Genetic variation of rice (*Oryza sativa* L.) germplasm in Cambodia

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Genetic variations of 179 rice (*Oryza sativa* L.) accessions from Cambodia were clarified based on the analyses for heading date, chromosome components, and blast resistance. The dominant accessions were found in three regions; early heading in North East (NE), medium in Central (CT), and late in South East (SE) along the Mekong River in the investigation at Ishigaki, Japan. In contrast, wide variations were observed in two regions, South West (SW) and North West (NW) located around Tonle Sap Lake. Polymorphism data of SSR markers showed that accessions were classified into Japonica Group (cluster Ib), and Indica Groups (IIa and IIb). In the NW and SW, the accessions of all three clusters were found, but these accessions in NE, CT, and SE, were limited to one or two clusters. Accessions were classified again into two clusters, A1 as having high resistance and A2 as having moderate resistance. Remarkable differences of these frequencies of clusters, A1 and A2, were found in the SE, SW, and NW, and similar with these of the whole accessions were in NE and CT. Rice accessions varied among the five regions, and there was a dramatic difference between the regions along Mekong River and the regions around Tonle Sap Lake.

Key Words: genetic variation, blast resistance, heading date, rice (*Oryza sativa* L.), Cambodia.

Introduction

Cambodia is one of the unique countries in Asia in that it has various distinct ecosystems each with different natural selection pressures, such as drought, flooding, and nutrient stresses (Javier 1997). The rice ecosystems in Cambodia have been categorized into (*i*) rainfed uplands mostly located in the North East of the country, (*ii*) rainfed lowlands (sub-grouped into upper, medium, and lower fields and early wet season fields), (*iii*) deep-water, and (*iv*) dry season fields (Chan 2011, Javier 1997, Men *et al.* 2001).

Upland rice (*Oryza sativa* L.) is cultivated on sloping land without bunds to retain water, in the northern and northeastern provinces (Nesbitt and Phaloeun 1997). The area of upland rice accounts for 0.8% of the total rice-growing land in Cambodia (MAFF 2019). Deep-water rice with strong sensitivity to photoperiod (long and late maturating) is cultivated in lowland areas located along big rivers and surrounding Tonle Sap Lake, and along the border with Vietnam. Deep-water rice ecosystems are con-

trolled greatly by the occurrence and strength of floods coming from the Mekong River, and it accounts for 1.0% of the cultivated rice area. Rainfed lowland rice with various degrees of photoperiod-sensitivity (medium–late maturing) is distributed in the higher areas surrounding the deep-water rice fields and accounts for 80.4% of total rice production area; these are located in the main rice production areas of the Central (CT), South East (SE), South West (SW), and North West (NW) regions (MAFF 2019). The remaining 17.8% is occupied by irrigated lowland rice insensitive to photoperiod, which is also cultivated in the highest areas or in dry fields of rainfed lowland areas in wet and dry seasons, and several modern cultivars from the International Rice Research Institute (IRRI) and other countries are also included.

Clarification of the genetic variations in rice germplasm is the first and the most important step towards understanding the potentials and problems with using this germplasm as breeding materials. To date, a total of 2,842 accessions from across four rice ecosystems have been collected and conserved in the gene bank of the Cambodian Agricultural Research and Development Institute (CARDI). Among those, 1,594 accessions were analyzed before the end of 1994; less than 2% were photoperiod insensitive (short maturity) and 98% were photoperiod sensitive (longer

maturation periods). There is variation in the degree of photo-period sensitivity, and strongly sensitive cultivars constitute around 93% of the collection. Lando and Mak (1994) and Fujisaka (1988) described the general characteristics, and cultivars are broadly grouped according to dates of flowering or harvesting. Early-maturing cultivars (*srau sral*, 43.9% of rice growing areas) are suited to the high fields, medium-maturing cultivars (*srau kandal*, 39.1%) grow well in the middle fields, and late-maturing cultivars (*srau thungun*, 17.0%) are best for the low fields. Early-maturing cultivars flower before 15 October, middle ones flower between 16 October and 15 November, and late ones flower afterward in Cambodia, and almost all middle- and late-maturing landraces are photoperiod sensitive. Other studies conducted later by Cambodia IRRI Australia Project (CIAP) (CIAP 1993, 1994, Sahai *et al.* 1992) characterized 2,109 landraces; those studies evaluated heading dates and several morphological traits including culm and panicle length, culm number, leaf length and width, and grain length and width.

Thus, studies such as those above have investigated variations in heading date and several morphological traits in Cambodia. However, traits of biotic and abiotic stresses were not included. Jahn *et al.* (1997) listed blast disease (*Pyricularia oryzae* Cavara) as a major pest of upland rice and irrigated rice in the dry season. Unfortunately, systematic study of fungal diseases, such as blast, is quite limited in Cambodia. The only such study is that of Fukuta *et al.* (2014) who clarified the pathogenicity of 122 blast isolates collected from regions surrounding Tonle Sap Lake and along the Mekong River, and categorized them into 92 races in Cambodia.

To build up a breeding program for stable rice production in Cambodia, the genetic variations in blast resistance in rice germplasm and the relationships between blast races and resistance of rice germplasm will need to be clarified.

