Introduction

The north and along the Vietnam border in Laos are mountainous areas. Rainfed upland rice (*Oryza sativa* L.) is grown in the upland fields, and rainfed lowland rice is grown in flat valley bottoms or on terraced hillsides. Most rice, however, is grown in the lowlands of central and southern Laos. In irrigated lowlands, rice is cropped twice a year: from November–December to May and from June to October–November. Rainfed rice cultivation is limited to the wet season (Linquist *et al.* 2006): from June to October–November in the lowlands and from May to September–October in the uplands.

The adoption of improved cultivars has generally been associated with the abandonment of most of the traditional landraces (Schiller *et al.* 2006). By the early 2000s, many landraces were no longer being grown or available in the central and southern regions (Appa Rao *et al.* 2006a). The loss of upland landraces is an expected consequence of the introduction of improved upland cultivars and the gradual decline in the areas of upland rice planted throughout Laos, as a result of government policy to move from annual cropping (rice cultivation) to more sustainable agricultural practices (plantation of gum trees) in upland areas. Thus, it is important to collect and evaluate the landraces in northern Laos before the genetic diversity in Laos is lost.

Rice germplasm was collected several times from 1970 to 1990 (Appa Rao *et al.* 2002a). From 1991 to 1994, another 1000+ samples were collected in the uplands of six

Genetic variation in rice (*Oryza sativa* L.) germplasm from northern Laos

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We assessed genetic variation in rice germplasm in northern Laos and Vientiane province from polymorphism data of SSR markers. We classified 314 accessions into three clusters; Ia (corresponding to the lowland Japonica Group), Ib (upland Japonica Group) and II (Indica Group). The accessions of cluster Ib grew mainly in mountainous fields, and those of cluster II grew commonly in basins and along rivers. The few accessions of cluster Ia grew in only three provinces: Houaphanh, Xiangkhouang and Vientiane. Lowland cultivars in cluster II were predominant in Vientiane. Variations in heading date under short-day conditions in 2014 and long-day conditions in 2015 indicate that many accessions were sensitive to the photoperiod on account of complex genetic mechanisms underlying both photoperiod sensitivity and basic vegetative growth. A total of 219 among whole accessions were classified into 6 groups: E1–3 and L1–3. E2 and E3 were dominant in clusters Ib and II; E1 and L1–3 were minor groups. These results demonstrate characteristic distributions of the Indica and Japonica Group’s germplasms in northern Laos and their genetic variation in heading date.

**Key Words:** genetic variation, heading, geographic distribution, landrace, lowland, upland, rice (*Oryza sativa* L.).
northern provinces (Roder et al. 1996). The International Rice Research Institute regarded a set of 13,192 samples collected from 1995 to 2000 as being representative of the genetic resources throughout Laos (Appa Rao et al. 2006a). Classified by maturity, 28.3% of those collected in rainfed uplands were early, 42.7% were intermediate and 29.0% were late. These accessions matured earlier than irrigated lowland accessions, some much earlier. Of the lowland accessions, 21.7% were early, 52.7% were intermediate and 25.7% were late, and the early and intermediate accessions were usually grown in the upper parts of terraces where drought stress is more likely immediately after the end of the wet season. Late accessions were grown mainly in low-lying areas on the beds of watercourses or in valley swamps in areas with variable water levels. Thus, rice cultivars with a variety of heading dates have been grown in each ecosystem according to the environment.

The heading date in rice is a complex trait with a variety of underlying genetic factors that control basic vegetative growth, photoperiod sensitivity and temperature sensitivity. Basic vegetative growth and photoperiod sensitivity play important roles as indicators of heading ability (Hosoi 1978, 1981). Various combinations of genetic factors that control both elements (Matsuo et al. 1960) have produced various cultivars with a range of heading traits (Tanisaka et al. 1992). It is important to understand heading in rice cultivars for stable rice production. Thus, it is necessary to elucidate the relationships between heading variation and local adaptations to use local landraces as genetic resources.

