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Research Paper

Genetic diversity and population structure of 'Khao Kai Noi', a Lao rice (*Oryza sativa* L.) landrace, revealed by microsatellite DNA markers

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Rice (*Oryza sativa* L.) is the main food for people in Laos, where it has been grown and eaten since prehistory. Diverse landraces are grown in Laos. 'Khao Kai Noi', a landrace favored for its eating quality, is held in the nationwide collection of traditional landraces in the Lao national genebank. Genetic diversity is crucial for sustainable use of genetic resources and conservation. To investigate the genetic diversity of 'Khao Kai Noi' for conservation, we genotyped 70 accessions by using 23 polymorphic simple sequence repeat markers. The markers generated 2 to 17 alleles (132 in total), with an average of 5.7 per locus. The total expected heterozygosity over all 'Khao Kai Noi' accessions was 0.271. Genetic variation was largest among accessions and smallest within accessions. Khao Kai Noi accessions were classified into three different genetic backgrounds, but there was unclear association between the three inferred population and name subgroups and geographical distribution. Most of the accessions were clustered with temperate *japonica* and showed genetic relatedness to rice from neighboring provinces of Vietnam, suggesting a Vietnamese origin. The results of this study will contribute to the conservation, core collection and future breeding of the Khao Kai Noi population.

Key Words: 'Khao Kai Noi', Lao rice, diversity, SSR markers.

Introduction

Rice (*Oryza sativa* L.) is a staple food of nearly half the people on the planet (Mohanty 2013). In Laos, most people depend on it as their staple food, and rice dominates agricultural production (Eliste and Santos 2012). Although Lao farmers grow improved cultivars for their high yields, some farmers still grow local landraces because of their adaptation to the local environment and preferred traits, such as aroma. Despite their lower yields, these landraces can serve as useful resources for breeding programs.

'Khao Kai Noi' (KKN) is a rainfed lowland glutinous rice grown in northern and northeastern Laos, with good grain quality, softness, and aroma (Rao *et al*. 2006a, 2006b). The name means "small chicken rice" in Lao. Its spelling in English is not standardized and is variously written as 'Khao Kay Noi' or 'Khao Kai Noi' (Rao *et al*. 2006b) or 'Khao Kai Noy' (Worklivelaos 2014). We spell it as 'Khao Kai Noi'. KKN is grown not just for domestic consumption but also for export to France and Vietnam. It is also used for beer brewing (Worklivelaos 2014).

From 1995 to 2000, the Lao Ministry of Agriculture and Forestry and the International Rice Research Institute (IRRI) collected 13,192 local accessions of rice throughout the country (Rao *et al.* 2006c). The collection, which includes KKN, is held in the national genebank of the Agriculture Research Center, National Agriculture and Forestry Research Institute. Another KKN-specific survey and collection was performed in 2008 in the provinces of Houaphan (HP) and Xiengkhouang (XK) (Bounphanousay *et al*. 2009).

Knowledge of the genetic diversity of KKN is required for the sustainable use of germplasm, efficient collection management, plant variety rights protection, core collection development, and breeding. Many studies have used simple sequence repeat (SSR) markers to examine the genetic diversity, population structure, and genetic variation within rice landraces (Bajracharya *et al*. 2006, Das *et al*. 2013, Pusadee *et al*. 2009, Roy *et al*. 2013, Zhang *et al*. 2013). However, no reports of Lao rice landraces are available, with the exception of an evaluation of black glutinous rice (Bounphanousay *et al*. 2008). Kanyavong (2012) described the agromorphological variation in KKN, but the molecular diversity has not been reported on.

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In this study, we evaluated the genetic diversity of accessions of KKN by SSR markers. We then studied genetic relatedness among accessions of KKN collected from different geographical locations. Finally, we compared genetic relatedness between KKN and other landraces within Laos and between Laos and Vietnam.

Materials and Methods

Plant materials

We examined 70 accessions of KKN from the Lao national genebank (**Table 1**). The collection of this variety consists of different subgroups, with some of them having an additional descriptor in the varietal name to reflect these special traits with differences in glume color and other characteristics, such as 'KKN Deng (red)', 'KKN Khao' or 'KKN Khaw (white)', 'KKN Leuang (yellow)', 'KKN Lai (striped)', and 'KKN Dam (black)'. These names were given at the collection time by farmers (Rao *et al.* 2006a). One Lao improved cultivar, 'TDK11', was also included in this study. 'Nipponbare' and 'Kasalath' were provided by the National Institute of Agrobiological Sciences (NIAS), Japan, and used as representatives of the *japonica* and *indica* types, respectively. We also included 18 accessions of other landraces from Houaphan Province (HP), Xiengkhouang Province (XK), and a neighboring province of Vietnam (**Table 1**). These materials were provided by the IRRI. Eight seeds of each accession were used.