Molecular markers can be used to reveal differences between rice accessions at the DNA level and thus provide a more direct, reliable, and efficient tool for analysis of genetic diversity, and many studies have been conducted to evaluate genetic diversity among rice cultivars by using microsatellite (SSR) markers (Chen *et al.* 1997, Temnykh *et al.* 2000). Okoshi *et al.* (2004) classified 73 Japanese landraces into Indica and Japonica Groups and into upland and paddy rice subgroups. Yamasaki and Ideta (2013) clarified the differences in genetic diversities in Japanese landraces and in improved cultivars using 104 accessions. Kawasaki-Tanaka and Fukuta (2014) clarified the genetic variation of 324 Japanese rice accessions using polymorphism data on 64 SSR markers across the genome chromosomes, and classified them into two cluster groups, I and II. Japonica Group accessions from irrigated lowland areas were included mainly in cluster I, and upland and Indica Group accessions were mainly in cluster II. Following those Japanese studies, several genetic analysis studies using the same set of SSR markers were conducted to clar-

ify genetic variations of rice germplasm in other parts of Asia and in Africa. Wunna *et al.* (2016) investigated 175 accessions from Myanmar; Khan *et al.* (2017) investigated 334 Bangladeshi rice accessions; Odjo *et al.* (2017) investigated 195 accessions comprising three species of the AA genome complex (Asian rice [*O. sativa* L.], African rice [*O. glaberrima* Steud.], and wild rice [*O. barthii*]); Fukuta *et al.* (2019) investigated 47 accessions in Kenya; and Muto *et al.* (2019) investigated 314 accessions in Laos. Thus, SSR markers are a useful tool for the clarification of genetic variations and for differentiation among AA genome species and between Indica Group and Japonica Group cultivars, between lowland and upland cultivars, and between landrace and improved types in rice.

The interaction between host resistance and fungus virulence in the rice blast patho-system can be explained by the gene-for-gene theory: for every resistance gene in the host, there is a corresponding avirulence gene in the pathogen (Flor 1956, Silué *et al.* 1992). Based on the gene-for-gene theory, Telebanco-Yanoria *et al.* (2008) found a wide variation in resistance to blast disease caused by *Pyricularia oryzae* Cavara among 922 rice cultivars collected mainly from Asia, based on inoculation tests using standard differential blast isolates (SDBIs) which were used to clarify the pathogenicity. These cultivars were classified into six varietal groups based on the patterns of reaction to 20 SDBIs from the Philippines, and the relative frequencies of these six varietal groups varied according to geographical regions. Several studies followed, clarifying the genetic variations for blast resistance in rice germplasm based on inoculation tests using SDBIs. Kawasaki-Tanaka and Fukuta (2014) found a wide variation of blast resistance in 324 Japanese rice accessions, including landrace, improved, and weedy types, using 16 SDBIs from Japan and the Philippines; Odjo *et al.* (2017) studied blast resistance in 195 accessions comprising three species of the AA genome complex, based on 32 SDBIs from Japan and West Africa; Khan *et al.* (2017) studied blast resistance in 334 Bangladesh accessions from four major ecotypes (Aus, Aman, Boro, and Jhum) using 20 SDBIs collected from Bangladesh, Japan, and Kenya; and Fukuta *et al.* (2019) studied blast resistance in 47 Kenyan accessions using 15 SDBIs from Japan, the Philippines, and Kenya. Thus, the genetic variation of resistance in rice germplasm has been clarified based on the reaction patterns to SDBIs.

In this study, we used a set of SSR markers across 12 rice chromosomes to examine polymorphism data from 179 rice accessions and tried to classify them based on the genotypes' data. Heading dates and blast resistance by inoculation tests using SDBIs were also investigated. The relationships among genotypes, heading date, and blast resistance Were then compared considering the geographical distributions. Based on these investigations, an outline of the genetic variation in rice germplasm from Cambodia is discussed.

Materials and Methods

Plant materials

A total of 179 accessions from Cambodia were used in this study (**Table 1**, **Supplemental Table 1**). These accessions (87.7% in the whole) were cultivated mainly in rainfed lowland areas in five regions (South East [SE], South West [SW], Central [CT], North West [NW], and North East [NE]), but included several upland (5.0%) and irrigated lowland (7.3%) accessions from the NE and SW regions respectively. The selection of accessions from each ecosystem and region were considered that these frequencies of rice accessions were corresponded with those of rainfed lowland rice (80.4% of the total rice-growing land in Cambodia), upland (0.8%), deep-water (1.0%), and irrigated lowland (17.8%) and these geographical distributions (MAFF 2019). It means that the 179 accessions were selected as the representatives among popular cultivars from each region and rice ecosystem in Cambodia.

They had been categorized previously at the Cambodian Agricultural Research and Development Institute (CARDI), into three heading types: early-heading (days to heading [DTH] ≤90 days, 20 accessions, 11%), middle-heading (91–120 days, 90, 50%), and late-heading (>120 days, 69, 39%).

Of the 179 accessions, 176 were cultivated and their DTHs investigated at Tropical Agricultural Research Front (TARF), Japan International Research Center for Agricultural Sciences (JIRCAS), Ishigaki, Japan in the second season from July to November 2017. The remained three accessions didn't germinate and could not investigated the DTHs in the season. The latitude at Phnom Penh, Cambodia, is 11° 54′ 48″, and locate in Tropical region. Rice cultivation are carried out from May to November and the day lengths are changed from to 12.5 h to 11.7 h. It means that the day length in Phnom Penh is always short and not so vary. That at TRAF, JIRCAS, Ishigaki, Okinawa is 24° 37′ 94″ in Sub-Tropical region, and the day lengths during the second season changed from 13.6 h to 11.2 h. In Ishigaki, the day length changes form long to short. Thus, the conditions of day lengths are slightly different between Phnom Penh and Ishigaki.

