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Research Paper

Assessment of genetic diversity and genetic structure of wild rice populations in Myanmar

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To evaluate genetic diversity and genetic structure of wild rice (*Oryza rufipogon*) populations in Myanmar, seven research sites were selected based on various ecological conditions. A large number of samples under natural growth conditions were collected and studied using six simple sequence repeats (SSRs) and two chloroplast DNA markers. A total of 77 alleles were detected from 1559 samples over six SSR loci. The mean number of alleles per population ranged from 3.167 to 8.667, and the mean expected heterozygosity ranged from 0.140 to 0.701. Wild rice populations survived under various environmental conditions and retained different levels of genetic diversity. The large number of samples was effective to confirm the spatial genetic structure of wild rice populations in a relatively small area. Regarding chloroplast DNA polymorphisms, four populations possessed only one pattern, while the other three showed two or five combinations of haplotypes, even within the same population. Additionally, the existence of a new genotype was revealed. Considerable variations in chloroplast DNA exist in the wild rice populations of Myanmar. A high proportion of genetic variation was detected within, rather than among, populations. To ensure maintenance of allelic diversity, it is advisable to preserve many individuals from a large population.

Key Words: wild rice (*O. rufipogon*), genetic diversity, genetic structure, SSR marker, chloroplast DNA marker, Myanmar.

Introduction

The common wild rice, *Oryza rufipogon* Griff., is the ancestor of Asian cultivated rice (*O. sativa* L.), and has been recognized as an important reservoir of agronomically useful genes to counter biotic and abiotic stresses and for sources of cytoplasmic male sterility (Vaughan 1994). Several quantitative trait locus (QTL) studies revealed the genetic potential of *O. rufipogon* for the improvement of yield-related traits. Alleles originating from the low-yielding *O. rufipogon* had shown beneficial effects in different elite cultivars of *O. sativa* (Fu *et al.* 2010, Thomson *et al.* 2003, Xiao *et al.* 1998, Xie *et al.* 2008). Furthermore, Thanh *et al.* (2010) detected wild rice alleles that contributed to early spikelet opening time. This trait will be useful for changing cultivar flowering time to avoid pollen sterility at high temperatures. Neelam *et al.* (2017) reported novel alleles for enhanced phosphorus uptake efficiency from three accessions of *O. rufipogon*. Transferring these wild rice alleles to elite cultivars can allow crop production on poor and problematic soils. *O. rufipogon* probably possesses many unknown genes that will be useful to improve the traits of cultivars as needs arise in the future.

The present distribution range of *O. rufipogon*, however, is considerably decreased and fragmented, and the existing populations are threatened in their natural habitats because of the modernization of agriculture, rapid urbanization, and accelerated development of greenfield sites (Akimoto *et al.* 1999, Vaughan 1994, Zhao *et al.* 2013). Myanmar has been recognized as a central part of rice germplasm, and extraordinary genetic diversity has been conserved not only in rice landraces but also in related wild species. However, these rice genetic resources are decreasing due to the disruption

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of their natural environments by various human activities.

Conservation efforts and related studies of *O. rufipogon* have been intensively conducted in China. The genetic structures of *in situ* conserved and reintroduced *O. rufipogon* populations with different status were analyzed using DNA markers and the genetic consequences and efficiency of conservation practices were evaluated based on the results obtained from the analyses (Qian *et al.* 2005, Song *et al.* 2003, 2005). However, few studies on the genetic condition of the existing wild rice population in Myanmar have been reported (Shishido *et al.* 2006, Uga *et al.* 2005, 2006). It is urgent for us to comprehend the genetic structures of existing natural populations and to put this information to practical use for the conservation of indigenous genetic resources.

Thus, in the current study, a large number of DNA samples in extant wild rice populations in their natural habitats were collected in Myanmar, and their genetic diversity and population genetic structure were evaluated using six simple sequence repeat (SSR) and two chloroplast DNA markers. A conservation management strategy for *O. rufipogon* populations based on this genetic information is also discussed.

Fig. 1. Seven sampling locations of *Oryza rufipogon* populations from Myanmar.