Simple sequence repeat (SSR) assay

We genotyped 24 nuclear SSR markers distributed across the rice genome (**Table 2**). Its primer sequence information is available at http://archive.gramene.org/markers/microsat/50_ssr.html.

DNA was extracted from single brown rice seeds. Seeds were ground in a multi-bead shocker (YASUI Kikai, Japan) at 2000 rpm for 1 min, and DNA was extracted by using the cetyl trimethyl ammonium bromide (CTAB) method (Doyle and Doyle 1987). Polymerase chain reaction (PCR) was performed as described in the Multiplex PCR strategy. For 24 SSR primer pairs, forward primers were labeled with 1 of 4 fluorophores (6FAM, NED, PET, or VIC). They were later divided into 4 groups, called "panels", of 6 markers each (**Table 2**) selected by Multiplex Manager v. 1.0 software to design an efficient combination of primers in a PCR reaction (Holleley and Geerts 2009). Each reaction contained 1× Type-it Multiplex PCR Master Mix (Qiagen, Hilden, Germany), 2–5 pmol of the forward and reverse primers of each of the 6 markers in a panel, and 50–100 ng of genomic DNA in a total volume of 15 µL. PCR amplification was performed in the GeneAmp PCR System 9700 (Applied Biosystems, CA, USA) as follows: 95°C for 5 min; 30 cycles of 95°C for 30 s, 58–60°C (depending on the panel, **Table 2**) for 90 s, and 72°C for 30 s; and finally 60°C for 30 min.

The PCR products were diluted 1:20 with sterilized Milli-Q water. Thereafter, 1 µL of each was added to 9.5 µL of Hi-Di formamide plus 0.5 µL of GeneScan 600-LIZ size standard marker (Applied Biosystems). The mixture was heated at 95°C for 3 min, immediately chilled on ice for 5 min, and then run in an ABI 3500xL genetic analyzer (Applied Biosystems). Data were collected by 3500xL data collection v. 1 software (Applied Biosystems). Fragment analysis or allele calling was performed with GeneMapper v. 5 software (Applied Biosystems).

Data analysis

Gene diversity or expected heterozygosity (H_e), observed heterozygosity (H_o) of each loci, and polymorphism information content (PIC) over 70 accessions (560 individuals) were calculated in PowerMarker v. 3.25 software (Liu and Muse 2005). Expected (H_{EA}) and observed (H_{OA}) heterozygosity for each accession over loci was calculated in GenAlEx v. 6.5 software (Peakall and Smouse 2012). Next, accessions were classified into groups based on their name subgroup. Expected (*HE*) and observed heterozygosity (*HO*) for each of them, average (H_S), total expected heterozygosity (H_T) overall name subgroups, and genetic differentiation among name subgroups (F_{ST}) were calculated in GenAlEx v. 6.5. Analysis of molecular variance (AMOVA) was also performed in GenAlEx v. 6.5 to determine hierarchical partitioning of genetic variation among the name subgroups, among accessions, and within accessions. Finally, we grouped accessions by geographical provinces and calculated all parameters in the same way as that done in analysis of name subgroups. Phylogenetic reconstruction used data of 70 accessions of KKN and other control accessions which each consisted of 8 individuals was based on the unweighted pair-group method for arithmetic mean (UPGMA; Nei *et al*. 1983) in PowerMarker 3.25 with 1,000 bootstrap replications.

The KKN population structure was assessed by a model-based method in STRUCTURE v. 2.3.4 software (Pritchard *et al*. 2000). The number of populations was tested from $K = 1$ to 10 with admixture and correlated allele frequencies models. Ten separate runs were performed for each K with a burn-in period of 100,000 and a run of 100,000. The optimum K value was determined from log probability of data (ln $P(D)$) and ad hoc statistic ΔK of Evanno *et al*. (2005) by using the STRUCTURE HARVESTER website and software (Earl and vonHoldt 2012).

