Rice (Oryza sativa L.) is a staple 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.
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 genebank (Table 1). The collection of this variety was performed in the GeneAmp PCR System 9700 (Applied Biosystems, CA, USA) as follows: 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 (HE) 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 (HE) and observed heterozygosity (HO) for each accession over loci was calculated in GenAIEx 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 (Hs), total expected heterozygosity (HT) overall name subgroups, and genetic differentiation among name subgroups (FST) were calculated in GenAIEx v. 6.5. Analysis of molecular variance (AMOVA) was also performed in GenAIEx 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. HE ranged from 0.037 (RM408) to

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). Fragment analysis or allele calling was performed with GeneMapper v. 5 software (Applied Biosystems).
**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 |
|-----|---------------|----------------|----------------------------------|--------|
| 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                             | XK     |
| 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    | stripped                         | 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     |
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 (Table 3).

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, H_O varied from 0.001 (‘Khao Kai Noi Dam’) to 0.038 (‘Khao Kai Noi Hay’). H_E 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, H_E = 0.286 and H_O = 0.014; in XK, H_E = 0.147 and H_O = 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
acccesion from the analysis, and 69 accessions were then used for the second analysis. The values of ln \( P(D) \) had great change and \( \Delta K \) 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
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.

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) (Bouphannouay 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.

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; Bouphannouay 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.

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**Heterozygosity of KKN—implications for germplasm management**

Only 6% of the total variation occurred within accessions (Table 3). $H_{EA} > H_{DA}$ 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 Chompee’, 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 paid 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 Chompee’, 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 ‘Nipponbabe’, 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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