distance-derived groups indicated by UPGMA (Figure 2). Our analyses produced clustering similar to that previously observed for U.S. weedy red rice, tropical japonica rice, and indica rice (Eizenga et al. 2009; Gealy et al. 2009; Londo and Schaal 2007; Shivrain et al. 2010a; Thomson et al. 2007).

Population structure analysis (Figure 3) clearly revealed a close relationship in the genetic backgrounds of the LhtsA− and StdRRA− accessions and the LhtsA+ and StdRRA+ accessions. LhtsA− and StdRRA− belonged primarily to group *K*4 and accessions LhtsA+ and StdRRA+ belonged primarily to group *K*5 (Figure 3). Most Sawn accessions shared a majority of their genetic background with both group *K*4 (awnless) and *K*5 (awned) red rice. Fifteen of the 17 Sawn accessions shared a 45 to 65% background with the *K*5 group (Figure 3). The two exceptions, AR02-1099 and AR02-1312, shared more than a 70% background with *K*4. The two Sawn accessions, AR02-1201 and AR02-1254, shared a ~45% background with indica rice and 13 of the 17 shared an ~8% background with *K*2 (typical of tropical japonica "medium grain" rice). Rice cultivars belonged primarily to groups *K*1 (tropical japonica long grain), *K*3 (tropical japonica medium grain or temperate japonica) and *K*2 (indica). As expected, the hand-crossed F_1 hybrids (StdHyb; CL161×RR and RR×CL121) were confirmed to share genetic backgrounds with awnless strawhull red rice and tropical japonica rice (Figure 3).

Based on intermediate positioning on the PCoA graphs (Figures 1A and 1B) and ties to genetic backgrounds of awned and awnless red rice types (Figure 3), our Sawn accessions appear to have been derived from crossing between typical awnless and awned red rice types. Furthermore, several SSR markers produced heterozygous alleles that were consistent with both awnless and awned red rice types, or homozygous alleles that were consistent with either awnless or awned red rice standards (RM271, RM551, RM162, RM44, RM316, RM413, RM55, RM124, RM174, RM215, RM488, RM317, RM146, and RM1339; Supplemental Table 2). Elevated levels of heterozygosity were particularly evident in AR02-1099 (#16) and LA02-1312 (#17), which clustered closely with StdRRA− accessions in the PCoA graph (Figure 1A). Their mixed ancestry is clearly observable from the population structure analysis (Figure 3).

Implications of Awned Brownhull Phenotype. A small number of red rice accessions were classified as "brownhull" phenotypes including the Sawn lines, AR02-1202 (#31), AR02-1271 (#28), MO02-1300 (#24), and AR02-1099 (#16), plus LA02-1431 (#47) (Table 2). Brownhull or other "intermediate" phenotypes sometimes have been associated with hybrid progeny in red rice (Delouche et al. 2007).

Reagon et al. (2010) found that a brownhull (BRH) weedy red rice population including MO02-1300, AR01-1183, and AR01-1111 (coded BRH_1C12, BRH_1D12, and BRH_1C09, respectively, in their study), might have been derived from hybridization between strawhull (SH) and blackhull awned (BHA) weedy rice groups. These plants contained diversity that was consistent with hybridization among U.S. weedy groups, had the same cytotype as strawhull red rice (suggesting a maternal lineage from SH types), and lacked heterozygosity, indicating that they were not early-generation hybrid progeny.