The genetic differentiation of the Indica-Japonica Groups has been studied using molecular markers, such as simple sequence repeat (SSR) and isozyme markers for evaluation of the diversity of genetic resources. Second (1982) reported that these two groups were domesticated independently from different ancestral species by using 40 isozyme loci among 1,948 strains of cultivated, wild and weedy rice strains from Asia and Africa. Glaszmann (1987) reported the classification of Indica, Japonica and four additional population structures by using 15 isozyme loci among 1,688 traditional accessions from Asia. Garris et al. (2005) investigated the genetic differentiation among 234 rice accessions by using 169 SSR markers, and classified them into five cluster groups: Indica, Temperate Japonica, Tropical Japonica, Aus and Aromatic. Thus, rice accessions were basically classified into the Japonica and Indica Groups with minor changes of groupings according to the rice materials and molecular markers used. Recent studies using common SSR markers in Japan (Kawasaki-Tanaka and Fukuta 2014), Myanmar (Wunna et al. 2016), Bangladesh (Khan et al. 2017) and West Africa (Odjo et al. 2017) divided rice germplasms into the lowland Japonica, upland Japonica and Indica Groups. The polymorphism data of these SSR markers is useful for understanding the genetic variations and differentiation of rice accessions in Laos.

Genetic analyses of upland rice landraces in northern Laos have been carried out before. Roder et al. (1996) collected 318 landraces from six northern provinces from 1991 to 1994, and classified 98% by isozyme analysis into the Japonica Group. Ishikawa et al. (2002) collected accessions at 27 sites in Luang Namtha and Oudomxay provinces and classified 106 as the Japonica Group and 16 as the Indica Group or intermediate by isozyme analysis. Yamanaka et al. (2002) collected 140 accessions in eight provinces throughout Laos and classified most as the Japonica Group according to a G-T substitution in the Wx gene. These results indicate that most upland landraces in northern Laos are the Japonica Group and glutinous rice.

On the other hand, lowland rice landraces have not been investigated, and the relationships among rice ecotypes (upland and lowland) and cultivar Groups (Indica and Japonica), the genetic differentiation of agricultural traits and adaptations to environmental conditions are not understood. In this study, we investigated the relationships among the Japonica and Indica Groups through the genetic structure of the chromosomes by SSR polymorphism data, variations in heading date as a key trait for adaptation and geographical distribution to elucidate the genetic diversity within rice accessions collected in northern Laos.

**Materials and Methods**

**Plant materials**

From 2003 to 2010, researchers from the Rice Research Center, National Agriculture and Forestry Research Institute (Laos), Institute of Humanity and Nature (Japan) and Hirosaki University (Japan) collected accessions of local rice landraces from upland and lowland areas in seven provinces (Oudomxay, Bokeo, Luang Namtha, Phongsaly, Luang Prabang, Houaphanh and Xiang Khouang) located in northern Laos and in Vientiane province where rice is cultivated mainly in the lowlands. (Fig. 1). With the help of local farmers of various ethnicities, they collected 1000+ accessions, of which 314 accessions, including several improved cultivars, were used in this study (Table 1, Supplemental Table 1). The study included ‘Nipponbare’ as the Japonica Group’s control and ‘Kosalath’ as the Indica Group’s control.

**Classification of rice accessions by polymorphism data of DNA markers**

Total DNA was extracted from young leaves of individual plants by the CTAB method, and polymorphism data of 67 SSR markers distributed among the 12 chromosomes (Supplemental Table 2) were collected. Kawasaki-Tanaka and Fukuta (2014), Wunna et al. (2016), Khan et al. (2017) and Odjo et al. (2017) also used the SSR markers, and they demonstrated that these polymorphisms could differentiate among the upland Japonica, lowland Japonica and Indica Groups. PCR was performed in a 10-μL mixture containing 1 μL sterile H₂O, a total 1.5 μL of forward and reverse primers (each 2 μM), 7.5 μL of 2× Quick Taq HS Dye Mix (Toyobo Co., Ltd. Japan) and 5 μL DNA concentrated to...
rose gel in 1× TAE buffer at 150 V for 90 or 120 min, and DNA polymorphisms were detected by ethidium bromide staining. The banding patterns were scored based on the presence (1) or absence (0) in each SSR markers’ allele. These polymorphism data were used to classify the accessions by Ward’s hierarchical clustering method in JMP software (v. 7.0.2; SAS Institute, Inc., Cary, NC, USA).