DNA extraction and genotyping using SSR markers

To clarify the genetic variation among rice accessions in Cambodia, a set of 64 SSR markers across the 12 rice chromosomes was used for polymorphism analysis. These were selected from a public database (http://www.gramene.org) (**Supplemental Table 2**). Genomic DNA was extracted from a young leaf from each rice accession. Leaf tissue was ground in 100 µL of 0.25 N sodium hydroxide (NaOH) with zirconium beads in 2.0 mL tubes. A volume of 400 µL of 100 mM Tris-HCl (pH 7.5) was added and the sample was then well mixed and centrifuged for 10 min at 10,000 rpm. The supernatant was transferred into 1.5 mL autoclaved tubes. PCR was performed in a 10 µL PCR mixture containing 1 µL sterile H_2O, 1.5 µL of forward (2 µM) and reverse (2 µM) primers of SSR markers, 7.5 µL of 2× Quick Taq TM HS DyeMix (Toyobo Co., Ltd. Japan), and 5 µL DNA concentrated to around 5–10 ng/µL. PCR amplification was carried out with the following profile: 94°C for 2 min, followed by 40 cycles of 30 s at 94°C, 30 s at 55°C, and 1 min at 68°C. PCR products were electrophoresed on 2% agarose gels in 1× TAE buffer at 150 V for 90–120 min and DNA fragments were visualized with ethidium bromide. The polymorphism data of each accession was recorded based on the banding pattern and compared with those of control cultivars Nipponbare and Kasalath. The numbers of alleles were counted as different polymorphisms in each SSR marker among rice accessions.

Inoculation of blast isolates and evaluation of resistance

To determine putative resistance genes in 176 among 179 rice accessions, a set of 25 monogenic lines (Tsunematsu *et al.* 2000) or LTH NILs (Telebanco-Yanoria *et al.* 2010) harboring 23 resistance genes as differential varieties (DVs) and two susceptible controls, an Indica Group line, US-2, and a Chinese Japonica Group cultivar, Lijiangxintuanheigu [LTH]), were used at Tropical Agriculture Research Front, Japan International Research Center Agricultural Sciences, Ishigaki, Okinawa, Japan. Three accessions, Santepheap1, Kong Yol and Rimke from SW, were not investigated, and not included. In addition, Nipponbare and Kasalath were used as controls for Japonica Group and Indica Group cultivars, respectively. A total of 16 standard differential blast isolates (SDBIs) from Japan (ten isolates), Cambodia (one), Bangladesh (one), Benin (one), Nigeria (one), the Philippines (one), and Kenya (one) were used to evaluate the variation of blast resistance among rice accessions. The evaluations were done with three seedings from each

Table 1. Rice accessions used in this study

Region	Landrace or improved	Ecosystem for rice cultivations			Total
		Irrigated lowland	Rainfed lowland	Upland	
NE	Landrace	0	3	7	10
CT	Landrace	0	51	0	51
SE	Landrace	0	26	0	26
SW	Landrace	0	47	0	47
	Improved	13	3	1	17
NW	Landrace	0	27	1	28
Whole	Landrace	0	154	8	162
		(0.0)	(95.1)	(4.9)	(100.0)
	Improved	13	3	1	17
		(76.5)	(17.6)	(5.9)	(100.0)
Total		13	157	9	179
		(7.3)	(87.7)	(5.0)	(100.0)

NE: Northeast, CT: Central, SE: Southeast, SW: Southwest, NW: Northwest.

accession with two replications. The seeds were sown in a plastic cell tray (14 × 32 cells; cells 16 mm diameter, 25 mm deep) and then placed in a greenhouse at 25°C for two weeks. Two-week-old seedlings were inoculated with each of the SDBIs at a volume of 80 mL with the spore concentration standardized to $30–50 × 10^4$ spores/mL. The tray od plants was placed on a swivel chair and rotated while being misting with the spore suspension until droplets were visible. The inoculated plants were put in an incubator room at 25°C with high relative humidity (>90%) for 20 h, and then transferred to a greenhouse with temperature of 25°C and 60% relative humidity for 1 week. Infection was evaluated at 7 days after inoculation. The degree of infection of each seedling was scored based on a scale of 0 to 5 as described by Hayashi and Fukuta (2009).

The resistance genes in each rice accession were postulated based on their patterns of reaction to SDBIs in comparison to the patterns of reaction of the 25 DVs.

Genetic variations of rice accessions classified based on polymorphism data of SSR markers and patterns of reaction to SDBIs were computed with Ward's hierarchical clustering method in JMP7.0.2 software (SAS Institute, Inc., Cary, NC, USA).

Results

Genetic variation in days to heading (DTH)