Results

Genetic diversity values of KKN collection

Of the 24 markers, one (RM133) was monomorphic, so it was excluded from analysis. In total, 132 alleles of the 23 polymorphic markers were detected in 560 seeds of KKN. The number of alleles ranged from 2 (RM171 and RM455) to 17 (RM259), with an average of 5.7 per locus (**Table 2**). The PIC ranged from 0.036 (RM408) to 0.526 (RM259), with an average of 0.199. H_e ranged from 0.037 (RM408) to

Table 1. Passport data of 70 accessions of Khao Kai Noi, a Lao rice landrace group, and 18 control rice accessions from IRRI used in this study

No.	Accession no.	Accession name	Grain morphology implied by name	Origin
Khao Kai Noi from Lao national genebank				
1	LG13251	Kai Noi	—	BK
2	LG13480	Kai Noi	—	BK
3	LG13535	Kai Noi	—	CS
4	LG13771	Kai Noi	—	LN
5	LG6493	Kai Noi	—	LP
6	LG9212	Kai Noi	—	LP
7	LG7488	Kai Noi	—	VM
8	LG10035	Kai Noi	—	VM
9	LG5845	Kai Noi	—	XK
10	LG6795	Kai Noi	—	XK
11	LG12360	Kai Noi	—	XK
12	LG12584	Kai Noi	—	XK
13	LG12923	Kai Noi	—	XK
14	LG13970	Kai Noi	—	XK
15	LG10195	Kai Noi	—	XS
16	LG10898	Kai Noi	—	XS
17	LG2755	Kai Noi Dam	black	HP
18	LG10133	Kai Noi Dam	black	XK
19	LG14024	Kai Noi Dam Mihang	black with awn	XK
20	LG14112	Kai Noi Dam Lay	black striped	XK
21	LG14027	Kai Noi Khaw Mihang	Khaw with awn	XK
22	LG2793	Kai Noi Deng	red	HP
23	LG6644	Kai Noi Deng	red	HP
24	LG14126	Kai Noi Deng	red	HP
25	LG6762	Kai Noi Deng	red	XK
26	LG14018	Kai Noi Deng	red	XK
27	LG14023	Kai Noi Deng	red	XK
28	LG14095	Kai Noi Deng	red	XK
29	LG14113	Kai Noi Deng	red	XK
30	LG2790	Kai Noi Lai	striped	HP
31	LG2794	Kai Noi Lai	striped	HP
32	LG2841	Kai Noi Lai	striped	HP
33	LG6665	Kai Noi Lai	striped	HP
34	LG14124	Kai Noi Lai	striped	HP
35	LG14077	Kai Noi Lai	striped	unknown
36	LG6760	Kai Noi Lai	striped	XK
37	LG6838	Kai Noi Lai	striped	XK
38	LG14110	Kai Noi Lai	striped	XK
39	LG10899	Kai Noi Lai	striped	XS
40	LG14016	Kai Noi Lai Dam	striped and black	XK
41	LG14020	Kai Noi Lai Dam	striped and black	XK
42	LG6746	Kai Noi Hay	upland	HP
43	LG14028	Kai Noi Khaw/Khao	white	XK
44	LG2746	Kai Noi Leuang	yellow	HP
45	LG2792	Kai Noi Leuang	yellow	HP
46	LG2806	Kai Noi Leuang	yellow	HP
47	LG6732	Kai Noi Leuang	yellow	HP
48	LG14116	Kai Noi Leuang	yellow	HP
49	LG14117	Kai Noi Leuang	yellow	HP
50	LG14118	Kai Noi Leuang	yellow	HP
51	LG14120	Kai Noi Leuang	yellow	HP
52	LG14121	Kai Noi Leuang	yellow	HP
53	LG14122	Kai Noi Leuang	yellow	HP
54	LG14123	Kai Noi Leuang	yellow	HP
55	LG14125	Kai Noi Leuang	yellow	HP
56	LG14076	Kai Noi Leuang	yellow	unknown
57	LG14017	Kai Noi Leuang	yellow	XK
58	LG14021	Kai Noi Leuang	yellow	XK
59	LG14022	Kai Noi Leuang	yellow	XK
60	LG14026	Kai Noi Leuang	yellow	XK

Table 1. (continued)

