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Population structural analysis of an *in-situ* conservation site for wild rice in Laos

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An *in-situ* conservation site in Laos for a mixture of annual and perennial wild rice, LV27, which is a single swamp with an observation pier has been developed. In order to develop a strategy for evaluation of natural resources, systematic leaf sampling has been conducted and their genetic characteristics measured with 16 SSR loci. In order to determine population structure, a small number of individuals localized together were regarded as sub-populations belonging to a single mother population. Annual individuals were clustered at particular peripheral areas of the pond. Perennial individuals were close by and growing within deeper pond water. Scores of observed heterozygosity (*Ho*) were not significantly different between annual and perennial sub-populations, but relatively lower in annual ones. Genetic distance among annual and perennial sub-populations in close juxtaposition at peripheral sites showed that annuals were clustered against perennials. In addition, comparison of perennial sub-populations peripheral areas and inside the swamp, found they clustered together and were some distance from annual ones. When the genetic components were compared in detail, private alleles were frequently found in annual plants, suggesting there might be restriction of gene flow between annual and perennial types. Partitions of deep water perennial sub-populations identified private alleles in particular areas, suggesting there were some areas with unique polymorphisms. Combining peripheral perennial sub-populations led to the disappearance of most private alleles which implied there is frequent gene flow among perennial sub-populations. This *in-situ* conservation site allowed us to observe the succession of populations and also to research detailed population structure of a typical wild population and this found wild rice genetic structure in this single swamp is complex. The data obtained will provide valuable insight about how to evaluate wild populations genetically and how to deal with such populations as field collections.

Key words: annual-perennial differentiation, *in-situ* conservation, life history, *Oryza rufipogon*, population structure

INTRODUCTION

Cultivated rice (*Oryza sativa* L.) feeds more people than any other species largely because it is the staple food of Asia, home to over 60% of the world's population (FAO stat, http://faostat.fao.org/). Asian rice cultivars have been domesticated from the wild ancestral species, *O. rufipogon*, which shows an annual-perennial continuum (Oka, 1988). Sharma and Shastry (1965) defined an annual form as *O. nivara*, however, because the perennial and annual forms lie on a continuum there is no clear

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distinction between *O. nivara* and *O. rufipogon* and so *O. nivara* is generally regarded as annual type of *O. rufipogon*. Collection records which include information on ecological life history demonstrate various morpho-ecological characteristics exist for perennial and annual types. In general, perennial individuals inhabit swamps and other areas where water is abundant and they propagate both sexually, setting seed, and vegetatively. In contrast, annual individuals inhabit the peripheral areas of swamps and propagate their progeny as seeds in the dry seasons. Perennial types tend to exhibit relatively higher outcrossing rates than annual types (Oka, 1988), but there is little difference in isozyme alleles frequency between the two (Oka, 1988). They may exchange some amounts of genetic constitution between them by change. So, although the annual and perennial types differ in morpho-ecological traits, they do not show huge differences in genetic traits. Mainly their morphological differences are due to their different resources allocation, probably controlled by multiple loci, but does not result in major reproductive barrier (Sano and Morishima, 1982; Cai and Morishima, 2002). It suggests that they belong to a single large gene pool.

Asian cultivated rice is divisible into *indica* and *japonica* subspecies. Compared to these cultigens, only Chinese wild relatives show some degree of differentiation while the common Asian wild rice species *O. rufipogon* do not tend to differ in their combination of characteristics (Morishima and Gadrinab, 1987). The process of domesticating wild ancestors to cultivated rice has involved strong selection through genetic bottlenecks to create the *indica* and *japonica* subspecies, and this has resulted in a loss of the wide genetic diversity found in the wild ancestors (Oka, 1988). The ancestral species, *O. rufipogon,* may harbour a huge degree of variation because most individuals are independent from domesticated cultivars and thus wild rice may be a reservoir of useful genes for future crop improvement. However, the natural habitats of wild rice (*O. rufipogon*) are being destroyed by human activities and genetic erosion through hybridization with cultivated rice (Suh et al., 1997; Tang and Morishima, 1997). Because of these pressures, methods of conservation must be developed quickly, informed by a complete knowledge of wild rice (Chitrakon, 1994; Gao et al., 1998; Morishima, 1994; Sato, 1994). In order to facilitate such studies, wild rice needs to be conserved and maintained in its natural condition. One idea proposed for the maintenance of wild populations is *in-situ* conservation (Vaughan and Chang, 1992; Xie et al., 2001). Successful management and conservation of wild rice in *in-situ* conservation areas requires a comprehensive knowledge of population genetic structure.

Various studies on the genetic structure of wild plant species in their natural habitats have been conducted to date (Gao et al., 2000; Kuroda et al., 2005a,b). However, those studies did not provide sufficient understanding of the extent of variation generally expected within single natural swamps, how the genetic structure of single wild populations could be fully conserved, or how to maintain wild rice resources as reservoirs for future rice breeding. In addition, a comprehensive knowledge of population genetic structure would also improve the sampling strategy for "*ex-situ* conservation" prior to extinction of natural vegetation, which is predicted because global environmental changes may affect natural vegetation drastically.

The development of molecular techniques has made it possible to evaluate the genetic structure of populations through statistical parameters such as expected heterozygosity (Nei, 1973). Molecular markers are capable of detecting genetic diversity and aiding the management of genetic resources (Song et al., 2003). A large number of studies have demonstrated SSR markers are particularly useful for the study of population structure because their significantly higher degree of polymorphism in rice facilitates the detection of diversity fine structure more efficiently than RFLP, RAPD, AFLP, or SNP (Wu and Tanksley, 1993; Yang et al., 1994; Gao et al., 2002; Garris et al., 2005; Huang et al., 2012). The high degree of polymorphism also means they are especially suitable for evaluation of genetic diversity among closely related species.

The wild rice in the *in-situ* conversation area located on the Vientiane plain of Laos designated LV27 (N 18.14.00, E 102.41.26) (Yamanaka et al., 2003; Kuroda, et al., 2005a), is a complex population consisting of annual and perennial types (Kuroda et al., 2005b). Annuals are defined as those germinating directly from seeds by any stimulation or disturbance at peripheral areas of swamps. Perennials tend to propagate vegetatively and are defined as those surviving in deep water which can be distinguished from seed-originating plantlets. As the water depth at LV27 increases in the rainy season and the area is completely submerged except at the fringes, perennials are abundantly supplied with water inside the swamp which persists through the dry season. Perennial seeds are buried in mud below the water and are hard to germinate. In comparison, germination of seeds dispersed from annual plants occurs when fringe areas are disturbed, often by water-buffalo. In a swamp such as this, pollen mediated gene flow is one of mechanism of sharing genetic polymorphism between annual and perennial types, and within each type. Molecular markers allow evaluation of genetic similarity and genetic distance, and whether more than one population is present in the swamp. Because details of population structure and the differences between annuals and perennials in LV27 are lacking, a systematic sampling and analysis are required to learn how to best maintain the *in-situ* conservation site and collect natural plants to maintain genetic polymorphism in an *ex-situ* collection such as a seed-bank.