A total of 176 accessions were cultivated and their DTHs investigated at Ishigaki, Okinawa, Japan, in the second season from July to November in 2017. The DTHs of these accessions varied from 81 days to 128 days with a mean of 107 days (**Fig. 1**). The distribution of DTHs was divided into three groups with ranges of early (<95 days), middle (from 96 days to 115 days), and late (≥116 days). The DTHs of the 20 accessions categorized in Cambodia as early-heading varied from 80 days to 110 days in Japan with a mean of 93 days; these were distributed from the early to the middle DTH groups in Japan with 14 accessions (70.0%) falling into the early group and six (30.0%) in the middle group, showing that accessions in the early DTH group were dominant and those in the middle DTH group were minor. The DTHs of 88 accessions categorized in Cambodia as middle-heading varied from 88 days to 123 days with a mean of 108 days, and these were distributed across all three DTH groups in Japan. The peak was located at 105–110 days, and 75 accessions (85.2%) among them fell into the middle DTH group. The DTHs of 68 accessions categorized in Cambodia as late-heading also varied across a wide range from 81 days to 126 days with a mean of 112 days. Those accessions were distributed across all three DTH groups, with 38 accessions (55.9%) among them falling into the late DTH group. These results indicate that the category for heading date of rice accessions allocated in Cambodia basically corresponded with those at Ishigaki, Japan, but included several exceptions. And wider and detail genetic variations of heading data among rice
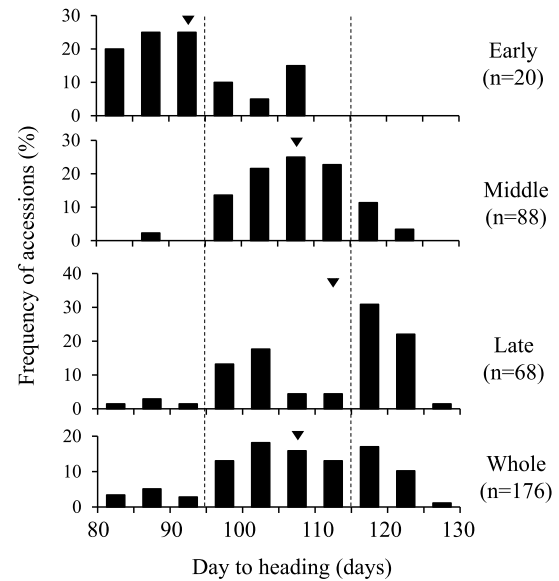

Fig. 1. Relationships between maturity type in Cambodia and genetic variation of day to heading at Japan. Three categories for heading date, Early, Middle, and Late, is the classification under the environmental condition at CARDI, Phnom Penh, Cambodia. Investigation was carried out at Ishigaki, Okinawa, Japan, in the second rice cultivation season from July to November of 2017. A total of 176 accessions were investigated for heading date among whole materials. Triangle: Mean.

accessions were shown in Ishigaki than those in Cambodia.

These variations in DTHs were compared among the five geographical regions of Cambodia, and different variations were found (**Fig. 2**). The DTHs of the 10 accessions from the NE region varied from 81 days to 105 days with a mean of 98 days. Of these, two accessions (20.0%) were categorized into the early DTH group and eight (80.0%) into the middle DTH group. The DTHs of the 51 accessions in the CT region varied from 96 days to 124 days with a mean of 107 days. These were categorized into middle and late DTH groups, with 48 accessions (94.1%) falling into the middle DHT group. The DTHs of the 26 accessions from the SE region varied from 93 days to 123 days with a mean of 113 days. Only four accessions (15.4%) fell into the early and the other 22 accessions (84.6%) were middle and late heading accessions. In the SW region, DTHs ranged from 81 days to 125 days with a mean of 107 days, and of the 61 accessions from this region 16 (26.2%) fell into the early DTH group, 22 (36.1%) in the middle DTH group, and 23 (37.7%) in the late DTH group. Included among these were local and introduced improved cultivars. Landrace accessions were included in all heading groups, and the improved accessions were included only in the early and late heading groups. In the NW region, accessions varied from 90 days to 126 days with a mean of 111 days. The numbers of accessions were 1 (3.6%) in the early DTH group, 14 (50.0%) in the middle DTH group, and 13 (46.4%) in the late DTH group. Included among these were
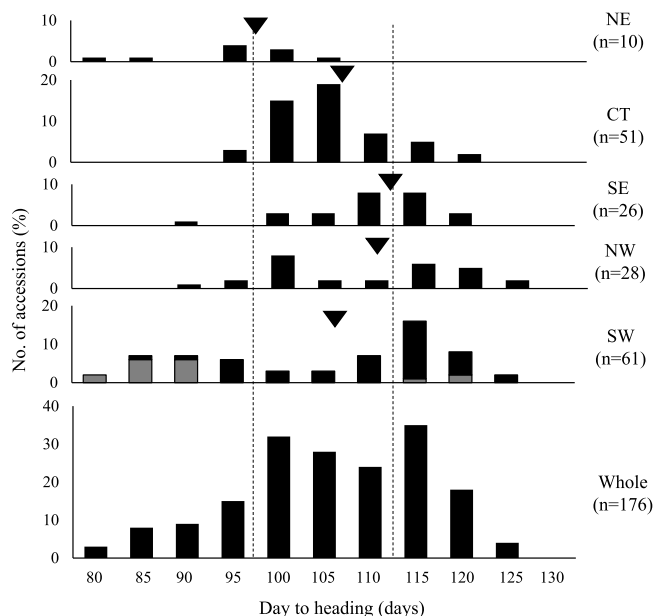
Fig. 2. Genetic variation of days to heading in each region of Cambodia. Investigation was carried out at Ishigaki, Okinawa, Japan in the second season from July to November 2017. A total of 176 accessions were investigated for heading date among whole materials. Black: Local accessions in Cambodia. Gray: Improved accessions introduced by IRRI and other foreign countries. Triangle: Mean.