Materials and Methods

Research sites and plant samples

A total of seven research sites were established in Myanmar, four in the western zone (AK1, AK18, AK29, and AK30) and three in the southern zone (PT1, YG23, and YG36) (**Fig. 1**). We distinguished two ecotypes on the basis of several characteristics. Perennial plants were characterized by short awns, long anthers, tall stature, and well-developed roots, often with stolons. In contrast, annual plants were characterized by long awns, short anthers, and short stature. At the AK1 site, perennial plants were observed along a roadside irrigation ditch of approximately 400 m in length (**Supplemental Fig. 1**). At AK18, typical annual plants occurred for approximately 200 m along the edge of the paddy field, growing in a narrow space between the paddy field and the roadway (**Supplemental Fig. 2**). At AK29, both annual and perennial plants were observed along the paddy field edges at both sides of the road (**Supplemental Fig. 3**). This is a rare situation because the perennial and annual populations of Asian common wild rice are usually allopatric (Oka 1988). In addition to typical forms, we observed individuals that could not be distinguished between annual and perennial. To evaluate the population as a first step, we did not analyze the annual and perennial separately in order to avoid sampling complications. At AK30, perennial plants were distributed along the irrigation ditch (**Supplemental Fig. 4**). At PT1, perennial plants were growing along the paddy ditch in the open wetland (**Supplemental Fig. 5**). These plants had a deep-water habitat with a floating ability. At YG23, perennial plants were observed in the swamp along the road, where small paths divided the population into seven patches (**Supplemental Fig. 6**). At YG36, annual plants were growing along the roadside swamp (**Supplemental Fig. 7**). Perennial plants inhabited areas with year-round water, whereas annual plants occurred in ponds and swamps that dried up completely in the dry season. The environmental and geographical information on these research sites is shown in **Table 1**.

At each site, wild rice leaves were collected from different plants at 1–4 m intervals according to the population

Table 1. Collection sites of wild rice populations used

Site	Ecotype	Samples	Habitat	Latitude	Longitude	State
Western zone						
AK1	Perennial	150	Road-side ditch	20°08′N	92°52′E	Rakhine
AK18	Annual	200	Paddy side	20°15′N	92°49′E	Rakhine
AK29	Annual + Perennial	240	Paddy side	20°13′N	92°49′E	Rakhine
AK30	Perennial	130	Road-side ditch	20°09′N	92°51′E	Rakhine
Southern zone						
PT1	Perennial	180	Open wetland	17°03′N	95°35′E	Ayeyarwady
YG23	Perennial	320	Road-side swamp	17°08′N	96°17′E	Yangon
YG36	Annual	360	Road-side swamp	17°36′N	95°47′E	Bago

size (**Supplemental Figs. 1–7**). In total, 1580 samples were collected in Myanmar. The number of samples ranged from 130 (AK30) to 360 (YG36), with an average of 225.7 (**Table 1**). Leaves from each plant were placed on an FTA® card (GE Healthcare, Buckinghamshire, UK), covered with a small piece of plastic wrap, and then squashed with a wooden hammer. The leaf extracts were fixed on the card by air-drying. Small discs (1.2 mm in diameter) from the cards were subjected to PCR amplification, as indicated below.

Molecular analysis of SSR and chloroplast DNA markers by PCR

Six SSR markers (RM29, RM31, RM60, RM201, RM208, and RM237) and two chloroplast DNA markers were used in this study. The SSR markers were developed by Chen *et al.* (1997). Chloroplast DNA markers were previously reported to detect polymorphisms between *japonica* and *indica* cultivars; most *indica* cultivars have a 69-bp deletion and 32-bp insertion in the ORF100 and ORF29-*trn*C regions, respectively (Kanno *et al.* 1993, Tang *et al.* 2004). The six SSR markers are useful for the investigation of genetic diversity in wild rice populations, producing polymorphic allele amplification and showing clear band patterns without background noise on silver-stained polyacrylamide gels. The two chloroplast markers were employed to identify chloroplast DNA haplotypes easily. In addition to the abovementioned advantages, we chose these markers to allow our research results regarding genetic diversity to be compared with those obtained from wild rice growing in other countries. Amplification reactions were performed in a final volume of 10 µl containing 0.5 µM of primers, 0.25 units of *Taq* DNA polymerase (BIOTAQ™ HS DNA Polymerase; Bioline, London, UK), 5 µl of 2× Ampdirect Plus (Shimadzu, Kyoto, JPN), and an FTA® small disc for the template DNA. The cycling conditions for the PCR amplification consisted of 95°C for 10 min, followed by 35 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min, with 7 min at 72°C as the final extension. The PCR products of the SSR markers were electrophoresed on 4% polyacrylamide denaturing gels. The banding patterns were visualized using the silver staining procedure described by Panaud *et al.* (1996). The size of the most intensely amplified product was determined for each SSR marker, using a 10-bp DNA ladder (Invitrogen, Paisley, UK) as a reference. In contrast, the PCR products of the chloroplast markers were analyzed by agarose gel electrophoresis. For both SSR and chloroplast markers, alleles were defined on the basis of fragment size.