The objectives of the present study were: 1) to use

molecular markers to identify how much genetic diversity exists in annuals and perennials in the *in-situ* conservation site, and 2) to obtain a better insight into natural wild rice populations to maintain polymorphism for future rice breeding applications.

MATERIALS AND METHODS

Plant materials A wild rice population in and at the fringe of a single pond located in the Vientiane plain of Laos (LV27 *in-situ* conservation site) was examined (Fig. 1A) in January, 2006. Annual individuals were recognized at the fringe of the pond beside perennial individuals (Fig. 1B).

Groups of individuals from both types which were presumed as sub-populations belonging to the single mother population were collected and compared for their genetic characteristics. A total of 191 individual wild rice plants

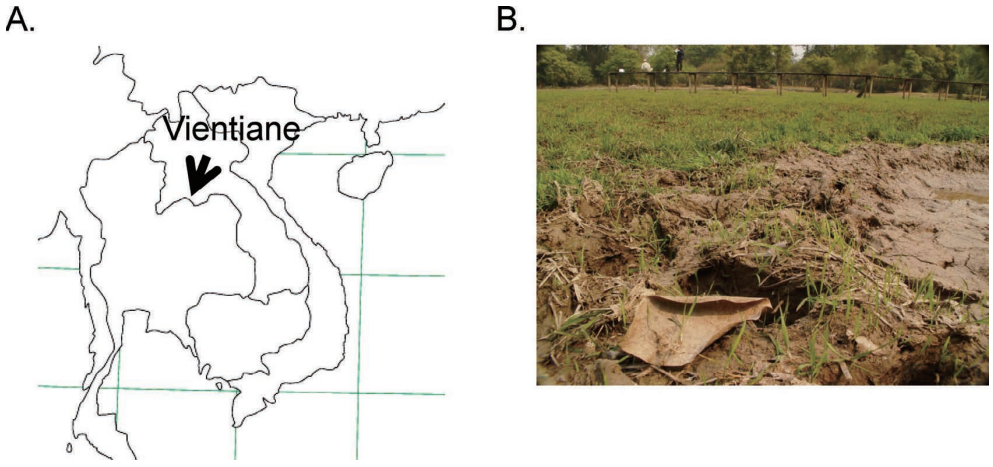

Fig. 1. LV27 as *in-situ* conservation site. Panel A : Location of the site in Vientiane. Panel B : annual and perennial individuals in the LV27 site. Annual plants (front) and perennial plants (back) are shown. Annual plants grow in sandy ditches disturbed by water buffalo.

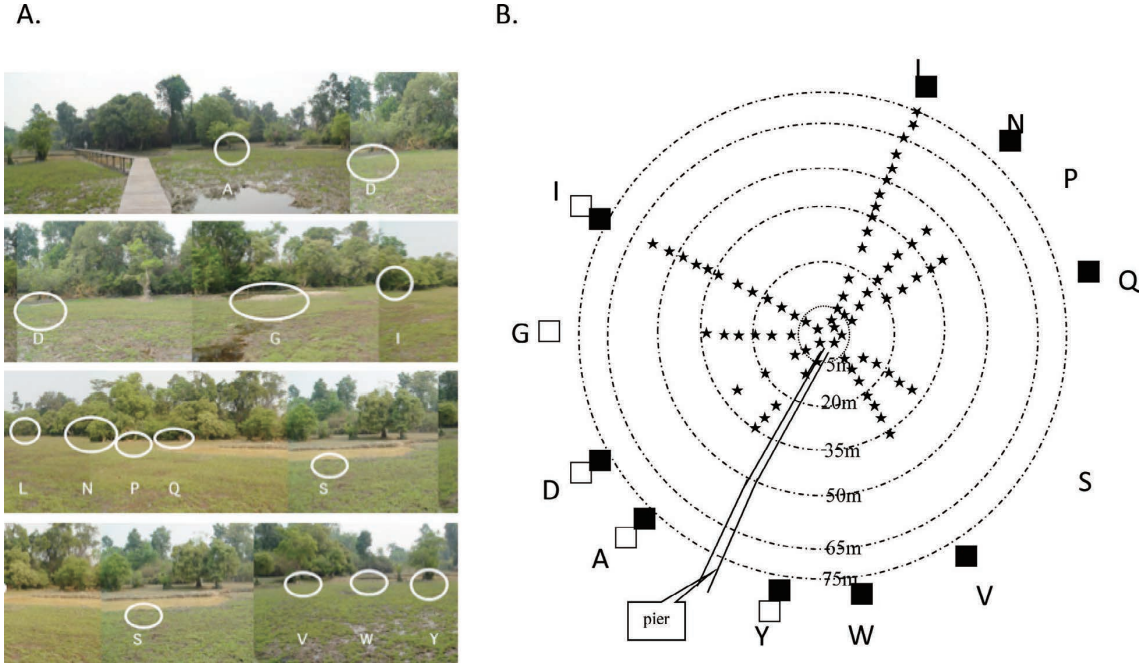

Fig. 2. Panorama photo of the i*n-situ* conservation site and illustration of sampling sites. A. A pier was constructed in the pond which allows researchers to take samples in rainy season when the water is deep. B. Illustration of samples. Solid squares represent perennial and clear squares annual sub-populations on the periphery of the swamp. Stars represent perennial strains in the swamp at the "LV27" *in situ* conservation site. Sampling sites on each line were termed as radA to radV along radiating lines toward the outside sub-populations.