No.	Accession no.	Accession name	Grain morphology implied by name	Origin
61	LG14029	Kai Noi Leuang	yellow	XK
62	LG14030	Kai Noi Leuang	yellow	XK
63	LG14031	Kai Noi Leuang	yellow	XK
64	LG14033	Kai Noi Leuang	yellow	XK
65	LG14043	Kai Noi Leuang	yellow	XK
66	LG14103	Kai Noi Leuang	yellow	XK
67	LG14107	Kai Noi Leuang	yellow	XK
68	LG14109	Kai Noi Leuang	yellow	XK
69	LG14114	Kai Noi Leuang	yellow	XK
70	LG14115	Kai Noi Leuang	yellow	XK
Control rice accessions from IRRI				
71	IRGC 89625	Do Keo		Laos
72	IRGC 89941	Khen Seua		Laos
73	IRGC 92257	Tam		Laos
74	IRGC 98381	Muang Souy		Laos
75	IRGC 107576	Leuang Kham		Laos
76	IRGC 111233	Do Lao Soung		Laos
77	IRGC 111242	Hang Ngoua Kom		Laos
78	IRGC 56034	Chao		Vietnam
79	IRGC 79554	Mac Duoi		Vietnam
80	IRGC 82649	Khau Chan Hay		Vietnam
81	IRGC 82651	Khau Mo		Vietnam
82	IRGC 82653	Khau Non Hay		Vietnam
83	IRGC 90679	Khau Tan		Vietnam
84	IRGC 90689	Khau Tan Nong		Vietnam
85	IRGC 90721	Tan Nhe		Vietnam
86	IRGC 96930	Khau Cham Hang		Vietnam
87	IRGC 112709	Ngo Gan		Vietnam
88	IRGC 113858	Khau Phroung		Vietnam
Control improved variety from Lao national genebank				
89	TDK11	Thadokkham11		Laos
Control rice accessions from NIAS				
90	Kasalath	Kasalath		
91	Nipponbare	Nipponbare		

BK, Borikhamxay; CS, Champasak; HP, Houaphan; LN, Luang Namtha; LP, Luang Prabang; VM, Vientiane Municipality; XK, Xiengkhouang; XS, Xaisomboun; IRRI, International Rice Research Institute.

0.574 (RM514). H_o ranged from 0.000 (RM455) to 0.061 (RM447; **Table 2**). AMOVA revealed that 79% of the total variation occurred among accessions, 15% among KKN name subgroups, and 6% within accessions (**Table 3**).

Genetic diversity values of individual accessions

The number of alleles detected per accession ranged from 25 (10 accessions) to 62 (LG14117; **Supplemental Table 1**). H_{EA} ranged from 0.01 (LG14125) to 0.49 (LG14117). H_{OA} was highest (0.09) in 2 accessions (LG12923 and LG5845 (**Supplemental Table 1**)).

Genetic diversity among KKN name subgroups

Within the 70 KKN accessions, we found 7 KKN name subgroups: 'Khao Kai Noi' (with no descriptor), 'Khao Kai Noi Leuang' (yellow), 'Khao Kai Noi Deng' (red), 'Khao Kai Noi Lai' (striped), 'Khao Kai Noi Hay' (upland), 'Khao Kai Noi Dam' (black), and 'Khao Kai Noi Khaw' (white). Among the subgroups, *HO* varied from 0.001 ('Khao Kai Noi Dam') to 0.038 ('Khao Kai Noi Hay'). *HE* ranged from 0.040 ('Khao Kai Noi Khaw') to 0.315 ('Khao Kai Noi Deng'). H_S among subgroups was 0.175. H_T was 0.271. Genetic differentiation of the KKN name subgroups was confirmed by F_{ST} (0.353; **Table 4**).

Population differentiation between Houaphan and Xiengkhouang provinces

Of the 70 KKN accessions, 81.4% originated from two provinces—Houaphan (HP, 22) and Xiengkhouang (XK, 35)—where the production of KKN is predominant (Rao *et al*. 2006a). To study the phylogeography of KKN, we compared genetic diversity values between HP and XK. In HP, $HE = 0.286$ and $HO = 0.014$; in XK, $HE = 0.147$ and $HO = 0.012$ (**Table 4**). H_S was 0.216 and H_T was 0.222 over the two provinces. F_{ST} between HP and XK was 0.025.

Dendrogram clustering and STRUCTURE analysis

Using the genetic-distance-based UPGMA method, the

Table 2. Diversity of 23 polymorphic SSR markers in 70 accessions of 'Khao Kai Noi' rice