**Cultivation and investigation of heading date**

Plants were grown in a greenhouse at the Tropical Agricultural Research Front (TARF), Japan International Research Centre for Agricultural Sciences (JIRCAS), Ishigaki, Okinawa, Japan, in 2014 and 2015. Heading dates were investigated under different day lengths in 2014 and 2015. In 2014, seeds were sown on 4 September in 1/2000◦a Wagner pots and grown under short-day length (10.8–12.5 h). In 2015, seeds were sown on 18 May and grown under long-day length (11.3–13.0 h). Days to heading (DTH) from the sowing date were calculated.

**Results**

**Rice samples collecting**

Among the 314 accessions, 207 (65.9%) were collected from mountainous upland fields, 91 (29.0%) from lowlands and 16 (5.1%) from unknown sources (Fig. 1, Table 1, Supplemental Table 1). Among the 280 northern accessions, 204 (72.9%) were collected from uplands (53.3% to 88.2% by province), 65 (23.2%) from lowlands (4.4% to 44.0% by province) and 11 (3.9%) from unknown sources. These values indicate that upland cultivars were predominant, but lowland cultivars were common in some regions. Among the Vientiane accessions, in contrast, only three (8.8%) were collected from uplands, 26 (76.5%) from lowlands and five (14.7%) from unknown sources. These results correspond with the predominance of slash-and-burn cultivation in mountainous uplands and lowland culture in basins or along rivers in the north and the predominance of lowland culture in Vientiane province reported by Appa Rao et al. (2002a) and Linquist et al. (2006).

**SSR marker polymorphisms and cluster analysis**

Among the 67 SSR markers, 234 bands were detected and used in cluster analysis (Supplemental Table 2). The accessions were classified into three groups, Ia, Ib and II (Supplemental Fig. 1, Table 2). Among the 314 accessions, 14 (4.5%) were placed in cluster Ia, 193 (61.5%) in Ib and 107 (34.1%) in II. Cluster Ia included both ‘Nipponbare’ (Japonica Group control) and ‘Khoai Kai Noi’, a glutinous Japonica cultivar from the lowlands of Houaphan and Xiangkhouang provinces. Cluster II included ‘Kasalath’ (Indica Group control) and many improved Indica Group cultivars, including ‘TDK1’, ‘TDK2’ and ‘TDK6’, from irrigated lowlands.

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Genetic variation in rice from northern Laos

Table 1. In 2014, DTH of 283 accessions ranged from 49 to 113 days (mean, 76.5 days), with a division between early (E) and late (L) at 90 days. In 2015, DTH of 224 accessions ranged from 70 to 163 days (mean, 121.8 days), with divisions between early (1) and intermediate (2) at 85 days, and between intermediate and late (3) at 135 days. There were 219 accessions in common in 2014 and 2015. The variations in DTH clearly differed between years: the range was wider and the mean was higher in 2015 than in 2014.

Using the results of both years, we reclassified the 219 accessions into six heading groups: E1 (13 accessions, 5.9%, ≤90 days in 2014, ≤85 days in 2015), E2 (90, 41.1%, ≤90 days in 2014, 85–135 days in 2015), E3 (90, 41.1%, ≤90 days in 2014, ≥135 days in 2015), L1 (11, 5.0%, ≥90 days in 2014, ≤85 days in 2015), L2 (13, 5.9%, ≥90 days in 2014, 85–135 days in 2015) and L3 (2, 0.9%, ≥90 days in 2014, ≥135 days in 2015). By this classification, E2 and E3 were major DTH groups, and E1, L1, L2 and L3 were minor.