Table 2. Relationship of rice accessions between cluster groups by polymorphism data of SSR markers and ecosystem for rice cultivation

Cluster group	Landrace or improved	No. of accessions (%)			
		Ecosystem			Total
		Irrigated lowland	Rainfed lowland	Upland	
Ia	Improved	1[a]	0	0	1
		(100.0)	(0.0)	(0.0)	(100.0)
Ib	Landrace	0	1	2	3
	Improved	0	0	1	1
	Sum	0	1	3	4
		(0.0)	(25.0)	(75.0)	(100.0)
IIa	Landrace	0	89[b]	6	95
	Improved	0	3	0	3
	Sum	0	92	6	98
		(0.0)	(93.9)	(6.1)	(100.0)
IIb	Landrace	0	65	0	65
	Improved	13	0	0	13
	Sum	13	65	0	78
		(16.7)	(83.3)	(0.0)	(100.0)
Total		14	158	9	181
		(7.7)	(87.3)	(5.0)	(100.0)

A total of 179 rice accessions form Cambodia and a Japonica Group rice cultivar, Nipponbare, and an Indica Group cultivar, Kasalath, were included.
[a] Nipponbare and [b] Kasalath were categorized into Ia and IIa, respectively.

landrace and introduced improved cultivars. Landrace accessions were included in all heading groups, and the improved accessions were included only in the early and late heading groups. The numbers of accessions were 1 in the early DTH group, 14 in the middle DTH group, and 13 in the late DTH group. These results indicate that the dominant rice accessions from the NE, CT, and SE regions varied from early heading in the NE region, medium heading in the CT region, and late heading in the SE region. In contrast, wide variations from early to late heading date were found in the other two regions, SW and NW. Thus, the distributions of DTHs varied among the five regions of Cambodia.

Genetic diversity among rice accessions based on polymorphism data of SSR markers

Based on polymorphism data of 64 SSR markers, a total of 252 alleles were detected among 181 rice accessions including a Japonica Group cultivar, Nipponbare, and an Indica Group cultivar, Kasalath; the mean number of alleles per locus was 3.9. The overall genetic diversity in 179 accessions was 0.48. The Polymorphic Information Content (PIC) ranged from 0.03 to 0.78 with a mean of 0.46 (**Supplemental Table 3**). The accessions were first classified into two cluster groups, I and II, and then classified again into sub-clusters, Ia and Ib and IIa and IIb (**Supplemental Fig. 1**, **Table 2**). Cluster Ia consisted only of the Japonica Group cultivar Nipponbare, and cluster IIa included the Indica Group cultivar Kasalath. These results suggest that

clusters I and II correspond to Japonica and Indica Groups, respectively. Cluster group Ib had four accessions: three from upland and one from rainfed lowland ecosystems. Cluster group IIa included 88 landrace accessions and three improved types (deep-water rice) from rainfed lowland ecosystems and six from upland ecosystems. Cluster group IIb consisted of 13 improved type accessions from irrigated lowland ecosystems and 65 landraces from rainfed lowland ecosystems. These results indicate that accessions of different types and from different ecosystems were included in each cluster group.

Geographic distribution of rice accessions in cluster groups based on polymorphism data of SSR markers

Accessions in the three cluster groups Ib, IIa, and IIb, classified on the basis of polymorphism data of SSR markers, were found in differing frequencies in the five regions (**Fig. 3**). In the NW and SW regions located around Tonle Sap Lake, accessions in all three of these cluster groups were found, but accessions in the other three regions, NE, CT, and SE along the Mekong River, were limited to one or two cluster groups. In the CT region, accessions fell into two cluster groups, IIa and IIb. The proportion of accessions falling into cluster group IIb was higher than those of IIa in CT and the whole of Cambodia. In the NE region, accessions were categorized into two cluster groups, Ib and IIa, and the dominant was cluster group IIa. In the SE
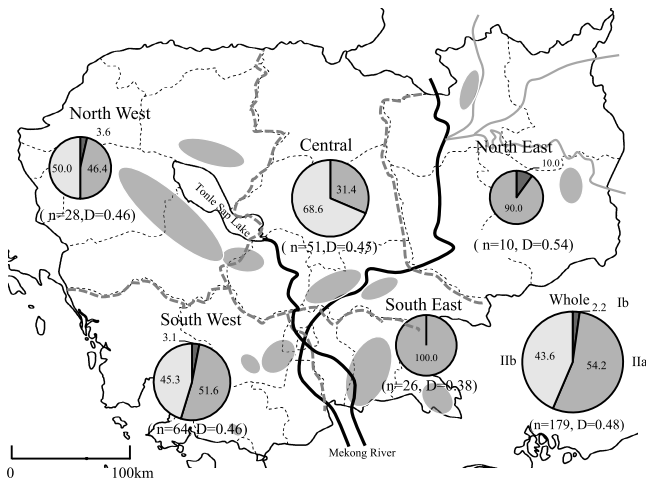
Fig. 3. Geographic distributions of rice accessions from Cambodia in each clusters group classified by polymorphism data of SSR markers. Grau region: Sits for collection of rice accessions.

region, accessions fell into only cluster group IIa. These results indicate that the distributions of rice accessions differed among the five regions in Cambodia, and there was a dramatic difference between three regions along Mekong River and the two regions around Tonle Sap Lake. Three regions, NE, NW, and SW, included Japonica Group accessions and were different from the other two regions, CT and SE. These results indicate that different types of accession are cultivated in each region according to its geographical conditions.

Genetic variation of heading date in each cluster group based on polymorphism data of SSR markers

The Japonica Group cultivar Nipponbare in cluster Ia showed early heading with a DTH value of 55 days (**Table 3**, **Supplemental Table 1**). DTH of four accessions in cluster Ib varied from 91 days to 100 days with a mean of 94.5 days, and these were categorized into the early and middle heading group in Japan. In cluster IIa, DTHs varied from 81 days to 128 days with a mean of 111.5 days. The accessions in cluster IIb varied from 81 days to 124 days with a mean of 103.9 days, and these were included in all three groups and the middle heading group was dominant. All improved cultivars with early heading were categorized into cluster group IIb. These results indicate that cluster groups Ia and Ib corresponding to the Japonica Group were early to middle heading. Cluster groups IIa and IIb corresponding to the Indica Group varied widely from early to late heading, and accessions in IIa were later than those in IIb.