Marker	Chr.	Label	Panel[a]	Tm (°C)[b]	A	H_e	H_o	PIC
RM1	1	PET	A	60	8	0.350	0.007	0.323
RM259	1	VIC	A	60	17	0.563	0.013	0.526
RM495	1	NED	C	58	7	0.137	0.045	0.132
RM452	2	NED	B	58	3	0.045	0.004	0.044
RM514	3	NED	A	60	6	0.574	0.005	0.482
RM55	3	PET	D	60	4	0.086	0.004	0.082
RM489	3	6FAM	D	60	4	0.426	0.009	0.357
RM307	4	6FAM	D	60	5	0.102	0.002	0.099
RM507	5	VIC	D	60	3	0.104	0.057	0.101
RM162	6	NED	B	58	6	0.122	0.005	0.119
RM11	7	PET	B	58	7	0.563	0.005	0.47
RM455	7	VIC	B	58	2	0.045	0.000	0.044
RM152	8	6FAM	A	60	3	0.389	0.002	0.313
RM408	8	6FAM	B	58	3	0.037	0.005	0.036
RM447	8	PET	C	58	6	0.090	0.061	0.085
RM215	9	VIC	C	58	5	0.148	0.011	0.143
RM316	9	6FAM	C	58	6	0.110	0.011	0.108
RM171	10	NED	D	60	2	0.047	0.005	0.045
RM287	11	NED	B	58	5	0.188	0.005	0.182
RM536	11	PET	C	58	6	0.115	0.016	0.112
RM552	11	NED	D	60	13	0.299	0.018	0.292
RM19	12	PET	A	60	6	0.051	0.005	0.05
RM277	12	NED	A	60	5	0.534	0.004	0.427
Average					5.7			0.199
Total					132			

Chr., chromosome; [a], panels managed by Multiplex Manager software; [b], optimum annealing temperature for each panel in this study; *A*, number of alleles; H_e, expected heterozygosity; H_o, observed heterozygosity; *PIC*, polymorphism information content.

Table 3. Analysis of molecular variance of 70 accessions of 'Khao Kai Noi' rice

Sources	d.f.	SS	Variance components	Variation (%)
Among KKN subgroups	6	363.707	0.404	15
Among accessions	553	2422.449	2.116	79
Within accessions	560	83.500	0.149	6
Total	1119	2869.645	2.669	100

d.f., degrees of freedom; SS, sum of squares.

Table 4. Genetic diversity parameters of 7 subgroups of 'Khao Kai Noi' rice on the basis of 23 SSR markers

	No.	*HE*	*HO*	H_S	H_T	F_{ST}
Name subgroups	70			0.175	0.271	0.353
Khao Kai Noi	16	0.252	0.023			
Khao Kai Noi Leuang (yellow)	28	0.164	0.008			
Khao Kai Noi Deng (red)	8	0.315	0.017			
Khao Kai Noi Lay/Lai (striped)	11	0.166	0.009			
Khao Kai Noi Hay (upland)	1	0.199	0.038			
Khao Kai Noi Dam (black)	4	0.089	0.001			
Khao Kai Noi Khaw (white)	2	0.040	0.005			
Provinces	57			0.216	0.222	0.025
HP	22	0.286	0.014			
XK	35	0.147	0.012			

No., number of accessions; *HE*, expected heterozygosity; *HO*, observed heterozygosity; H_S, average expected heterozygosity; H_T, total expected heterozygosity; F_{ST}, genetic differentiation.

accessions were divided into 2 major clusters, *indica* and *japonica* (**Fig. 1**). The *indica* cluster contained 'Kasalath', 'TDK11' (Lao improved cultivar), 6 control traditional landraces, and 1 KKN accession (LG6644). The *japonica* cluster was divided into 2 subclusters. The smaller subcluster consisted of 3 KKN accessions (LG6746, LG6493, and LG9212) and 8 control landraces from Laos and Vietnam, including 2 placed in the *tropical japonica* (*javanica*) cultivar group (IRGC111242 and IRGC111233) according to passport data in the Genesys Gateway to Genetic Resources (www.genesys-pgr.org, accessed March 6, 2015). The larger subcluster consisted of 'Nipponbare', 4 traditional landraces from Vietnam, and most KKN accessions.

In model-based population structure analysis, we first tested all 70 accessions of KKN, and one accession was grouped in the *indica* type (LG6644). We omitted this accession from the analysis, and 69 accessions were then used for the second analysis. The values of ln $P(D)$ had great change and ΔK were highest when the number of populations was $K = 4$ followed by $K = 7$ (**Supplemental Fig. 2A**, **2B**). In the STRUCTURE result with $K = 4$, accessions assigned to the *tropical japonica* (*javanica*) clusters in the dendrogram were clearly separated from other KKN accessions (green; **Fig. 1**). Most of accessions were assigned to 3 inferred populations (blue, yellow, and red in **Fig. 1**). Subsequently, we removed three accessions grouped with *tropical japonica* accessions (LG9212, LG6493 and LG6746) and carried out construction of dendrogram and STRUCTURE analysis only with *temperate japonica* KKN accessions. Clustering patterns of the dendrogram did not change significantly without the three *tropical japonica* accessions. However, the STRUCTURE results demonstrated a distinct pattern (optimum $K = 3$, **Supplemental Figs. 3**, **4**). Although KKN has been considered to be intermediate between the *indica* and *tropical japonica* types (Rao *et al*. 2006b), our results showed that most KKN accessions have a *temperate japonica* background. Studying effect of *tropical* and *temperate japonica* genetic background to be important revealing general genetic structure of KKN, and thus, we decided to include both *tropical* and *temperate japonica* accessions for further analysis and discussions.