Altitudinal distributions of DTH groups
We found characteristic distributions of altitude among the six heading groups (Fig. 4). The altitudes of group E1 ranged from 267 to 664 m (mean, 447 m). Those of E2 ranged from 165 to 1257 m (mean, 567 m). Those of E3 ranged from 165 to 1,257 m (mean, 577 m). The altitudes of E2 and E3 varied widely within the same range, but those of E1 covered a narrower range of lower altitudes. The distributions of groups L1, L2 and L3 were limited, ranging from 185 to 761 m (mean: L1, 438 m; L2, 415 m; L3, 473 m), in the same range as E1. These results indicate that the major heading groups E2 and E3 covered a wider range of altitudes, and the minor groups E1, L1, L2 and L3 covered a narrow range of low altitudes.

Genetic variation in days to heading
Wide variations in DTH were found among accessions in 2014 and 2015 (Fig. 3, Supplemental Fig. 2, Supplemental Table 1). In 2014, DTH of 283 accessions ranged from 49 to 113 days (mean, 76.5 days), with a division between early (E) and late (L) at 90 days. In 2015, DTH of 224 accessions ranged from 70 to 163 days (mean, 121.8 days), with divisions between early (1) and intermediate (2) at 85 days, and between intermediate and late (3) at 135 days. There were 219 accessions in common in 2014 and 2015. The variations in DTH clearly differed between years: the range was wider and the mean was higher in 2015 than in 2014.

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Fig. 2. Distribution of rice accessions in each SSR polymorphism cluster group by altitude. n = 314. ▼ Mean.
mean, 81.8 days); that of cluster Ib ranged from 54 to 113 days (mean, 75.7 days); and that of cluster II ranged from 49 to 106 days (mean, 77.1 days) (Fig. 3). In 2015, DTH of cluster Ia ranged from 119 to 152 days (mean, 135.4 days); that of cluster Ib ranged from 70 to 151 days (mean, 118.8 days); and that of cluster II ranged from 77 to 163 days (mean, 125.8 days). These results indicate that all three clusters had wide variations in DTH, and cluster Ia had the latest mean.

Fig. 3. Distribution of days to heading in rice accessions from Laos. Accessions were classified into cluster groups Ia, Ib and II by SSR polymorphism data. n = 283 in 2014, 224 in 2015. ▼ Mean. Plants were grown in a greenhouse of TRAF, JIRCAS, Ishigaki, Okinawa, from 4 September 2014 (short-day condition) and 18 May 2015 (long-day condition).

Fig. 4. Distribution of rice accessions in each cultivar group by altitude. Accessions were classified into “E 1–3” and “L 1–3” on the basis of days to heading in 2014 and 2015. ▼ Mean.
Table 3. Relationship between cluster groups based on SSR polymorphism data and cultivar groups classified by days to heading.

| Cluster groups | Cultivar groups classified by days to heading | Total |
|----------------|---------------------------------------------|-------|
|                | E1  | E2  | E3  | L1  | L2  | L3  |     |
| Ia             | 0   | 2   | 6   | 0   | 0   | 0   | 8   |
| (0.0)          | (25.0) | (75.0) | (0.0) | (0.0) | (0.0) |     |
| Ib             | 12  | 52  | 54  | 8   | 7   | 0   | 133 |
| (9.0)          | (39.1) | (40.6) | (6.0) | (5.3) | (0.0) |     |
| II             | 1   | 36  | 30  | 3   | 6   | 2   | 78  |
| (1.3)          | (46.2) | (38.5) | (3.8) | (7.7) | (2.6) |     |
| Total          | 13  | 90  | 90  | 11  | 13  | 2   | 219 |
| (5.9)          | (41.1) | (41.1) | (5.0) | (5.9) | (0.9) |     |