Sequencing of chloroplast DNA

In the ORF29-*trnC* region, the amplified fragments were further analyzed by sequencing. Ultrafree®-DA Centrifugal Filter Units (Merck Millipore, Carrigtwohill, Ireland), each containing a Gel Nebulizer, Ultrafree®-MC microcentrifuge filter (0.45 µm Durapore), and microcentrifuge filtrate vial, were used to extrude DNA rapidly from the agarose gel matrix. These purified PCR fragments were used as templates, and sequencing reactions were performed using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). Data were collected using the Applied Biosystems 3130xl Genetic Analyzer. The sequences reported in the current study were aligned using the ClustalW2.1 program (Larkin *et al.* 2007).

Statistical analysis

Parameters of genetic diversity, mean number of alleles (*Na*), mean number of effective alleles (*Ne*), observed heterozygosity (*Ho*), expected heterozygosity (*He*), Shannon's information index (*I*), and fixation index (*F*) were calculated using GenAlEx 6 software (Peakall and Smouse 2006). The genetic diversity for all populations (H_T), genetic diversity within subpopulations (H_S), gene diversity among subpopulations (D_{ST}), and coefficient of gene differentiation (G_{ST}) were estimated (Nei 1973). These values were calculated using FSTAT 2.9.3 software (Goudet 2001).

Relationships among the *O. rufipogon* populations were estimated from the SSR data using unweighted pair group method with arithmetic mean (UPGMA) clustering on the basis of Da genetic distance (Nei *et al.* 1983). The UPGMA tree was constructed using the Populations 1.2.30beta program (Langella 2007) and subsequently visualized with TreeExplorer software (Tamura 1999).

Results

Genetic diversity within populations

Since eight AK1 samples and 13 YG36 samples did not give good PCR products, they were excluded from the analysis. As a result, a total of 77 alleles were detected from 1559 samples over six SSR loci. The highest number of alleles was scored from the locus RM237 (19 alleles) and the lowest was scored from the locus RM60 (8 alleles). The mean number of alleles (*Na*), mean number of effective alleles (*Ne*), observed heterozygosity (*Ho*), expected heterozygosity (*He*), Shannon's index (*I*), and fixation index (*F*) were used to demonstrate the level of population genetic diversity and are listed in **Table 2**. The *Na* and *Ne* ranged from 3.167 (AK18) to 8.667 (PT1) and from 1.253 (AK18) to 3.639 (PT1), respectively. The mean *Ho* and *He* ranged from 0.059 (AK18) to 0.539 (PT1) and from 0.140 (AK18) to 0.701 (PT1), respectively. The value of *F* ranged from 0.027 (YG23) to 0.892 (YG36). The highest genetic diversity was scored in PT1. The average *Ho* of the annual populations (0.063) was considerably lower than that of the perennial populations (0.444). On an average, perennial populations showed higher levels of genetic diversity compared to annual populations. Except for AK18, the populations included in this study showed a relatively high level of genetic diversity with *Na* = 5.452, *Ne* = 2.443, *He* = 0.496, and *I* = 0.962.

Table 2. Summary of genetic diversity in seven *Oryza rufipogon* populations revealed by SSRs

Site	*Na*	*Ne*	*Ho*	*He*	*I*	*F*
AK1	4.500	1.971	0.238	0.415	0.801	0.399
AK18	3.167	1.253	0.059	0.140	0.274	0.263
AK29	5.167	2.311	0.419	0.538	0.947	0.281
AK30	5.000	2.651	0.496	0.550	1.027	0.134
PT1	8.667	3.639	0.539	0.701	1.531	0.252
YG23	5.500	2.028	0.504	0.476	0.863	0.027
YG36	6.167	3.244	0.067	0.653	1.295	0.892
Western zone	4.458	2.047	0.303	0.411	0.762	0.269
Southern zone	6.778	2.970	0.370	0.610	1.230	0.390
Annual	4.667	2.249	0.063	0.397	0.784	0.578
Perennial	5.917	2.572	0.444	0.536	1.056	0.203
Overall	5.452	2.443	0.332	0.496	0.962	0.321

Na, mean number of alleles; *Ne*, mean number of effective alleles; *Ho*, observed heterozygosity; *He*, expected heterozygosity; *I*, Shannon's Information index; *F*, fixation index; SSRs, simple sequence repeats. Annual populations, AK18 and YG36; Perennial populations, AK1, AK30, PT1, and YG23.