The overall clustering pattern of the dendrogram coincides with the STRUCTURE results. The inferred populations indicated in red and yellow with $K = 4$ were localized in two clusters (*tropical and temperate japonica*) of the dendrogram, but these populations were separated into distinct inferred populations with $K = 7$. Accessions LG14117, LG13535 and LG14118, which were in the *japonica* cluster but were not grouped with other KKN accessions, contain genetic compositions of *tropical japonica* type. It is noteworthy that there were only two accessions, LG10133 and LG14018, that possessed three genetic backgrounds (blue, yellow and red) inferred by STRUCTURE analysis.

The population of KKN used in this study consisted of

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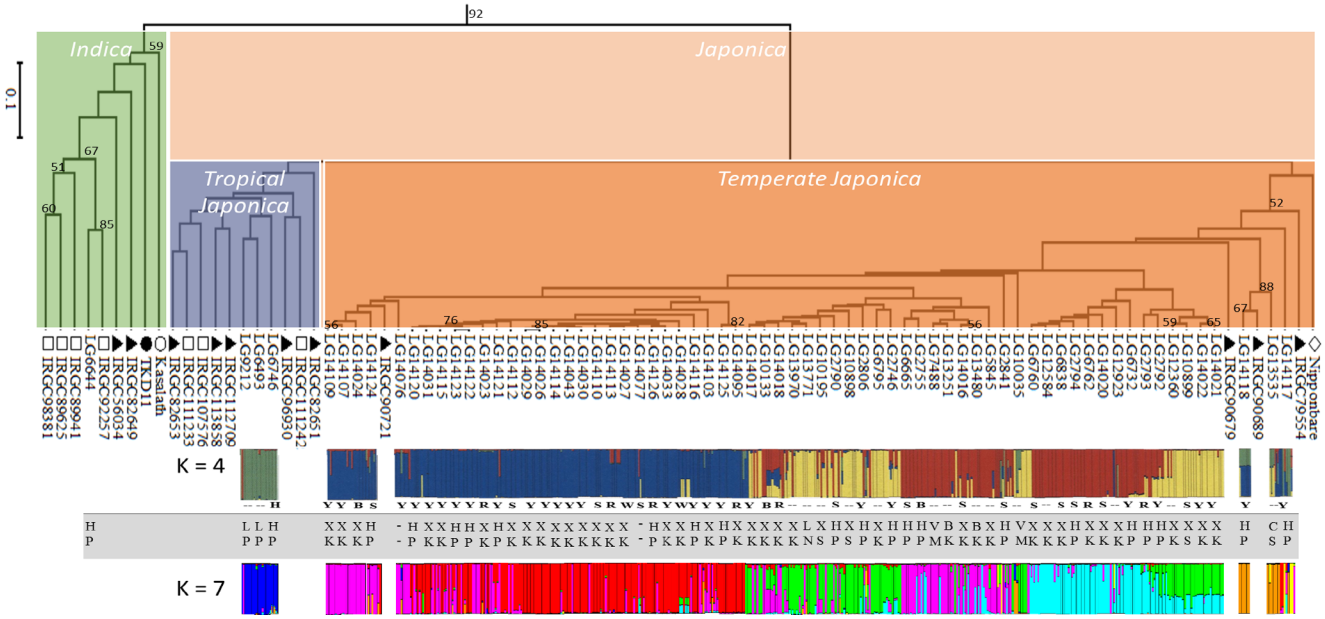