were analyzed, of which 46 were annual and 72 perennial individuals at fringe of the pond and 73 perennial individuals inside the pond (Table 1). Groups of annual individuals were found in disturbed sandy ditches with the original seeds at the basal part (Fig. 1B). They germinated in that season directly from seeds. Outside of the ditches, there were perennial individuals. A pier extending from the shallow peripheral area to the swamp center does not disturb water flow and also vegetation, and it was used as a landmark to categorize sub-populations (Fig. 2, A and B). The sub-populations were named in a clockwise direction from PER-A. Nine perennial populations, PER-A, PER-D, PER-I, PER-L, PER-N, PER-Q, PER-V, PER-W and PER-Y, were set based on their locations relative to each other, and eight individuals were collected per site. Five annual sub-populations, ANN-A, ANN-D, ANN-G, ANN-I, and ANN-Y, were taken from the periphery of the swamp. They were composed of 12, 7, 8, 9, and 10 individuals, respectively. Many perennial individuals were observed inside the swamps. Samples termed as radA to radV were collected along lines radiating from site A to site V, respectively. Distance from a center in the pond was indicated , for example, as radA5 which was 5 m from the center. Seventy-two perennial individuals from within the swamp were collected. The center was presumed to be the deepest point so we classified into five sub-populations from center to outside named PER0-5, PER10-15, PER20-25, PER30-35 and PER40-75 (the number in each case indicating the distance in meters from the center, as measured along the edge of the pier). From single sites, single individuals were collected. Later those individuals were rearranged along radial lines from the center such as radA, radD, radG, radI, radL, radN, radP, radS, and radV. Details of relationships among individuals were represented as phylogenetic relationships. Twenty *indica* and 20 *japonica* cultivars classified as *indica* and *japonica* described previously (Imai et al., 2009) were used as diversity measure control populations.

SSR analysis A total of 16 SSR primer pairs were chosen for PCR amplification in this study (Table 2). Some loci were defined as diagnostic markers for distinguishing *indica* or *japonica* types, such as RM6301, +29CAT and ACP1+2K (Kawasaki et al., 2009). PCR was performed in a 20 μl reaction mixture containing 10–30 ng DNA, 0.2 μM dNTP, 15 pmol each primer, and 0.5 unit of Taq DNA polymerase. Amplification was carried out under the following conditions: 94°C for 3 min., 32 cycles of 95°C for 10 sec., 55°C for 30 sec., and then 72°C for 5 min. The number of cycles and annealing temperature were adjusted according to the specific requirements of each primer combination. The amplified products were mixed with loading dye and separated on 6% denaturing polyacrylamide gel, and electrophoresis was performed at 1500 V for 1.5–3 h in 1×TBE. The gels were finally subjected to silver staining procedure to visualize DNA fragments.

Statistical analysis Parameters for evaluating genetic variations in loci and populations were calculated, including the number of different alleles per locus (*Na*), the num-

Table 1. Wild rice collected from a single pond as *in-situ* conservation site, and summary of their ecological condition

Site direction	No. of individuals at peripheral areas			No. of individuals every each distanc from a center of the pond (m)																	
	Perennial	Annual	Sub-total	PER0-5*		PER10-15		PER20-25		PER30-35		PER40-75								Sub-total	Rearranged sub-population for inside individuals
				0	5	10	15	20	25	30	35	40	45	50	55	60	65	70	75		
A	8	12	20	0	1	1	0	0	1	1	1	0	0	0	0	0	0	0	0	5	radA
D	8	7	15	1	1	1	0	1	0	1	0	0	0	0	0	0	0	0	0	5	radD
G	0	8	8	0	0	1	1	1	1	1	1	0	0	0	0	0	0	0	0	6	radG
I	8	9	17	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	13	radI
L	8	0	8	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	15	radL
N	8	0	8	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	9	radN
P	0	0	0	1	1	0	0	1	1	1	1	1	0	0	0	0	0	0	0	7	radP
Q	8	0	8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
S	0	0	0	0	0	1	1	1	1	1	0	0	0	0	0	0	0	0	0	5	radS
V	8	0	8	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	8	radV
W	8	0	8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
Y	8	10	18	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
Total	72	46	118	6	7	8	6	7	8	9	7	4	2	2	2	2	1	1	1	73	

*Individuals belonging to each site direction were termed with the site name such as radA5, radA10, and so on when they were collected along radiating direction to site A.

Table 2. Data summary for SSR markers

SSR marker	Sequence	Chr.	Genome position	Total no. of alleles	*Na*				LV27	ANN	PER
					Cultivars	ALL	ANN	PER	*Ho* - *He*	*Ho* - *He*	*Ho* - *He*
RM3604	5'-ATGTCAGACTCCGATCTGGG-3'	1	5137073	8	3	6	6	6	0.665 - 0.774	0.543 - 0.797	0.703 - 0.763
	5'-TCTTGACCTTACCACCAGGC-3'										
RM1347	5'-AACAAATTAAACTGCCAAG-3'	2	5314190	8	8	6	5	6	0.492 - 0.703	0.261 - 0.646	0.566 - 0.706
	5'-GTCTTATCATCAGAACTGGA-3'										
RM3180	5'-GGGTCGGATAGCCACACAC-3'	3	1.8E+07	7	7	6	5	6	0.414 - 0.658	0.457 - 0.665	0.400 - 0.652
	5'-GAGGTAATCTCGCGGAGTTG-3'										
RM6301	5'-CGCTACCTTATGCTGCTGTC-3'	3	2651356	3	2	3	3	3	0.225 - 0.265	0.109 - 0.250	0.262 - 0.270
	5'-TCGGCTACAACCTCTCCTTC-3'										
AC084748	5'-GAAACAGGTTCATATGGTCAC-3'	3	3.2E+07	3	3	3	3	3	0.236 - 0.565	0.043 - 0.542	0.297 - 0.565
	5'-GGTGTGTTAGTGTTAGTAGTGG-3'										
RM8213	5'-AGCCCAGTGATACAAAGATG-3'	4	3512769	12	9	6	6	5	0.550 - 0.705	0.283 - 0.700	0.634 - 0.689
	5'-GCGAGGAGATACCAAGAAAG-3'										
AL606650	5'-CACATAGACCGAAATCGGGG-3'	4	3.2E+07	7	7	6	4	5	0.016 - 0.691	0.065 - 0.659	0.000 - 0.688
	5'-GACGGTAGGTAAAGTACAATC-3'										
RM3476	5'-GATTCTCGTCGTAATCAAGA-3'	5	2.4E+07	15	12	6	5	5	0.330 - 0.414	0.217 - 0.383	0.366 - 0.420
	5'-ATCCACGGTTAAGATAAATG-3'										
AP005527	5'-CAGTCCAAGAATCGTACTTC-3'	6	8324649	4	4	1	1	1	0.000 - 0.000	0.000 - 0.000	0.000 - 0.000
	5'-GCTTTTGCTGTGATTTGGTG-3'										
+29CAT	5'-CACGATCTAGAAGACGAGAG-3'	6	3.1E+07	4	4	4	2	4	0.000 - 0.604	0.000 - 0.440	0.000 - 0.511
	5'-CCAAATTACGCCTTCCTACC-3'										
RM125	5'-TCAGCAGCCATGGCAGCGACC-3'	7	5478776	3	2	3	3	3	0.424 - 0.543	0.304 - 0.491	0.462 - 0.556
	5'-ATGGGGATCATGTGCCGAAGGCC-3'										
RM8019	5'-CAAGACAGATAAAGCATTAA-3'	8	7487569	9	8	8	5	7	0.369 - 0.696	0.133 - 0.457	0.444 - 0.737
	5'-GTAGTTTTGAAGTGATGGAA-3'										
RM311	5'-TGGTAGTATAGGTACTAAACAT-3'	10	9487264	7	7	5	4	5	0.239 - 0.616	0.159 - 0.563	0.264 - 0.630
	5'-TCCTATACACATACAAACATAC-3'										
RM17	5'-TGCCCTGTTATTTTCTTCTCTC-3'	12	2.7E+07	6	6	2	2	2	0.000 - 0.198	0.000 - 0.227	0.000 - 0.188
	5'-GGTGATCCTTTCCCATTTCA-3'										
RM7102	5'-TTGAGAGCGTTTTTAGGATG-3'	12	1.3E+07	10	6	9	9	9	0.635 - 0.829	0.717 - 0.819	0.608 - 0.827
	5'-TCGGTTTACTTGGTTACTCG-3'										
ACP1+2K	5'-CCAATAGTCCATGGCAGAGG-3'	12	2.7E+07	3	3	1	1	1	0.000 - 0.000	0.000 - 0.000	0.000 - 0.000
	5'-CTGCTGCTTCCTCTGAAAATATATC-3'										