Genetic differentiation among populations

In order to examine the geographical differences among wild rice populations, coefficient of gene differentiation (G_{ST}) values were calculated for both western and southern wild rice populations (**Table 3**). The overall genetic diversity (H_T) of the southern populations (0.745) showed approximately the same value as that of the western populations (0.722). The genetic diversity within subpopulations (H_S) of the southern zone (0.612) was higher than that of the western zone (0.412). Consequently, the G_{ST} for the southern populations (0.179) was lower than that for the western populations (0.429).

In addition to geographical differentiation, ecotypic differentiation was investigated. G_{ST} values were calculated for both annual and perennial populations (**Table 3**). The AK29 data were excluded from the calculation because annual and perennial types coexisted in the population. The overall genetic diversity (H_T) of the annual populations (0.682) showed roughly the same value as that of the perennial populations (0.719). The genetic diversity within subpopulations (H_S) of the perennial populations (0.537) was greater than that of the annual populations (0.398). As a result, the G_{ST} for the perennial populations (0.252) was lower than that for the annual populations (0.416).

The UPGMA tree for the seven populations revealed two distinct groups (**Fig. 2**). The AK18 population showed considerable differentiation from the other populations. The two perennial populations from the western zone (AK1 and AK30) were clustered in one group, with relatively low differentiation between them, whereas the two perennial populations from the southern zone (YG23 and PT1) were clustered in another group, showing relatively high differentiation between the populations.

Table 3. Partitioning of SSR diversity for subpopulations, classified according to their location and ecotype

Population	No. sites	H_T	H_S	D_{ST}	G_{ST}
Western zone	4	0.722	0.412	0.309	0.429
Southern zone	3	0.745	0.612	0.133	0.179
Annual	2	0.682	0.398	0.284	0.416
Perennial	4	0.719	0.537	0.181	0.252
Overall	7	0.805	0.498	0.307	0.381

H_T, genetic diversity for all populations; H_S, genetic diversity within subpopulations; D_{ST}, gene diversity among subpopulations (H_T-H_S); G_{ST}, Coefficient of gene differentiation (D_{ST}/H_T) (Nei 1973); SSR, simple sequence repeat.

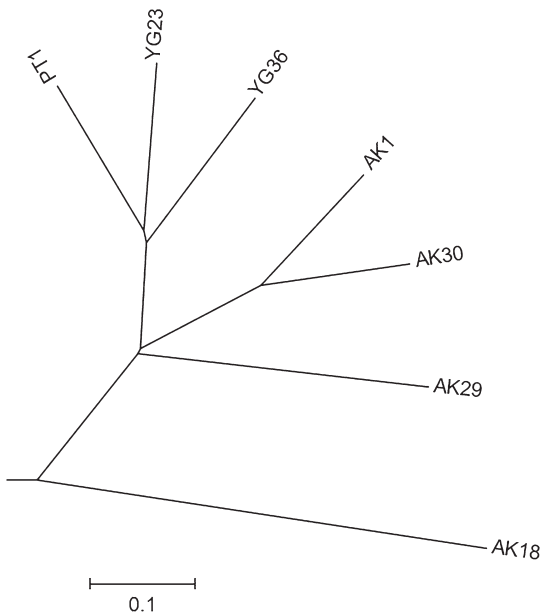

Fig. 2. An unweighted pair group method with arithmetic mean (UPGMA) tree for the seven wild rice populations based on Nei's genetic distances.

Chloroplast DNA polymorphism

Six PT1 samples did not give good PCR products of the ORF29-*trn*C region. Among seven wild rice populations, two and three band patterns were detected for the ORF100 and ORF29-*trn*C regions, respectively. They were classified into genotypes A, B, and C, representing markers A (original genotype, observed in *japonica* cultivars and distantly-related species), B (*indica*-specific genotype, mainly found among *indica* cultivars), and C (newly observed genotype in this study).