Fig. 1. Dendrogram of 70 accessions of 'Khao Kai Noi' (KKN) and 21 control accessions, clustered by the unweighted pair-group method for arithmetic mean (UPGMA). Bootstrap values with 1000 replications are shown in each branch. □ Traditional Lao landraces, ○ a representative of *indica* subspecies, ● improved Lao cultivars, ▲ Vietnamese landraces. Bar plots obtained from STRUCTURE analysis using 69 accessions in Japonica group (K = 4 and 7) are shown beneath KKN accession. The letters under bar plot indicate characteristics of each accession implied by accession name. H, upland; B, black; R, Red; S, striped; Y, Yellow; W, white; and –, no implication by accession name. The letters in the grey box is the name of provinces that samples were collected. HP, Houaphan; XK, Xiengkhuang; CS, Champasak; BK, Bolikhamxay; XS, Xaisomboun; LN, Luang Namtha; LB, Luang Prabang; VM, Vientiane Municipality; -, no record (unknown).

name subgroups (**Table 1**). Distribution of the name subgroups 'KKN leuang (yellow, Y)', 'KKN deng (red, R)', 'KKN Khao/Khaw (white, W)', 'KKN Lai/Lay (striped, S)', and 'KKN dam (black, B)' are shown in **Fig. 1**. Although accessions of the name subgroup Y (yellow) are distributed throughout temperate *japonica* clusters, most of them coincide with the blue inferred population by STRUCTURE. The other name subgroups did not correspond clearly with any cluster or inferred populations, and were present throughout the dendrogram.

Discussion

Genetic diversity of KKN accessions

The 23 SSR markers revealed relatively high genetic diversity in the 70 KKN accessions, with an average of 5.7 alleles per locus and H_T = 0.271 (**Table 4**). The number of alleles per locus was smaller than that of Indian landraces comprising different cultivar groups (7.9 and 7.8, respectively; Das *et al*. 2013, Jian *et al*. 2004), similar to that of Indian local aromatic cultivars (5.4; Roy *et al*. 2013), and greater than those of Indian aromatic rice from Orissa state (2.08; Meti *et al*. 2013) and black glutinous rice from Laos (3.1; Bounphanousay *et al.* 2008). H_e was similar to that of 'Balam', an indigenous cultivar from India (Choudhury *et al*. 2013).

By analysis of morphology, Rao *et al*. (2006a) identified 9 name subgroups within the KKN group; we found 7 of these in the KKN collection. The high diversity is consistent with the use of SSR markers. Nevertheless, the collection of additional accessions might be needed to obtain variant forms that are not currently held.

Within the 7 forms of KKN found in this study, the 'KKN Deng' (red) group had the highest genetic diversity (**Table 4**). As the name indicates, the grain is red. A red pericarp is ubiquitous in wild populations and is associated with resistance to biotic stresses (Sweeney and McCouch 2007). This trait is due to an SNP in the *Rc* gene that occurred during domestication, changing the pericarp from red to white (Sweeney *et al.* 2007). Since wild rice is common throughout Laos (Kuroda *et al.* 2006), the red pericarp of 'KKN Deng' may have been caused by gene flow from wild rice. Further study of the *Rc* haplotype of 'KKN Deng' and adjacent wild populations may clarify the issue.

Locus RM259 had the most alleles (17). Interestingly, the same locus had the most alleles in a group of Lao black glutinous rice accessions (7) (Bounphanousay *et al.* 2008). Pusadee *et al.* (2014) also reported high diversity at the same locus in Thai landraces. Marker RM259 is mapped on chromosome 1 (Chen *et al.* 1997) and has been used for quantitative trait locus analysis of early flowering (Thomson *et al*. 2006) and grain yield under drought stress (Sandhu *et al*. 2014). Rice germplasm collected from HP, XK, and nearby regions may contain novel alleles for traits linked with this marker.

Heterozygosity of KKN—implications for germplasm management

Only 6% of the total variation occurred within accessions (**Table 3**). $H_{EA} > H_{OA}$ when averaged across all loci in almost all accessions (**Supplemental Table 1**). This situation indicates inbreeding. Inbreeding is common in self-pollinated species, such as rice (Matsui and Kagata 2003), as seen in the high inbreeding coefficient (F_{IS}) of 'Bue Chomee', a Thai landrace (Pusadee *et al*. 2009), which ranges from 0.859 to 1. This observation reflects strong but not complete inbreeding in the KKN populations in farmers' fields.

Germplasm regeneration is a fundamental role of genebanks. Cross and Wallace (1994) recommended that accessions be held as pure lines on the basis of their simulation of allele loss during regeneration of heterogeneous self-pollinating accessions. A study of the effect of genetic integrity in bulked and pure line approaches to seed management demonstrated that selection or genetic drift may occur during rejuvenation in the bulked approach (Hirano *et al*. 2009). Thus, KKN accessions identified as heterogeneous populations, for example LG14117, should be subdivided into pure lines and preserved to maintain their genetic integrity during rejuvenation and conservation in the Lao genebank.