**Discussion**

We collected and investigated a total of 314 rice accessions from seven provinces in northern Laos and Vientiane province (Fig. 1, Table 1, Supplemental Table 1). Cluster analysis classified them into three clusters (Supplemental Fig. 1, Table 2); Ia (lowland Japonica Group), Ib (upland Japonica Group) and II (Indica Group). Cluster Ib accessions were predominant in the mountainous north, and cluster II accessions were also common. Cluster Ia accessions grew in a narrow range of lower altitudes than clusters Ib and II, which grew in the same range of altitudes, although cluster Ib grew at a higher mean altitude (Fig. 2). Cluster Ia accessions were restricted to Houaphan, Xiangkhouang and Vientiane provinces. In lowland Vientiane, cluster II accessions were predominant (Fig. 1). Ishikawa et al. (2002) and Yamanaka et al. (2002) reported that the Japonica Group was dominant in uplands and the Indica Group was dominant in the lowlands in Laos. Our results support this distribution. In contrast, the Indica Group accessions were predominant in Vientiane province. In addition, cluster Ia accessions (lowland Japonica) grew only in Houaphan, Xiangkhouang and Vientiane provinces, including the glutinous Khao Kai Noi, found previously by Appa Rao et al. (2002b, 2006b) and Vilayheuang et al. (2016), who considered it to have been introduced from Vietnam (Appa Rao et al. 2006b, Vilayheuang et al. 2016). We confirmed the cultivation of the Japonica Group rice in irrigated lowlands.

Evaluation of the accessions in Okinawa under short-day and long-day conditions revealed wide variations in DTH (Fig. 3, Supplemental Fig. 2). The distributions of 219 accessions grown in both years were clearly different: wider and higher in 2015 (long day) than in 2014 (short day). These results indicate that almost all accessions are photoperiod sensitive. Basnayake et al. (2006) similarly reported strong photoperiod sensitivity among landraces in Laos, which headed at day lengths of <12 h.

Reclassification of these 219 accessions based on these variations identified 6 groups: E1, E2, E3, L1, L2 and L3 (Supplemental Fig. 2). These groups reflect photoperiod sensitivity and differences in basic vegetative growth. The distributions of the accessions revealed characteristic variations (Fig. 4). DTH groups E2 and E3 were dominant, and grew at a wide range of altitudes. Groups E1, L1, L2 and L3 were minor, and grew at lower altitudes than E2 and E3. Among the three cluster groups, cluster Ia had only eight accessions from E2 and E3 (Table 3). In contrast, Ib included five of the six groups, and cluster II included all six groups. These results mean that the upland Japonica and lowland Indica Groups were distributed widely over a range of altitudes and had wide variation in heading date, but the lowland Japonica Group was more limited.

Appa Rao et al. (2006a) reported that late heading cultivars with a long vegetative stage were grown in fertile paddies, and that early heading cultivars were grown where water conditions were unreliable. The distributions of the late heading groups (L1–L3) were limited to lower altitudes. Group L3 (latest heading) was found only in cluster group II, corresponding to lowland rice. E1 (earliest heading) came from Luang Prabang, Houaphan and Xiangkhouang provinces, where the rainfall is lower than in the other provinces. Our results agree well with the relationships between distribution by altitude and variations in heading date. They suggest that the major groups E2 and E3 represent widely adaptable populations and that the 4 minor groups are adapted to specific regions and conditions in Laos.

Our results show that local farmers in northern Laos have conserved a wide variety of rice landraces adapted to specific environmental conditions, such as altitudinal differences, seasonal water availability and climatic variability, with a wide range of heading dates. Detailed analyses of geographical distribution and agricultural traits such as stress tolerance and yield components will allow us to identify differences in germplasms for use in breeding or as new cultivars.

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analyses were conducted under 3 research projects: “Collection and Characteristics Analysis of Plant Genetic Resources” (PGRAsia, GRC, NARO, Japan, 2014–2017), “Rice innovation for environmentally sustainable production systems” (2011–2015) and “Development of technologies for the control of migratory plant pests and transboundary diseases” (JIRCAS, 2016).