Five combinations of maker haplotypes were observed for ORF100/ORF29-*trn*C among the wild rice populations: A/A, A/B, B/A, B/B, and B/C (**Table 4**). In the annual populations (AK18 and YG36), the haplotypes of ORF100 and ORF29-*trn*C were fixed as the original genotype, A/A. In the western perennial populations (AK1 and AK30), they were fixed as the original and *indica*-specific genotypes, A/B. Two combinations of maker haplotypes, A/A plus A/B, and A/B plus B/B, were detected in AK29 and YG23,

Table 4. Number of plants having different chloroplast DNA (ORF100 and ORF29-*trnC*) haplotypes found among seven wild rice populations in Myanmar

Population	Haplotype (ORF100/ORF29-*trnC*)				
	A/A	A/B	B/A	B/B	B/C
AK1	0	142	0	0	0
AK18	200	0	0	0	0
AK29	6	234	0	0	0
AK30	0	130	0	0	0
PT1	8	40	1	96	29
YG23	0	201	0	119	0
YG36	347	0	0	0	0
Total	561	747	1	215	29

A, original genotype; B, *indica*-specific genotype; C, new genotype.

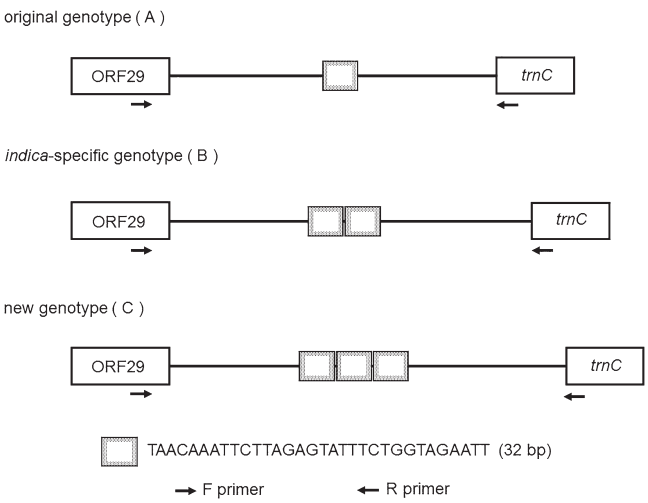

Fig. 3. Genotypic variation in the ORF29-*trn*C region. The 32-bp repeat regions are shown, along with the positions of the forward (F) and reverse (R) primers used in PCR genotyping.

respectively. In AK29, the minor haplotype A/A was not found in a specific area, and in YG23, one side of the road exclusively contained the B/B haplotype, while the other side held a mixture of both A/B and B/B (**Supplemental Fig. 6**). All five combinations of maker haplotypes were found in PT1, and there was no deviation of the haplotype distribution.

Regarding the ORF29-*trn*C spacer region, larger fragments were observed compared with those of the B genotype (32-bp insertion). Such a pattern was regarded as a new genotype (C). Regarding the newly observed banding pattern of the ORF29-*trnC* region, sequence analysis of the target fragment revealed that the length mutation was caused by a tandem repeat of this 32-bp sequence (**Fig. 3**).

Discussion

Genetic variation observed in seven O. rufipogon populations

Some samples could not be amplified, most likely because of the age of the leaves collected and the subsequent ability to fix their DNA onto the FTA cards. However, using SSR markers, distinctive genetic structures of seven *O. rufipogon* populations were revealed as follows.

At the AK1 site, the low level of genetic diversity was maintained by vegetative reproduction. In addition to a small number of alleles, the grazing disturbance by animals seems to have suppressed cross-pollination.

At AK18, most individuals were found to share some fixed homozygous genotype. Their seed propagation through self-pollination with a small number of alleles was the main factor that preserved the low genetic variation within the population.

At AK29, compared with other populations in the western zone, a few more alleles were detected and moderate genetic diversity was sustained.

At AK30, the relatively high genetic diversity was maintained by vegetative reproduction. Since few panicles were observed due to frequent grazing of animals, it is thought that the exchange of pollen happened in the distant past.

At PT1, the high level of genetic variation could be maintained by clonal propagation of various genotypes under stable environmental conditions. Wild rice widely grew without disturbance, including animal grazing and human cutting.

At YG23, the genotype distribution varied among the patches. In this site, the local people sometimes removed all vegetation, including the wild rice, to make small fish ponds. Heterozygote genotypes may have contributed to plant adaptations to the unstable environmental conditions (Kuroda *et al.* 2005).

At YG36, plants having various genotypes self-pollinated and produced seeds. Numerous seed-derived progenies maintained the genetic diversity of the population.