ALL, PER, and ANN indicating all individuals in LV27, only perennial individuals, and annual individuals, respectively.
Na: number of different alleles; *Ho*: observed heterozygosity; *He*: excepted heterozygosity.

ber of private alleles, expected and observed heterozygosity (*He* and *Ho*; *He* was also used as measurement of genetic diversity) using the GenAlEx 6.2 software package (Peakall and Smouse, 2006). The private alleles were defined as percentage of alleles detected only in single sub-populations. Genetic distances among sub-populations calculated by using GenAlEx 6.2, and dendrograms were constructed using the neighbor joining method based on Nei's unbiased genetic distances by Populations1.2.30 beta2 program which is free software downloaded at http://bioinformatics.org/~tryphon/populations/#ancre_bibliographie (Nei et al., 1983). All dendrograms were viewed in TreeExplorer software used to show and edit population dendrogram, supplied as a free software with MEGA at http://www.megasoftware.net/ (Kumar et al., 2008). Analysis of molecular variance (AMOVA; Excoffier et al., 1992) was conducted with groups of sub-populations such as peripheral and inside sub-populations as factors, using GenAlEx 6.

RESULTS

Polymorphism of SSR markers In order to confirm population structure in LV27 *in-situ* conservation site, annual and perennial samples were collected intensively (Fig. 1, Table 1). In a previous study, Kuroda et al. (2005a) found 16% of annual individuals were in fringe areas of LV27 in March, 1998. We could detect annuals only in areas disturbed by water-buffalo. Seed dormancy was probably broken earlier than usual by the disturbance. All individuals were presumed to compose a single mother population because annual and perennial types can cross-pollinate and produce fertile progenies.

A total of 16 SSR markers dispersed across the genome

were used to assess genetic diversity in LV27. In total 109 alleles were detected at the 16 SSR loci. The number of alleles per locus varied from three to 15. Some alleles were unique to either to wild rice or cultivars. With the exception of AP005527 and ACP1+2K, 14 SSR loci generated polymorphic patterns across annuals and perennials (Table 2). Wild rice at LV27 carried single *indica*-specific genotypes at both AP005527 and ACP1+2K. Relative to wild rice, cultivars had a higher number of alleles at ten loci and a smaller number of alleles at four loci, suggesting the wild rice in *in-situ* conservation site still possess polymorphism over genome. The 14 polymorphic loci were used for further analysis.

Ho had a lower value than *He* for the examined 14 loci, suggesting a majority of loci showed some degree of deviation from a Hardy-Weinberg equilibrium (HWE), possibly because of self-pollination in both annual and perennial sub-populations. When genotypes were compared among sub-populations inside the pond, concentric sub-populations tended to show private alleles except for PER40-75 (Fig. 3A). When data from within pond sub-populations were rearranged along axis, radD, radG, radI, radL and radS showed private alleles (Fig. 3B), suggesting there are patches of individuals carrying private alleles inside the pond. When peripheral and within pond perennial sub-populations were compared, only the inner most PER0-5 and intermediate PER20-25, had private alleles. Private alleles were rare in perennial individuals suggesting they tended to exchange their genetic components by pollen-flow.

When only peripheral sub-populations were compared, three of the five annual sub-populations and two of the nine perennial sub-populations carried private alleles (Fig. 3D). Comparison of all sub-populations in LV27 found only two annual sub-populations, ANN-A and ANN-I, and one perennial sub-population, PER20-25 had private alleles (Fig. 3E). Private alleles in annual sub-populations possibly reflected a preference for self-fertilization in annual individuals. The most inner PER0-5 lost the private allele when annual sub-populations were compared, which indicated PER0-5 shared similarity with parts of the peripheral annual sub-populations.

Genetic variation in annual and perennial sub-populations Data derived from all individual plants in