Relationship between name subgroups, geographical distribution, and genetic structure of KKN

The most popular name subgroup, 'KKN Leuang (yellow)', shared nearly 40% of total accessions of KKN used in this study, followed by 'KKN Lai (striped)' (14%) and 'KKN Deng (red)' (12%). Accessions of these name subgroups originated from both HP and XK provinces. Name subgroups present in HP are always present in XK, except 'KKN Hay (upland)'. 'Kai Noi Khaw/Khao (white)' and 'KKN Mihang (awned)' are present only in XK. Rao *et al*. (2006a) described eight out of nine name subgroups collected from HP and only 'KKN Mihang (awned)' was collected from XK. In this study, variation of name subgroup in HP and XK were almost the same.

Interestingly, names which describe more than two grain traits {e.g., Kai Noi Lai Dam (striped and black)} were present only in XK. These name combinations were not described in previous report (Rao *et al.* 2006a). Only one form of KKN name subgroup is grown in most of the fields of farmers (Rao *et al.* 2006b). This study showed the presence of heterogeneous accessions, which contain different genotypes within accessions; therefore, gene flow and hybridization are expected. Introduction of the new traits through hybridization may have caused establishment of combined grain morphology and successive arise of combined name. Attention should be payed to XK Province to cover a broader morphological and genetic diversity of KKN.

No clear genetic structure was observed in terms of name subgroups based on the dendrogram and inferred populations of STRUCTURE analysis. Not only the name subgroup, genetic background inferred by dendrogram and STRUCTURE analysis did not separate clearly HP and XK provinces (**Fig. 1**). Isolation by distance occurs in fragmented populations owing to a lack of gene flow (Wright 1943, 1946). For example, 'Bue Chomee', a Thai landrace, was significantly differentiated among villages (Pusadee *et al*. 2009). Genetic differentiation of KKN accessions from the two main production provinces, HP and XK, was very low ($F_{ST} = 0.025$). Therefore, accessions from these two provinces share a genetic background, maybe via human-mediated gene flow, such as seed exchange among farmers, as KKN was introduced first into HP and then into XK (Bounphanousay *et al*. 2009, Rao *et al*. 2006b). KKN became popular and therefore spread to other villages and provinces because of its good eating quality. The provinces of HP and XK, which share a border, on account of the promotion of production for industrial uses (Vientiane Times 2014).

Genetic background of KKN and its origin—implications for conservation and genetic improvement

The dendrogram revealed genetic relationships between KKN and other common cultivars and among KKN accessions. Rao *et al*. (2006b) described KKN to be intermediate between the *indica* and *tropical japonica* types based on their gross morphology. However, most of the KKN accessions were grouped with *japonica* 'Nipponbare', and *temperate japonica* background can be assumed for most of KKN. This group, together with the *tropical japonica* type, was clearly separated from the *indica* group in the dendrogram, reflecting the genetic structure and diversity in *O. sativa* reported by Garris *et al*. (2005). Only one accession, LG6644, was grouped with *indica* accessions. This clustering pattern was supported by a high bootstrap value, implying that LG6644 has an *indica* background. Possible explanations are an error during collection and substitution at the time of seed regeneration.

KKN accessions LG6493, LG6746, and LG9212 were grouped with upland *tropical japonica* accessions, whereas most KKN accessions are lowland. Since all the traditional Lao rice landraces used in this study were grouped in the *tropical japonica* type, these accessions may have some influences from other traditional varieties in the region.

Four traditional Vietnamese landraces were clustered in the *temperate japonica* type together with KKN accessions in the dendrogram, yet all the traditional Lao landraces were included in the *tropical japonica* group (**Fig. 1**). This shows that Vietnamese landraces are more closely related to KKN than other Lao landraces. KKN is believed to have been introduced into HP from Vietnam and later into XK (Rao *et al.* 2006b). Our results support this origin. The distribution of crop cultivars across borders is common where the local people share either sociocultural backgrounds (Kyndt *et al*. 2009) or climatic conditions (Forsberg *et al*. 2015). Rice landraces closely related to KKN in Vietnam may possess promising agromorphological characteristics. Therefore, such landraces should be added to the germplasm

collection, either by germplasm exchange between Laos and Vietnam or by joint collection expeditions, to enhance genetic diversity within the collection and for use in breeding programs.

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