Genetic variation of resistance

Based on their patterns of reaction to 16 SDBIs, the 176 accessions, 25 DVs, two susceptible controls (LTH and US-2), and the two control cultivars (Nipponbare and Kasalath) were classified into three clusters: A1, A2 and B

Table 3. Genetic variations of DTHs in each cluster groups classified by polymorphism data of SSR markers

Days to heading	No. of accessions (%)				
	Cluster group based on the polymorphic data of SSR markers				
	Ia	Ib	IIa	IIb	Total
−79	1[a]	0	0	0	1
80–84	0	0	1	2	3
85–89	0	0	2[b]	7	9
90–94	0	1	2	5	8
95–99	0	1	4	9	14
100–104	0	2	18	14	34
105–109	0	0	9	19	28
110–114	0	0	19	5	24
115–119	0	0	26	9	35
120–124	0	0	12	6	18
125–130	0	0	4	0	4
Total	1	4	97	76	178
	(0.6)	(2.3)	(54.5)	(42.7)	(100.0)

A total of 176 rice accessions from Cambodia were investigated at TARF, JIRCAS, Ishigaki Japan in the second season from July to November 2017.
Nipponbare and Kasalath were included in the categories, *a* and *b*, respectively.
DTHs of Nipponbare and Kasalath were 55 days and 85 days, respectively.

(**Fig. 4**, **Supplemental Fig. 2**, **Supplemental Table 1**). Cluster A1 consisted of 98 accessions (48.3% among whole accessions) and a DV, IRBL9-W, harboring high-resistance gene *Pi9*(t). Cluster A2 contained 92 accessions (44.9%), Kasalath, and 13 DVs for *Pib*, *Pia*, *Pit*, *Piz*, *Piz-5*, *Piz-t*, *Pita-2* (two lines), *Pi12*(t), *Pita* (three lines) and *Pi20*(t). Cluster B included the remained 15 control cultivars (7.3%) such as, Nipponbare, LTH, US-2, and 12 DVs for *Pish*, *Pii*, *Pi3*, *Pi5*(t), *Pik-s*, *Pik-m*, *Pi1*, *Pik-h*, *Pik*, *Pik-p*, *Pi7*(t) and *Pi19*(t), but included no accession from Cambodia. The mean infection scores of accessions were 1.3 in cluster A1, 2.1 in A2 and 3.4 in B, and 1.8 in the whole group. These results indicate that cluster A1 was categorized as having high resistance, A2 as having moderate resistance with specific reactions, and B as being susceptible; rice accessions from Cambodia were categorized only into the two clusters of high and moderate resistance.

The frequencies of clusters differed among the five regions. A total of 97 accessions (55.1%) were categorized into cluster A1 and 79 (44.9%) into cluster A2 (**Table 4**). And the frequencies in the NE and CT regions were not very different from the frequencies in the whole group of accessions. In the SE region, nine accessions (34.6%) were categorized into cluster A1 and 17 (65.4%) into A2; in the NW region there were 13 (46.4%) in A1 and 15 (53.6%) in A2. In contrast, in the SW region there were 41 (67.2%) in A1 and 20 (32.8%) in A2. These results indicate that the resistance of rice accessions in the SE, SW, and NW
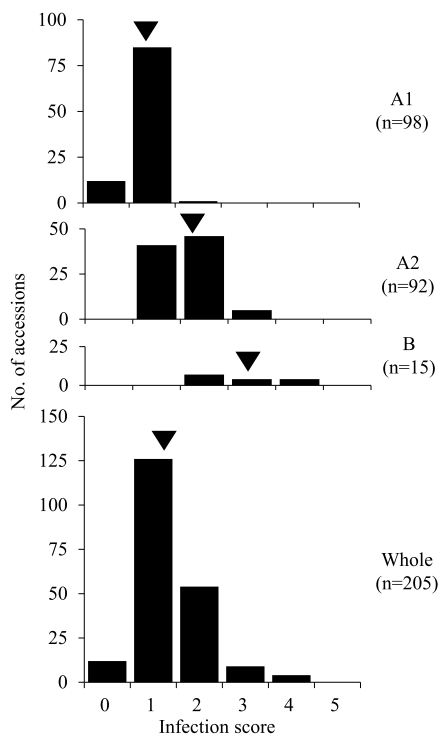
Fig. 4. Genetic variation for resistant of rice accessions in each cluster group classified based on reaction pattern of 16 standard differential blast isolates. A total of 179 rice accessions from Cambodia, 25 DVs for blast resistance genes, two susceptible controls, LTH and US-2, were included. The mean value of scores to 16 SDBLs was used as the representative data for resistant in each accession. Triangle: Mean.

Table 4. Distribution of cluster groups of rice accessions classified based on reactions to SDBIs in each region

Region	No. of accessions (%)			
	Blast resistant groups			Total
	A1	A2	B	
NE	5	5	0	10
	(50.0)	(50.0)	(0.0)	(100.0)
CT	29	22	0	51
	(56.9)	(43.1)	(0.0)	(100.0)
SE	9	17	0	26
	(34.6)	(65.4)	(0.0)	(100.0)
SW	41	20	0	61
	(67.2)	(32.8)	(0.0)	(100.0)
NW	13	15	0	28
	(46.4)	(53.6)	(0.0)	(100.0)
Sum	97	79	0	176
	(55.1)	(44.9)	(0.0)	(100.0)
Other	1[a]	13[b]	15[c]	30
	(3.3)	(46.7)	(50.0)	(100.0)
Total	98	92	15	205
	(48.3)	(44.9)	(7.3)	(100.0)