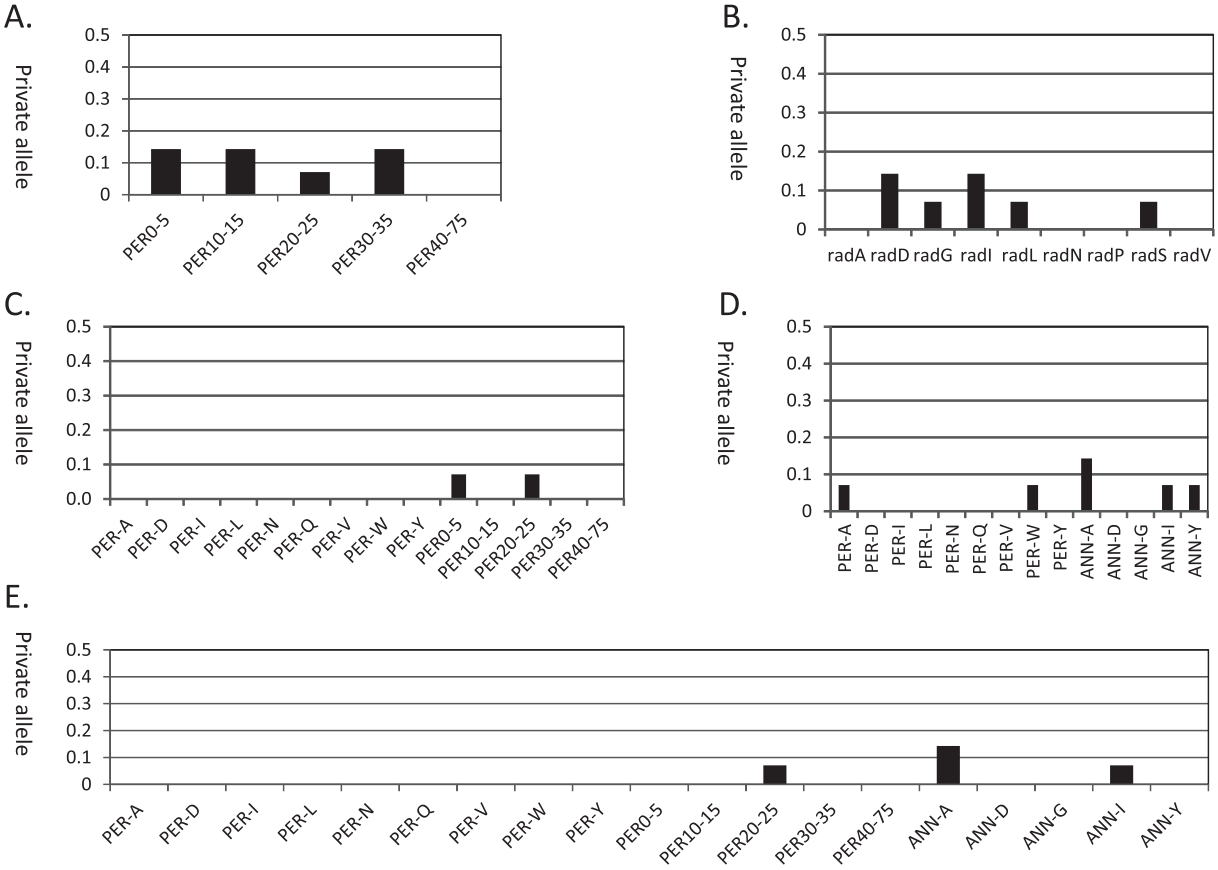

Fig. 3. Frequency distribution of private alleles. A. Within pond sub-populations were categorized by concentric circles from the distance of the center. B. Within pond individuals were rearranged along an axis. C. Perennial sub-populations at peripheral areas and within pond. D. Annual and perennial peripheral sub-populations. E. All sub-populations in LV27.

LV27 were pooled to calculate *Ho* and *He* (Table 3). *Ho* was 0.328 (Standard error, SE=0.059), *He* was 0.590 (SE=0.047). All perennial *Ho* scores were higher than annual sub-populations. Although *Ho* scores among within pond and peripheral perennial sub-populations appeared to gradually decrease from 0.376 to 0.339 and 0.235, respectively (Table 3), there was no significant difference in *Ho* scores. When the *Ho* score for within pond and peripheral perennial individuals were calculated together, *Ho* was 0.358 which was higher than that for annual individuals. The level of heterozygosity as measured by *Ho* suggests within pond perennial sub-populations outcross more frequently than the peripheral perennial and annual sub-populations. Five annual sub-populations showed similar *Ho* scores of 0.207 to 0.262, a difference in *Ho* which was lower than any partitions between perennial sub-populations. Although higher *Ho* scores were generally found at the inner perennial populations relative to outer sub-populations, there was no significant difference in *Ho* between perennial populations. Partitioned areas were rearranged along each axis. The highest *Ho* score of 0.444 was found in radN and the lowest one, 0.300 was found in radD. *Ho* scores of other perennial sub-populations at peripheral areas ranged from 0.277 (PER-A) to 0.406 (PER-N). There was also no significant difference among the rearranged subpopulations.

Table 3. Observed and expecrted heterozygosity in sub-populations and whole population with 14 SSR loci

Location	Annual/ Perennial	Sub-populations	*Ho* Mean ± SE	*He* Mean ± SE
Inside	Perennial	PER0-5	0.399 ± 0.083	0.579 ± 0.049
Inside	Perennial	PER10-15	0.359 ± 0.072	0.536 ± 0.063
Inside	Perennial	PER20-25	0.376 ± 0.073	0.542 ± 0.062
Inside	Perennial	PER30-35	0.353 ± 0.067	0.551 ± 0.053
Inside	Perennial	PER40-75	0.395 ± 0.090	0.542 ± 0.059
Inside	Perennial	radA	0.357 ± 0.073	0.496 ± 0.072
Inside	Perennial	radD	0.300 ± 0.093	0.496 ± 0.070
Inside	Perennial	radG	0.393 ± 0.104	0.478 ± 0.082
Inside	Perennial	radI	0.332 ± 0.075	0.546 ± 0.054
Inside	Perennial	radL	0.388 ± 0.082	0.543 ± 0.060
Inside	Perennial	radN	0.444 ± 0.085	0.486 ± 0.060
Inside	Perennial	radP	0.388 ± 0.084	0.452 ± 0.057
Inside	Perennial	radS	0.386 ± 0.088	0.437 ± 0.081
Inside	Perennial	radV	0.375 ± 0.069	0.513 ± 0.055
Inside	Perennial	All*	0.376 ± 0.073	0.577 ± 0.055
Peripheral	Perennial	PER-A	0.277 ± 0.075	0.480 ± 0.046
Peripheral	Perennial	PER-D	0.295 ± 0.075	0.455 ± 0.068
Peripheral	Perennial	PER-I	0.313 ± 0.071	0.425 ± 0.065
Peripheral	Perennial	PER-L	0.357 ± 0.086	0.444 ± 0.061
Peripheral	Perennial	PER-N	0.406 ± 0.087	0.503 ± 0.063
Peripheral	Perennial	PER-Q	0.339 ± 0.109	0.346 ± 0.071
Peripheral	Perennial	PER-V	0.321 ± 0.068	0.496 ± 0.063
Peripheral	Perennial	PER-W	0.366 ± 0.081	0.497 ± 0.065
Peripheral	Perennial	PER-Y	0.374 ± 0.090	0.570 ± 0.038
Peripheral	Perennial	All	0.339 ± 0.064	0.567 ± 0.048
Inside and peripheral	Perennial	All	0.358 ± 0.063	0.586 ± 0.050
Peripheral	Annual	ANN-A	0.207 ± 0.046	0.469 ± 0.053
Peripheral	Annual	ANN-D	0.245 ± 0.089	0.454 ± 0.070
Peripheral	Annual	ANN-G	0.241 ± 0.061	0.488 ± 0.051
Peripheral	Annual	ANN-I	0.262 ± 0.085	0.440 ± 0.060
Peripheral	Annual	ANN-Y	0.236 ± 0.077	0.479 ± 0.063
Peripheral	Annual	All	0.235 ± 0.057	0.545 ± 0.049
LV27	Annual and perennial		0.328 ± 0.059	0.545 ± 0.049

*All: all individuals in were regarded as members in a single sub-populations.