In the present study, we investigated four perennial and two annual populations. With regard to the two annual populations, the number of samples collected from and the degree of genetic diversity in each population varied considerably. Nevertheless, we examined the differences between genetic structures of the annual and perennial populations on the basis of their mean values. The average *Ho* of the annual populations was small (0.063), indicating that most of the individuals were homozygous. In contrast, the average *Ho* of the perennial populations was higher (0.444), indicating that the population had more heterozygous individuals. However, the average *He* (0.397) and *Ne* (2.249) for the annual populations were slightly lower than those for the perennial populations. This indicated that the annual populations consisted of individuals with various homozygous genotypes and they maintained a moderate genetic diversity.

While the two perennial populations from the western zone and the southern zone showed genetic similarity within each zone, the ecotypic factors seemed to have a greater influence on genetic differentiation among wild rice populations.

Chloroplast DNA variation

In previous studies, annual and perennial strains were reported to be readily discernible based on the presence of a deletion in the ORF100 region. The annuals tended to possess the deletion, whereas most of the perennials did not (Chen *et al.* 1993, Yamanaka *et al.* 2003). However, in two typical annual populations in the present study, AK18 and YG36, there were no plants that showed this deletion in the ORF100 region (**Table 4**). In addition, coexistence of the two genotypes (i.e., deletion and non-deletion) was observed even in the same population (YG23 and PT1). This suggests that the cytoplasmic variation is common in wild rice populations under natural conditions.

Orn *et al.* (2015) evaluated chloroplast genome variations among wild rice populations in Cambodia. They also detected variations in the ORF100 and ORF29-*trn*C regions, and the A/A, A/B, and B/B variants (i.e., ORF100/ORF29-*trn*C haplotype) were observed in five natural populations. Regarding the Cambodian wild rice plants, nearly 90% were B/B. In the case of the Myanmar plants, A/A, A/B, B/A, B/B, and B/C were observed in seven natural populations, and the most prevalent variants were A/B (48.1%), A/A (36.1%), and B/B (13.8%). To the best of our knowledge, genotype 'C' of the ORF29-*trn*C region had not been found to date, making it a newly identified, Myanmar-specific genotype. These results indicate that considerable variation in chloroplast DNA occurred in the Myanmar wild rice populations.

Implications for conservation of O. rufipogon populations

Usually, to evaluate the genetic structure of wild rice populations, a survey is conducted on 20–30 plants (Gao *et al.* 2002, Gao and Zhang 2005, Zhou *et al.* 2003). In the current study, a large number of DNA samples per population were collected, and the distribution of genotypes within the population was fully revealed. For conservation of endangered species, it is important to consider the distribution of unique genotypes.

YG23 consisted of seven patches (**Supplemental Fig. 6**). In this area, both SSR genotypes and chloroplast haplotypes were distributed differently among the patches. Neighboring patches tended to have similar genotypes; however, the population structure was considerably different between patch groups on different sides of the road. The large number of samples made it possible to detect such spatial genetic structure at a single site. In this case, at least two patch groups must be considered to preserve a sufficient amount of the genetic variability.

Both AK18 and YG36 were annual populations, and annual plants tend to engage in autogamy and reproduce by means of seeds. A successive self-pollination system does not produce new genotypes and drives their genotype to be homozygous. However, the degrees of genetic diversity at the two sites were quite different (**Table 2**), and their differences could be caused by the population sizes. Continuous habitats, such as YG36, enable the population to maintain various alleles. In contrast, self-pollination in a small population, such as AK18, does not give rise to high genetic variation because of the limited number of alleles. AK18 has potential genetic risks associated with its low level of genetic diversity, and it may have difficulty adapting to environmental changes in order to survive. Restoration seed materials from AK18 should be obtained and archived to protect against this potential extinction crisis.

In Myanmar, a high proportion of genetic variation is distributed within rather than among populations. Based on information regarding the population genetic structure, a conservation strategy should be considered. To ensure maintenance of allelic diversity, preserving more individuals from a large population is advisable. It is recommended to keep the currently studied populations *in situ*, except for AK18. Regarding the perennial populations, a distinct correlation between their genetic distance and geographical location was shown. In ancient times, AK1 and AK30 might have been a huge continuous habitat. Long-term research of the regional genetic structure of remnant *O. rufipogon* populations to assess both spatial and temporal changes will be essential for the future.

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