A total of 176 accessions in Cambodia, 25 DVs, two susceptible controls, LTH and US-2 and Nipponbare and Kasalath as Japonica and Indica Groups' cultivars, respectively, were included.
[a] DV, IRBL9-W.
[b] Kasalath and 12 DVs, IRBLb-B, IRBLt-K59, IRBLa-A, IRBLz-Fu, IRBLz5-CA-1, IRBLzt-T, IRBLta-CP1, IRBLta-K1[LT], IRBL12-M, IRBLta2-Pi, IRBLta2-Re and IRBL20-IR24.
[c] Nippobare, LTH, US-2 and 12 DVs, IRBLsh-B, IRBLi-F5, IRBL3-CP4, IRBL5-M[LT], IRBLks-F5, IRBLkp-K60, IRBL1-CL[LT], IRBLkm-Ts, IRBLk-K, IRBLkh-k3[LT], IRBL7-M and IRBL19-A.

Table 5. Relationships between cluster groups classified by reaction to SDBIs and by polymorphism data of SSR markers

Cluster groups by polymorphism data of SSR markers	No. of accession (%)			
	Cluster groups by blast resistance			Total
	A1	A2	B	
Ia	0	0	1[a]	1
	(0.0)	(0.0)	(100.0)	(100.0)
Ib	3	0	0	3
	(100.0)	(0.0)	(0.0)	(100.0)
IIa	47	50[b]	0	97
	(48.5)	(51.5)	(0.0)	(100.0)
IIb	47	30	0	77
	(61.0)	(39.0)	(0.0)	(100.0)
Total	97	80	1	178
	(54.5)	(44.9)	(0.6)	(100.0)

[a] Nipponbare and [b] Kasalath were included in the categories, Ia-B and IIa-A1, respectively.

regions was remarkably different from each other and from the other two regions.

Relationships between clusters of resistance and cluster groups by polymorphism data

The Japonica Group cultivar Nipponbare of cluster Ia was classified into group B (**Table 5**). Among four accessions in cluster Ib, only three were evaluated for resistances, and these three were all categorized into group A1. In cluster IIa, 47 (48.5%) accessions were categorized into group A1 and 50 (51.5%) were categorized into group A2, and the frequency in group A1 was lower and that in group A2 was higher than the frequencies in these groups for the whole group of accessions. In cluster group IIb, in contrast, 47 (61.0%) and 30 (39.0%) accessions were categorized into A1 and A2, respectively, and the frequencies of A1 and A2 were the opposite from those of IIa. These results indicate that the accessions in clusters Ib, IIa, and IIb had different variations of blast resistance.

Discussion

Based on polymorphism data of SSR markers, the mean number of alleles per locus was 3.9 and the overall genetic diversity in the 179 Cambodian accessions (excluding

Nipponbare and Kasalath) was 0.48. Hamada (1965) collected 276 rice accessions in Cambodia from 1957 to 1958; Orn *et al.* (2014) investigated these using 12 SSR markers and found their genetic diversity to be 0.55. Khan *et al.* (2017) investigated 334 accessions from Bangladesh using

74 SSR markers and showed the genetic diversity to be 0.49, and number of alleles per locus to be 3.0. Odjo *et al.* (2017) reported that the mean number of alleles per locus was 1.6 among 195 accessions from West Africa investigated using 61 SSR markers. Wunna *et al.* (2016) reported high genetic diversity of 0.84 and high numbers of alleles per locus of 6.4 in 175 accessions from Myanmar examined using 67 SSR markers. These results indicate that the genetic variation in Cambodian rice germplasm is similar to that of Bangladesh, higher than of West Africa, and lower than of Myanmar. Rice breeding in Cambodia has been carried out using local cultivars, and cultivation of improved cultivars from the International Rice Research Institute (IRRI) and other Asian countries is still limited. The civil war that occurred in Cambodia from 1970 to 1979 severely damaged the national agricultural systems, and much rice germplasm was lost during that time (Javier 1997). It means that the genetic diversity in rice cultivars might have been reduced by the war.

Rice accessions in Cambodia classified on the basis of polymorphism data of SSR markers fell into four cluster groups, Ia, Ib, IIa, and IIb (**Fig. 3**), with cluster groups Ia, and Ib corresponding to the Japonica Group and groups IIa and IIb corresponding to the Indica Group. Rice accessions of clusters Ib, IIa, and IIb occurred in differing frequencies in the five regions of Cambodia (**Fig. 4**). Upland accessions were cultivated in the limited areas of forest (mountainous area) of the NW, SW, and NE regions, and these were categorized into the Japonica Group. Accessions from rainfed lowland areas were classified into cluster groups IIa or IIb (Indica Group). The frequencies of clusters IIa and IIb in the whole set of accessions were 54.2% and 43.6%, respectively, and the frequencies in the NW and SW regions were quite similar. In the CT region, the frequency of cluster IIb was higher than that of cluster group IIa and that of the whole set of accessions. Cluster IIa was dominant in two regions, NE and SE. These results indicate that cluster groups IIa and IIb were distributed together in the three regions NW, SW, and CT surrounding Tonle Sap Lake. In contrast, the other two regions, NE and SE along the Mekong River, were limited to accessions of cluster IIa. Thus, the geographical variations of cluster groups demonstrate well the different situations of rice cultivation between areas surrounding Tonal Sap Lake and areas along the Mekong River, and upland cultivars were used in limited areas as a minor rice.