He is a measurement of genetic diversity based on allele frequencies. *He* of the LV27 population including all annual and perennial individuals was 0.590 (SE=0.049). *He* scores were similar among all sub-populations including annual and perennial individuals suggesting the annual sub-populations carry a degree of polymorphism similar to perennials (Table 3). The score of all annual individuals was 0.545 and those of five annual sub-populations ranged from 0.440 (ANN-I) to 0.488 (ANN-G). The *He* for the perennial sub-populations derived from pooled data from within pond and peripheral perennial individuals was 0.586. When all sub-populations inside the pond were combined together to calculate the data, the *He* was 0.577. That of peripheral ones was 0.567. Of PER0-5, PER10-15, PER20-25, PER30-35 and PER40-75, the inner most one, PER0-5 had the highest *He* of 0.579. Sub-populations in peripheral areas had *He* ranging from 0.346 (PER-Q) to 0.570 (PER-Y). Different arrangements of sub-populations from radA to radV showed similar scores. PER-Q had the lowest *He* score of 0.346. The averaged *He* score of annual subpopulations was 0.545 when all annual individuals were pooled as a single annual sub-population together. There were relatively higher differences for their allelic constitutions among annual sub-populations. The highest score among all sub-populations in LV27 was 0.579 shown by PER0-5. This implies the within pond sub-populations maintain higher genetic diversity by outcrossing. This is also supported by the presence of a private allele which was rarely found in perennial sub-populations.

Genetic relationships among annual and perennial sub-populations in peripheral area A phylogenetic tree for the outlying populations constructed by the neighbor joining method found that all the perennial sub-populations with the exception of PER-W, grouped into a single cluster (Fig. 4A). The annual sub-populations grouped into another cluster which included PER-W.

This phylogeny suggested gene flow is infrequent between annual and perennial sub-populations. As PER-W, PER-A, and ANN-Y were located physically near to each other, such physical factor may influence genetic similarity among those sub-populations via gene flow.

The unrooted neighbor-joining tree constructed for perennial sub-populations in the central and peripheral areas showed two clusters corresponding to both areas (Fig. 4B). Peripheral sub-populations (PER-A, PER-D, PER-I, PER-L, PER-N, PER-Q, PER-V, and PER-Y) were grouped into the single cluster, while PER-W was grouped together with the within pond sub-populations (PER0-5, PER10-15, PER20-25, PER30-35, and PER40-75). Attempts to construct a tree with rearranged central and peripheral perennial sub-populations (Fig. 4C) suggested the within pond and peripheral sub-populations are somewhat distant from each other. This was also suggested from the phylogenetic trees with all sub-populations (Fig. 4D).

Analysis of molecular variance was conducted among peripheral annual and perennial groups, and within pond groups. Proportions of genetic variation partitioned among these groups, among populations, and within individuals are shown in Table 4. In all combinations, more than 80% of total molecular variance was explained by variance among individuals within groups. Small parts of variance were attributed to comparisons among groups. All phigraph (Phi) scores were significant from randomized scores at 0.1% level and indicated that sub-populations maintain high genetic variance and there were no significant differences due to life history traits or physical location. Thus, it was concluded although there was some difference among groups, these sub-populations compose a single population where some degree of gene