Similar to results in our study, some brownhull biotypes (BR-Ark-27-Y, BR-Cra-1-Y, BR-Des-7-Y, and BR-Dre-2-Y) from another collection had the same blackhull awned (their *K*2 group; ≈ our *K*5) and strawhull awnless (their *K*3 or *K*4 group; ≈ our *K*4) genetic backgrounds in common with our Sawn lines (Shivrain et al. 2010). None of their biotypes appeared to have the trace amounts of medium grain rice backgrounds (their *K*5 group; ≈ our *K*3) present in our Sawn lines, suggesting that some Sawn lines might have been derived from crossing with medium grain rice. In contrast to our findings, a number of their red rice biotypes (e.g., BH-Whi-2-Y, BR-Stf-10-Y, GH-Ark-9-Y, SH-Cla-4-N, SH-Lin-9-Y, SH-Mis-5-N, SH-Poi-10-N) exhibited substantial shared genetic backgrounds with long grain rice cultivars (their *K*1 group; ≈ our *K*1), suggesting the possibility that these types were the result of earlier gene-flow events (Shivrain et al. 2010a).

Have Rice Genes Introgressed into the Atypical Red Rice Phenotypes? The 127-bp allele from RM1339 (located 184 kb upstream of the SD1 locus) has been associated with the sd1 semidwarfness allele (Fjellstrom et al. 2004). Thus, this 127-bp allele was detected in the semidwarf cultivars (e.g., Cypress and CL161), as well as in the major indica rice cultivars (Supplemental Table 2). It also was present in all 11 of the 13 StdRRA− accessions (144 cm average height), and six of the eight LhtsA− accessions (111 cm average height), despite the great height difference (~33 cm) between groups, but was absent from all awned red rice types (Supplemental Table 2). Thus, its presence in U.S. red rice appears more

