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RESEARCH

Comparison of the Genetic Structure between In Situ and Ex Situ Populations of Dongxiang Wild Rice (*Oryza rufipogon* Griff.)

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ABSTRACT

In situ and ex situ conservation are two main wild rice (*Oryza rufipogon* Griff.) protection strategies. Few studies have compared the genetic diversity and genetic structure of wild rice between ex situ and in situ populations. Thus in this study, 278 individuals collected from three in situ and nine ex situ populations of Dongxiang wild rice (DXWR) were genotyped using 32 microsatellite loci to compare their population genetic structure and genetic diversity. Model-based grouping, neighbor-joining tree, and principal coordinate analyses showed that there were significant differences in the population structure between in situ and ex situ populations. The in situ populations clustered into three major groups, which were in accordance with their geographical distribution (Anjiashan, Zhangtang, and Shuitaoshu). However, the nine ex situ populations of DXWR showed no differences among populations, and there was a high heterozygosity for each individual. In addition, one of the in situ populations (Zhangtang) was not represented in the ex situ conservation garden. Given the above results, we concluded that the germplasm of both in situ and ex situ populations are precious, and that the combination of these two conservation strategies is necessary to protect the germplasm resources of DXWR, but special emphasis should be placed on in situ conservation to better maintain their genetic identities. In addition, it is necessary to recollect genetic resources from in situ populations for ex situ conservation, especially from the Zhangtang population.

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Abbreviations: Ae, mean number of alleles per locus; AJ, Anjiashan; AMOVA, analysis of molecular variance; DXWR, Dongxiang wild rice; Fis, fixation index; Fst, gene differentiation index; He, expected heterozygosity; Ho, observed heterozygosity; *I*, Shannon–Weaver information index; NJ, neighbor-joining; PCA, principal component analysis; PCR, polymerase chain reaction; Ppl, percentage of polymorphic loci; SSR, simple sequence repeat; ST, Shuitaoshu; TL, Dongtangliangbian; TS, Dongtangshang; TX, Dongtangxia; ZT, Zhangtang.

In situ and ex situ conservation are two main wild rice (*Oryza rufipogon* Griff.) protection strategies (Nevo, 1998; Zhong et al., 2003; Volis and Blecher, 2010). In situ conservation, which allows species to evolve dynamically with the changing environment, involves the protection of natural populations of endangered species in their original habitat where random mating occurs (Henry et al., 2009). However, when in situ conservation is impractical because of habitat destruction making it too expensive to conserve the original habitat, ex situ conservation can be used to conserve population diversity and genetic variation effectively (Volis and Blecher, 2010; Lauterbach et al., 2012). Ex situ conservation is a resource-limited strategy in which many samples from as many different original habitats as possible are collected and grown together (Namoff et al., 2010). Consequently, it is

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important that ex situ collections contain a representative set of the extant in situ diversity, and that their population dynamics are monitored over time to ensure that the original genetic diversity is retained (Lauterbach et al., 2012). After several generations of ex situ cultivation, the population diversity and structure may have changed because multiple samples from different populations have been grown together or due to ecological shifts in mating and reproductive systems; gardener-induced selection may also cause the change (Krauss et al., 2002; Enßlin et al., 2011). However, long-term observation has not been done of ex situ populations in the same protective environment, and few studies have compared the genetic diversity and structure between ex situ populations and their in situ source populations after a defined period of time (Rucińska and Puchalski, 2011).

Evaluating the efficiency of ex situ conservation over a long time is complicated (Li et al., 2002), because enough samples of ex situ populations and their relevant in situ populations are required. Most studies comparing in situ and ex situ genetics have focused on tree or shrub species (Krauss et al., 2002; Goodall-Copestake et al., 2005). However, research on endangered herbaceous plant species, especially crops and their wild relatives, is scarce (Soleri and Smith, 1995; Parzies et al., 2000). Parzies et al. (2000) reported a decrease in genetic diversity within barley (*Hordeum vulgare* L. ssp. *vulgare*) accessions during the preservation and renewal process of ex situ populations, compared with the corresponding in situ population. Soleri and Smith (1995) also found phenotypic differences between in situ and ex situ populations of Hopi maize (*Zea mays* L.). A thorough understanding of the strengths and weaknesses of in situ and ex situ conservation could help experts to balance conservation strategies and make best use of protected wild crop species (Simberloff, 1988).

Common wild rice (*O. rufipogon*), as the recent common ancestor of cultivated rice (*O. sativa* L.), plays an important role in rice improvement (Sun et al., 2001; Kovach et al., 2007). The northernmost populations of common wild rice worldwide are Dongxiang wild rice (DXWR), which grows in Dongxiang County in China (Gao et al., 2000). Dongxiang wild rice has many invaluable resistance properties, such as highly cold tolerance (Mao et al., 2015). It has been the focus of wild rice conservation because it represents a rare genetic resource for the improvement of cultivated rice. During 1978 to 1982, nine wild rice populations growing at three sites in Dongxiang, including a total of 252 accessions, were collected and conserved in an ex situ conservation garden (Xie et al., 2010). By 2000, however, the nine natural wild rice populations had been reduced to three populations (Zhong et al., 2003; Yang et al., 2005). These three remaining populations were conserved in their original habitat (in situ conservation) in 2003 with the aim of avoiding DXWR extinction. Several studies have analyzed the genetic diversity of DXWR samples collected from the in situ and ex situ populations (Yang et al., 2005; Xie et al., 2010). Their results suggested that ex situ populations showed lower genetic diversity than in situ populations and did not represent the genetic diversity of the in situ populations. Xie et al. (2010) also found that in situ populations of DXWR showed greater heterozygosity than that of any cultivated rice. However, little is known about the genetic structure of ex situ and in situ populations of DXWR. To analyze the genetic relationships of in situ and ex situ populations of DXWR, we analyzed population genetic structure and genetic diversity of nine ex situ populations and three in situ populations using 32 pairs of simple sequence repeat (SSR) primers. This information will provide the scientific basis to develop the best strategies to conserve and exploit DXWR.