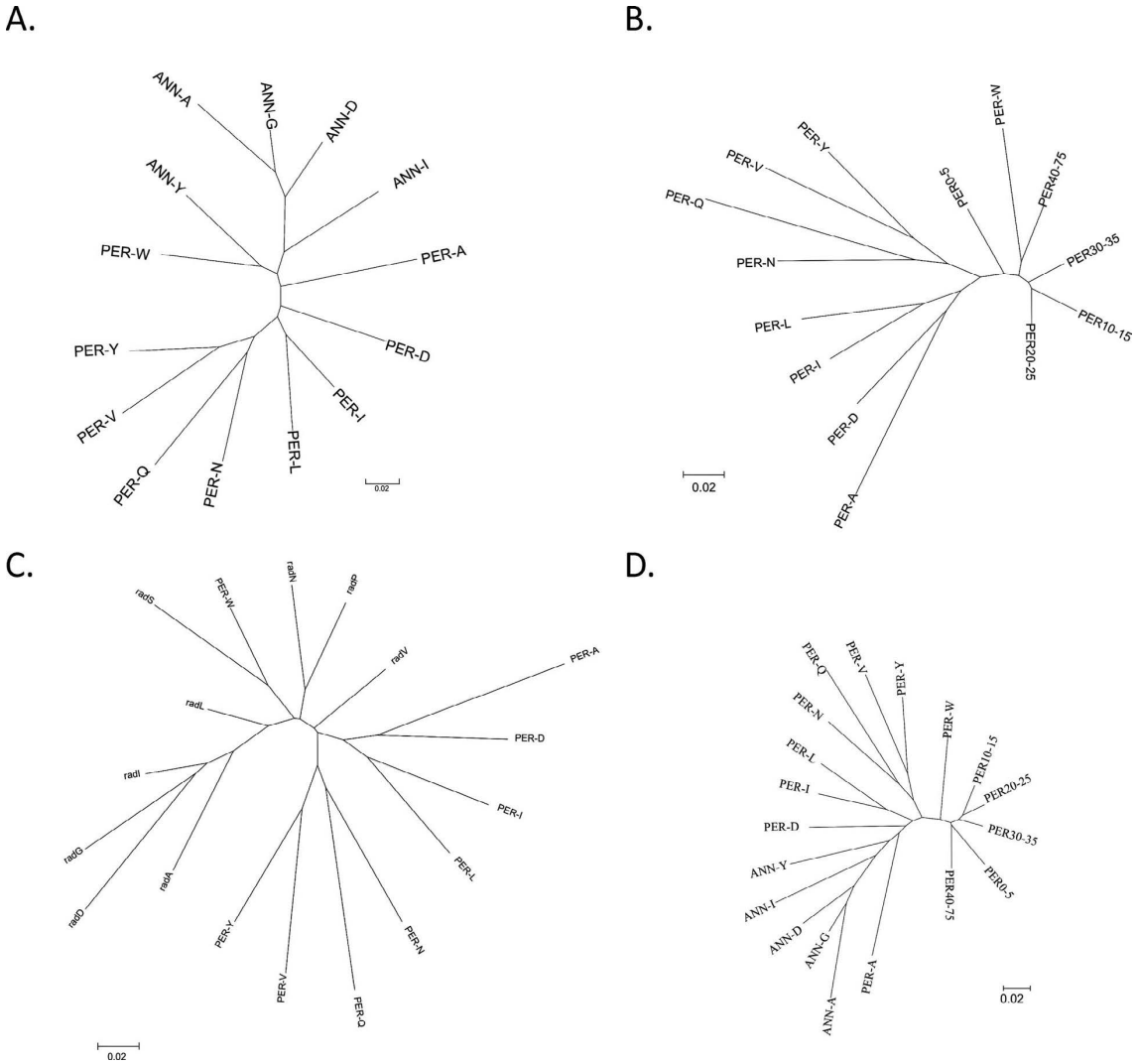

Fig. 4. Phylogenetic trees based on neighbor–joining method. A. Comparison of peripheral populations. B. Comparison of peripheral and within pond perennial sub-populations. C. Peripheral and rearranged within pond perennial sub-populations. D. Comparison of within pond and peripheral perennial, and annual sub-populations. Scores in each tree are genetic distance.

Table 4. Summary results of AMOVA among different combinations of groups including peripheral annual, peripheral perennial, and inside perennial groups

Source	df	SS	MS	Est. Var.	%	Statistics	Value	p *
Three groups; peripheral annual and perennial groups, and inside perennial group								
Among groups	2	93.865	46.933	0.407	3%	PhiRT	0.034	0.001
Among Pops within groups	16	332.090	20.756	1.038	9%	PhiPR	0.090	0.001
Among individuals	172	1797.201	10.449	10.449	88%	PhiPT	0.122	0.001
Total	190	2223.157		11.895	100%			
Two groups; peripheral and inside perennial groups								
Among groups	1	55.678	55.678	0.455	4%	PhiRT	0.038	0.001
Among Pops within groups	12	258.037	21.503	1.094	9%	PhiPR	0.096	0.001
Among individuals	131	1355.450	10.347	10.347	87%	PhiPT	0.130	0.001
Total	144	1669.166		11.896	100%			
Two groups; annual and inside perennial groups								
Among groups	1	69.265	69.265	0.882	7%	PhiRT	0.070	0.001
Among Pops within groups	8	157.896	19.737	0.737	6%	PhiPR	0.063	0.001
Among individuals	109	1198.856	10.999	10.999	87%	PhiPT	0.128	0.001
Total	118	1426.017		12.618	100%			
Two groups; peripheral annual and perennial groups								
Among groups	1	70.298	70.298	0.785	6%	PhiRT	0.061	0.001
Among Pops within groups	12	302.776	25.231	1.787	14%	PhiPR	0.148	0.001
Among individuals	104	1067.656	10.266	10.266	80%	PhiPT	0.200	0.001
Total	117	1440.729		12.838	100%			

*Probability, for PhiRT, PhiPR and PhiPT is based on permutation across the full data set. 999 permutation was performed.

flow is allowed and maintains genetic variation within the LV27 population.

DISCUSSION

The earth's human population has reached seven billion and based on State of World Population report (http://www.unfpa.org/swp/) will within this century reach ten billion. Population growth is very rapid within Asia where rice (*O. sativa)* is the staple food. *O. rufipogon* is the ancestral species from which *O. sativa* is descended and offers huge genetic diversity which has the potential to deliver a response to global climate change through rice breeding. As demonstrated here, *O. rufipogon* found in Laos which tends to carry similar alleles to that *indica* carries, show higher genetic diversity within populations and dissimilar characters in overall genotypic composition in comparison with both *japonica* and *indica* cultivars. Probably they might not be concerned to domestication events for *japonica* or *indica*.

Many studies have identified and reported differences between annual and perennial *O. rufipogon* populations (Morishima et al., 1961; Oka, 1976; Sano et al., 1980; Barbier, 1989; Morishima and Barbier, 1990; Kuroda et al., 2002). The annual and perennial forms of *O. rufipogon* have different life history traits, most significantly, the latter have a higher outcrossing rate and tend to propagate vegetatively (Oka, 1988). Propagation and mating systems such as these and related to factors, such as demographic characteristics (generation length and survivorship), distribution pattern (widespread or endemic; patchy or continuous), and gene flow from other populations (pollen flow and seed dispersal) affect the genetic structure of populations. The present study demonstrated despite the perennial sub-populations in the center of the swamp having relatively higher genetic variation than outlying annual and perennial sub-populations, LV27 is a single population composed of annual and perennial sub-populations, which share genetic material with little restriction of gene flow. In a previous study undertaken in 1998, 19 plants (nine and ten nodes from a fringe site and inside area, respectively) and 19 seeds (nine and ten

nodes from a fringe site and deep water area, respectively) and seven SSR loci were analyzed (*Ho* and *He*) (Kuroda et al., 2005a). In the present study, 14 polymorphic SSR markers were examined in 46 annuals and 73 within pond perennials. *He* scores of perennial individuals in both studies were little different. For example, they were 0.769 in all perennials and 0.688 within deep water in the previous report. Similar scores were obtained in this study, 0.586 (overall) and 0.577 (deep water). However, *Ho* scores in perennials were quite different between both studies, 0.609 (overall) and 0.614 (deep water) in the previous paper, and 0.358 (overall) and 0.376 (deep water) in this study. The lower level of heterozygosity observed in 2006 compared to 1998 may be due to the greater number of individuals sampled and of SSR loci assayed in 2006 which may more accurately reflect the true genetic composition of the population. Or there may be any factors preventing them from outcrossing and promoting self-pollination. Annual sub-populations tended to show relatively lower *Ho* scores (0.238 in the previous study and 0.235 in this study) than perennial sub-populations in both studies.

He in over all annuals was 0.545 in this study (0.536 in the previous study), which was not significantly different from that in over all perennials, 0.586. This means that although the frequency of heterozygotes was lower in annuals than perennials, the level of polymorphism in annuals across the entire population is little different to the perennials (Table 1). The relatively high polymorphism rate in annuals resulted partly from the existence of private alleles in annual sub-populations. Thus, average scores of the *He* in over-all annuals was higher than any single annual sub-populations. In addition, some mechanisms including rare gene flow events from perennials may maintain heterogeneity in annuals. In this study, deep water and peripheral individuals were partitioned into small sub-populations which allowed identification of LV27 genetic structure in detail.