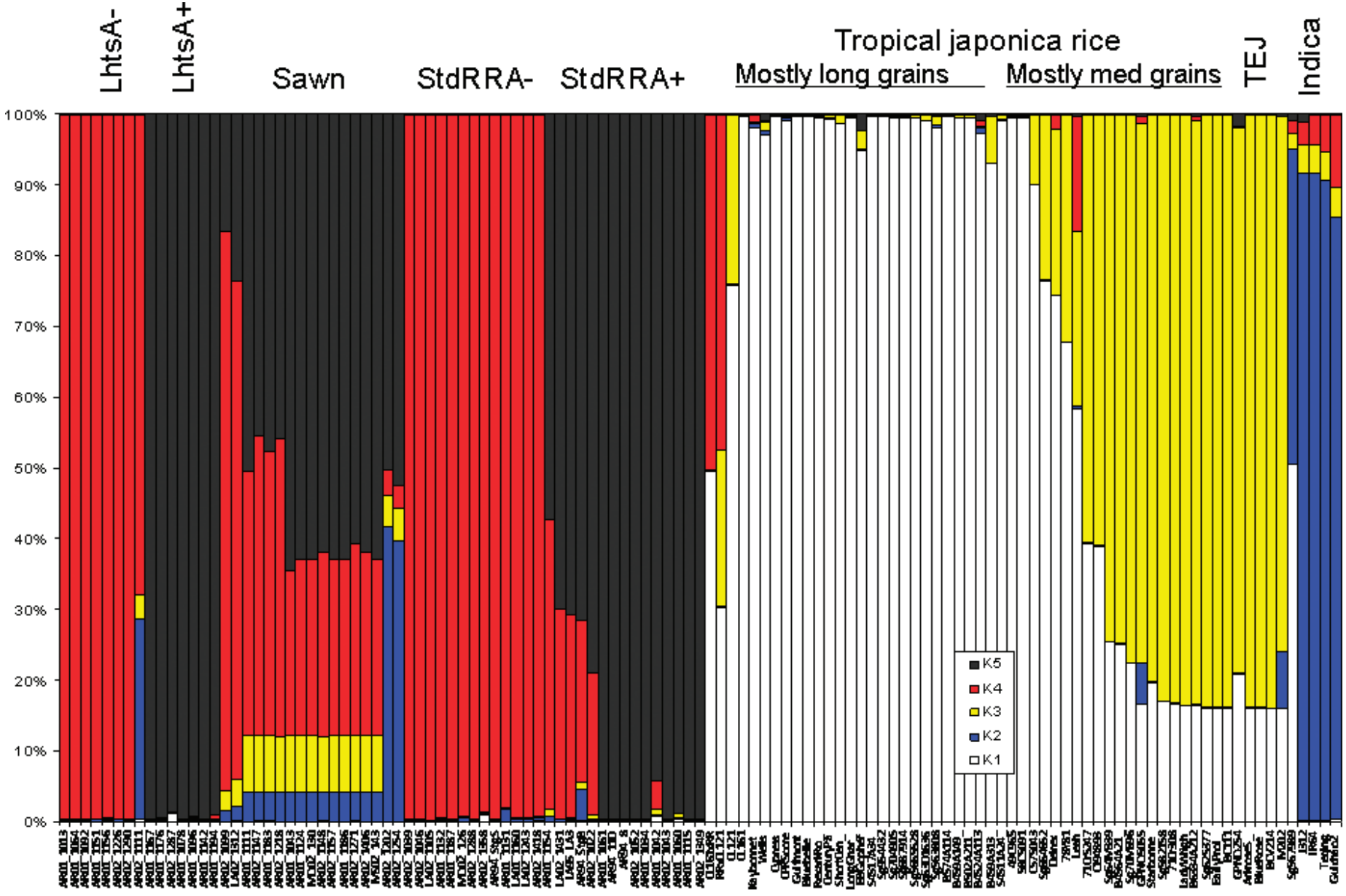

Figure 3. Population structure analysis of U.S. red rice groups and rice standards. TEJ (temperate japonica white rice); Indica (indica white rice). The five potential genetic backgrounds (*K* groups) contributing to each entry are represented by bars of different colors and lengths. Longer bars indicate a greater contribution from a particular genetic background.

likely to be due to a shared genetic background with indica rice than to gene flow from semidwarf rice cultivars in the United States.

Presence of rare RM1339 alleles among the red rice accessions, however, might be evidence of previous gene flow from rice (data not shown; see Supplemental Table 2). The 134-bp allele present in all of the LhtsA+ accessions is consistent with only a select few rice cultivars, 'Blue Rose Supreme', 'Lady Wright', and '71Cr-5247', all of which harbor the allele. Thus, these or similar cultivars might have been the source of the allele in LhtsA+ red rice accessions. Likewise, AR02-1349 (StdRRA+), which is heterozygous at RM1339 (134-bp and 125-bp alleles), could be the progeny from a cross between a 134-bp-containing rice cultivar and awned red rice. AR02-1099 (Sawn), which was heterozygous at RM1339 (127-bp allele; and 125-bp allele, present in most Sawn and StdRRA+ red rice types), might be the hybrid progeny from a cross between awnless × awned red rice (Figure 3), although a cross between red rice with a 125-bp allele and semidwarf rice with a 127-bp allele cannot be ruled out. In a molecular study of the SD1 locus, Reagon et al. (2011) showed that an *SD1* haplotype (H.J-13) similar to that in Drew, and older cultivars such as 'Blue Rose', 'Carolina Gold', 'Edith', 'Palmyra', 'Rexoro', and 'Zenith' had introgressed from tropical japonica cultivars, as the maternal parent, into nine awned U.S. red rice types, including AR01-1096 (PI 653430; LhtsA+) from the present study. Interestingly, however, AR01-1096 carried a 134-bp allele for RM1339 that was found only in our Sawn accessions (Supplemental Table 2). Reagon et al. (2011) indicated that the introgression most likely had occurred recently within the United States and that it appeared to be associated with shortened plant heights (also confirmed in Gealy unpublished data) and other biological traits typical of japonica rice. Recent functional genomics data has shown that the japonica SD1 allele has a measurable effect on gibberellin production, which is known to affect plant growth (Asano et al. 2011). Sequencing the SD1 locus, Reagon et al. (2011) also confirmed that two putative hybrid progeny types were descended from crosses between awned or awnless red rice types and tropical japonica cultivars that were consistent with haplotypes (H.GR-1; i.e., sd1, the green revolution allele) present in CL 161, CL 121, 'Lemont', Cypress, and 'Bengal'.