Rice accessions were categorized at CARDI, Phnom Penh, Cambodia into three heading types, early-heading (DTH <90 days), middle-heading (91–120 days), and late-heading (>120 days) (Javier 1997). The accessions from Cambodia were investigated at Ishigaki, Okinawa, Japan, under conditions of changing day lengths from long to short days (13.5–11.2 h) in the second season from July to November 2017. The distribution of DTHs at Ishigaki was divided into three groups: early (80–95 days), middle (96–115 days), and late (116–130 days) (**Fig. 1**). The mean

DTHs of the three groups at Ishigaki corresponded with the classifications at Phnom Penh, but there were many exceptions. The day lengths at Phnom Penh vary in the range from 12.5 h to 11.7 h during wet season, and rice is cultivated basically under short-day-length conditions. In Ishigaki, accessions were cultivated under slightly longer day lengths (around 13.6 h per day) from July to August, in the early rice growing stages. The longer day lengths at Ishigaki might account for the wider variations and differences from those at Phnom Penh. In the other words, the condition of second season at Ishigaki make possible to observe the detail and unique variation for heading date in rice. These findings indicate that various and different genetic factors for heading date were included in each heading type classified at CARDI, and these genetic mechanisms were complex. These variations of DTHs differed among the five regions in Cambodia (**Fig. 2**). In the SW and NW regions, accessions of all three heading groups were found in the Ishigaki environment. However, among accessions from the other three regions, heading groups were limited and the dominant heading groups varied from early in the NE region, to medium in the CT region, to late in the SE region in the Ishigaki environment. The main topographies also differ among the three regions. The NE region is mainly mountainous and hilly, SE is lowland, and CT is a combination of hilly and lowland areas. In the SW region, improved cultivars from foreign counties have been introduced, and these were classified into both early and late heading groups. These results indicate that various rice cultivars are cultivated in the two regions SW and NW around Tonle Sap Lake, and that variations in heading in the CT region and the two regions NE and SE along the Mekong River were limited according to topographical conditions. Japonica Group upland accessions of cluster Ib were limited to early and middle heading groups, and the Indica Group deep-water and rainfed lowland accessions in cluster IIa and IIb varied widely from early to late heading. Accessions in cluster IIa, which included many deep-water rice cultivars, showed a tendency of late heading compared with those in cluster IIb. Javier (1997) indicated that genetic variation among Cambodian cultivars is mainly influenced by topography, rainfall, and soil type, according to which rice needs to adjust its flowering time to survive in critical environments. Our results demonstrated well that the genetic variations in heading date of rice accessions in Cambodia corresponded to rice cultivating systems and to differentiation between Japonica and Indica Groups.

A total of 176 rice accessions from Cambodia, 25 DVs, two susceptible controls (LTH and US-2), and two control cultivars (Nipponbare for Japonica Group and Kasalath for Indica Group) were classified into three clusters, A1, A2, and B, based on patterns of reaction to 16 SDBIs; accessions from Cambodia were classified into only clusters A1 and A2 (**Fig. 4**, **Supplemental Fig. 2**). A total of 97 accessions plus the DV for *Pi9*(t) were classified into cluster A1, and 79 accessions plus the 13 DVs for *Pib*, *Pia*, *Pit*, *Piz*,

Piz-5, *Piz-t*, *Pita-2* (two lines), *Pi12*(t), *Pita* (three lines), and *Pi20*(t) were classified into cluster A2. The other 12 DVs for *Pish*, *Pii*, *Pi3*, *Pi5*(t), *Pik-s*, *Pik-m*, *Pi1*, *Pik-h*, *Pik*, *Pik-p*, *Pi7*(t), and *Pi19*(t), LTH, US-2, Kasalath, and Nipponbare were categorized into cluster B. Cluster A1 was characterized as high resistance and cluster A2 as medium resistance. Cluster B was characterized as susceptible and included no accessions from Cambodia. These results indicate that all accessions from Cambodia had high and medium resistance to blast. The geographical distributions of the rice accessions in clusters A1 and A2 were also investigated, and differences of frequencies between them were found in SE, SW, and NW regions (**Table 4**). The frequencies of accessions in cluster A1 were lower in the SE and NW regions than were frequencies of accessions in cluster A2 and the mean for the whole set of accessions. In contrast, the frequency of accessions in cluster A1 was higher than in cluster A2 in the SW region. These results indicate that genetic variations of blast resistance in rice cultivars were found, and especially unique differences occurred in the western and southern regions of Cambodia. In other words, blast resistance among rice accessions from Cambodia was strongly associated with ecotypes which were determined by topographical condition.

Thus, we could clarify the genetic variations of rice germplasm in Cambodia. Rice cultivation in Cambodia is basically divided into two areas: the area surrounding Tonle Sap Lake including NW and SW, and the areas along the Mekong River including the CT, NE and SE regions. In the NW and SW regions, rice is cultivated on rainfed lowlands using changes of water level in Tonle Sap Lake. The genetic variations of rice accessions in Cambodia corresponded with the geographical differentiations.

This is the first study to elucidate the genetic variations of Cambodian rice germplasm based on the analyses of rice genome chromosome components using molecular markers, heading dates comparing two sites in Cambodia and Japan, and blast resistance by inoculation test, considering the relationships with geographical regions and ecosystems for rice cultivation. This information will be useful for the development of breeding strategies for blast resistance in Cambodia and for utilizing germplasm materials more effectively.

Author Contribution Statement

YF and CO conceived of the study, designed the experiments, and wrote the manuscript. TV, SK and OM selected the rice accessions and collected the data for heading date in Cambodia. MAIK and MRN performed some parts of DNA works and inoculation test for blast resistance. HS edited and revised the manuscript.

Acknowledgments

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