When all sub-populations were compared, private alleles were detected in the annual sub-populations ANN-A and ANN-I and the perennial sub-population PER20-25 only. This may be attributed to the tendency of annuals to self-pollinate and perennials to cross-pollinate (Oka, 1988; Sharma et al., 2000). Lower *Ho* scores in annual subpopulations are also due to their preference for self-pollination. Such a difference of reproductive system leads to a population structure in wild rice where perennial individuals tend to be more polymorphic than annual individuals. However, LV27 annual sub-populations may exchange a small part of their genetic components with perennial sub-populations which may maintain genetic heterogeneity in the annual community, an example being the close relationship between ANN-Y and PER-W (Fig. 4A). Thus, genetic diversity as represented by *He* among annual sub-populations was not significantly lower than perennial sub-populations (Table 3).

Some deep water perennial sub-populations showed private alleles due to micro structure of each perennial community. However, the private alleles disappeared when peripheral perennial sub-populations were included in the analysis (Fig. 3C). The uniformity of polymorphism among perennial sub-populations would be due to gene flow among them. Gene flow may occur between annual and perennial individuals differing in life history traits, although the direction of gene flow may be restricted to one direction, from perennial to annual types, and so annual sub-populations tended to show private alleles. Ecological situations may keep any traits required for annuals when they survive at the peripheral locations. An exceptional case was the inner most polymorphic sub-population, PER0-5, which lost private alleles when compared to all annual sub-populations, demonstrating PER0-5 received gene flow from peripheral annuals and maintained polymorphisms.

A previous study of genetic variation based on qualitative and quantitative genetic characteristics suggested the highest level of genetic differentiation was between seeds at the fringe and seeds in deep water (Kuroda et al., 2005a). Our data inferred by genetic distances calculated by multiple SSR markers suggested that there is weak differentiation between annuals and deep water perennial (Fig. 4A). There is likely selection which keeps progeny in the peripheral area. Quantitative trait differences between typical annuals and perennials included panicle and spikelet number, regeneration ability from stem, degree of dormancy, and anther length. Loci controlling these quantitative traits (QTL) are scattered across the whole genome (Cai and Morishima, 2002). The limited number of SSR markers in this study could not detect such QTL and so significant differences in levels of selection between annuals and perennials were not detected with these SSRs. Gene flow may also weaken genetic differentiation between annuals and perennials, an example being the relationship between ANN-Y and PER-W. AMOVA analysis found sub-populations differed by life history did not demonstrate significant differences in genetic variance suggesting each sub-population maintains diversity by gene flow. If this was not the case, annual sub-populations would display lower diversity, however, single annual sub-populations differed significantly in genetic diversity.

Changes in environmental conditions between 1998 and 2006 may have changed the population structure of LV27. Any change in environment would require plants to adapt to the new condition assuming that there is sufficient diversity within LV27 to respond to the change. Annuals and perennials would be adapted to already different conditions and so any changes in environment would have impact these types differently. Annuals still inhabited in LV27 at 2006. However, if any barriers

between annuals and perennials are weakened, intermediate type between annual and perennial types would be appeared. Generally, such intermediate type could be observed in nature, which have a continuous variation of traits (Sano et al., 1980). Outcrossing between annuals and perennials can produce such intermediate types having a wide variation in quantitative traits in natural populations. Gene flow between annuals and perennials could be expected in LV27 and may result in traits variation concerning these two ecotypes. High genetic diversity, low frequency of private alleles, and weak differentiation among annuals and perennials would result from gene flow in LV27. However, there was still differentiation for example, annuals tended to carry private alleles and quickly germinate by stimulation of disturbance. Reinforcement of the differentiation would require environmental factors to exert some level of selection on different genotypes. Such reinforcement may work in these two groups differed in life history.

Then, what kind of natural factors affect *in-situ* conservation sites such as LV27 in order to reinforce groups differed in life history or what will affect to restructure the population in future ? Water is one factor that affects wild rice habitat and plays a role in changing population structure within the *in-situ* conservation site. Total precipitation, sudden flood, and drought damage influenced by irrigation activity in near-by paddies, might affect the structure in the site. Perennial plants have the ability of stem elongation forming what is known as floating rice which allows perennial wild rice to inhabit permanent ponds while annual plants inhabit the edges of these ponds. In January no annuals were evident but perennials, most of which were submerged, were growing inside the pond because the center of the pond was still inundated. Young plantlets were grown from seeds could be found on some dried peripheral areas. Currently, in rainy season at LV27 site, there is plenty of water available in the pond and the most inner of these sub-populations maintain the highest diversity. Also in dry season, LV27 keeps wet condition inside and dry condition in peripheral areas where annuals could inhabit. We do not know which level of changes will trigger reconstruction of the population structure in detail. We may know the changes after particular populations would extinct. Rice geneticists have paid little attention to transition of *in-situ* conservation sites. Both continuous observation of the site and periodic genotyping will trace changes to be monitored the extent to which these changes affect population structure determined.

Animal disturbance of peripheral areas is another factor of concerns with regard to diversity maintenance in a natural population. Water buffalo prefer to roll or lie down on sandy dry areas. These actions break seed dormancy of the seed-bank originating from annual individuals while perennial plantlets are uprooted in disturbed areas (Fig. 1B). This tends to favor propagation of annuals rather than perennials. Micro environmental conditions such as these factors impact annuals and perennials differentially and maintains their distinct characteristics such as regeneration ability from nodes, seed dormancy, and so on. Given surrounding environmental conditions affect plant genetic selection and structure. Such environment can be used to guide how to collect wide series of genetic variation, to monitor population structure to keep the population, or to maintain *in-situ* conservation sites with regulation of these factors artificially like natural condition.

Population structure affects efficiency for collection to evaluate "net" diversity. Deep evaluation of a single population informs us on how much diversity is to be expected, or how we can effectively collect with minimum loss of polymorphism, and how we safely maintain the diversity for *in-situ* conservation. Wild rice has huge natural diversity and we have not fully understood how to evaluate genetic diversity in each site. In usual field studies, we cannot collect the huge number of samples as we have here, and/or we do not have the capacity to capture the whole diversity as an *ex-situ* conservation collection such as a gene-bank. Therefore, *in-situ* conservation models are required for analyzing natural populations distributed in Asia, and for maintenance of genetic resources.

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