"Short" red rice biotypes with heights similar to LhtsA− and LhtsA+ plants in the present study have been reported previously. In Louisiana rice fields planted to semidwarf IMI-resistant cultivars, red rice biotypes 111 to 114 cm in height had average outcrossing rates of 0.44% (Zhang et al. 2006). In controlled outcrossing studies with red rice to IMI-resistant rice, two biotypes were as short as 112 and 119 cm and averaged 0.018% at two locations in Arkansas (Shivrain et al. 2009a). In neither case, however, were analyses conducted to determine whether the short stature of red rice plants had resulted from earlier crossing with rice or other red rice plants, or to ascertain the genetic backgrounds of the red rice types.

Reagon et al. (2011) showed that the *SD1* haplotype present in U.S. tropical japonica rice (e.g., Blue Rose) has been found in both "short" and "tall" red rice plant types in the same location, which supports the hypothesis that red rice genes have introgressed into rice. Their group previously reported introgression between red rice and tropical japonica rice with red rice as the apparent pollen donor (Reagon et al. 2010). An accession that was genetically and phenotypically ("short" awns) consistent with hybrid progeny from rice × awned red rice crosses also has been reported (Gealy et al. 2006). Introgression of a blast-susceptible *pi-ta* allele from rice into red rice also has been postulated (Lee et al. 2011).

Our study revealed that the three atypical red rice accessions shared little genetic background with U.S. rice cultivars evaluated in this test (Figures 1, 2, and 3) and the additional cultivars Rexoro, 'Newbonnet', Lemont, 'LaGrue', 'Drew', 'Dawn', 'Bluebonnet', Carolina Gold, Zenith, 'Saturn', 'Nato', 'Mars', and Bengal, 'M-204', and 'Koshihikari' (Lu et al. 2005; data not shown). However, red rice accessions from an independent collection appeared to have a higher degree of shared genetic background with cultivars historically grown in the region (Shivrain et al. 2010a). The reason for this difference is not clear, but might have been due in part to their analysis of a much larger group (137) of "typical" representative accessions compared to ours (28).

The short LhtsA− and LhtsA+ red rice phenotypes in our studies might have arisen from pre-existing genetic diversity within the red rice populations. However, because *SD1* haplotypes of commercial rice origin have been confirmed in short-statured weedy rice accessions, including AR01-1096 (#13; present study) (Reagon et al. (2011), it also seems plausible that gene flow from rice to red rice occurred, and that most of the rice alleles initially transferred to red rice subsequently were lost from these populations due to selection pressure or other causes. The genetic similarities observed within our short-statured accessions also might relate to a phenomenon observed by Cao et al. (2006) in which roguing by farmers over time tended to reduce genetic diversity among weedy rice infestations. Thus, many of the obvious (e.g., tall) and more diverse hybrid progeny from crosses between red rice and cultivated rice might have been depleted from these fields over time. Short red rice types have been shown to be less competitive against rice, and thus less productive, compared to tall types (Estorninos et al. 2005), which is also consistent with their low frequencies in our collection.

The accessions most recently acquired for our collection were obtained in the early 2000s. This was before the introduction of imidazolinone-resistant rice cultivars (Gealy 2005). Thus, our results can serve as a PRE-resistant rice baseline for atypical red rice types. Imidazolinone-resistant rice systems using true-breeding or hybrid rice cultivars, now are estimated to occupy nearly 70% of the rice area in Arkansas and Louisiana. Gene flow from rice into red rice in these fields could lead to increasing numbers of atypically short red rice types that are difficult to detect visually and are well-equipped to compete in these rice systems once they have acquired resistance to the imidazolinone herbicides.

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