MATERIAL AND METHODS

Plant Materials

In total, 252 accessions of DXWR collected from nine natural wild rice populations were conserved ex situ at the Jiangxi Academy of Agricultural Science in 1982. However, because some of the materials were difficult to cultivate and/or propagate, only 224 accessions are survived at the ex situ conservation garden. Due to vegetative propagation of wild rice, the plants are prone to the occurrence of the rhizome propagation during the preservation and renewal process, so it is easy to produce duplication accessions after years of conservation. We used the permutation test (Belkhir et al., 2002) to screen duplicate accessions. The duplicate accessions were only reserved one and finally reserved 190 accessions. Of these, 190 accessions of ex situ were used for this study. The 190 ex situ accessions consisted of 58 accessions from Anjiashan (AJ), 36 from Zhangtang (ZT), 21 from Shuitaoshu (ST), 12 from Kanxialong (KX), seven from Linchang (LC), nine from Dongtang (DT), six from Dongtangxia (TX), 35 from Dongtangshang (TS), and six from Dongtangliangbian (TL). For the in situ populations, 88 individuals were sampled from three geographically distant populations. In each population, samples were collected at intervals of at least 5 m to avoid collecting repeated accessions. Of the 88 in situ accessions, 54 were from AJ, 29 were from ZT, and five were from ST. The three in situ populations corresponded to the AJ, ZT, and ST ex situ populations.

DNA Extraction, Primer Screening, and PCR Amplification

Genomic DNA was extracted from tender plant tissues by the modified cetrimonium bromide (CTAB) method (Tel-zur et al., 1999). The DNA concentration was quantified by ultraviolet spectrophotometry, and the quality of DNA was checked by 1.5% agarose gel electrophoresis. The extracted genomic DNA was diluted to 20 ng μL^{-1} in TE buffer and stored at −20°C. We selected 32 SSRs from 84 alternative microsatellite primer pairs (Wang et al., 2014), which were randomly distributed on the 12 rice chromosomes and were able to produce clear

bands in our samples. Then, polymerase chain reaction (PCR) amplification was performed using a PTC-200 thermal cycler (MJ Research) in a 15-μL reaction mixture containing 7.35 μL double-distilled H_2O, 1.5 μL 10× SSR buffer, 1.5 μL 2 mmol L^{-1} deoxynucleotides, 1.5 μL 25 mmol L^{-1} $MgCl_2$, 1.8 μL 10 mmol L^{-1} primers (0.9 μL each of forward and reverse primer), 5 U μL^{-1} Taq polymerase (0.15 μL), and ~1.2 μL template DNA. The PCR cycling conditions were as follows: 94°C for 2 min, followed by 35 cycles of 94°C for 45 s, 55°C for 1 min, and 72°C for 1 min, with final extension at 72°C for 8 min. Purified PCR products were used for fragment analyses, and the fragments were separated based on size by capillary electrophoresis using a 3500 genetic analyzer (Applied Biosystems). Data were analyzed and genotyping was conducted using GeneMapper software (Applied Biosystems, 2006).

Statistical Analyses

The original SSR data were preprocessed using DataTrans 1.0, which transforms SSR-format files to input files for STRUCTURE, PowerMarker, Tassel, and Popgene (Ge and Ren, 2011). The programs STRUCTURE, PowerMarker, and Tassel, which are based on three different theoretical principals, were used to analyze the phylogenetic relationship of individuals and population structure. STRUCTURE was used to infer genetic clusters (K) of DXWR based on the model-based clustering method (Falush et al., 2003). We assumed K values from 1 to 10 by five independent runs for each K value, and the model was run with a 10,000 burn-in period and 10,000 Monte Carlo Markov chain repetitions. Then, we computed the LnP(D) value and Evanno's ΔK (Evanno et al., 2005) to explore the optimal K value. We used the program CLUMPP version 1.1 (Jakobsson and Rosenberg, 2007) to obtain the optimal alignment of clusters for each K. Phylogenetic relationships were analyzed using the neighbor-joining (NJ) method based on Nei's (1972) standard genetic distance by PowerMarker (Liu and Muse, 2005). The phylogenetic trees were viewed and edited using FigTree 1.4.0 (Rambaut, 2012). A principal component analysis (PCA) was implemented using Tassel 3.0 (Bradbury et al., 2007) to summarize the major patterns of individual genetic differences.

We used Popgene32 (Yeh et al., 2000) to calculate the following genetic diversity parameters: mean number of alleles per locus (Ae), percentage of polymorphic loci (Ppl), Shannon–Weaver information index (I), observed heterozygosity (Ho), expected heterozygosity (He), and fixation index (Fis). Analysis of molecular variance (AMOVA) was implemented using Arlequin 3.0 software (Excoffier et al., 2005).

RESULTS

Microsatellite Polymorphisms and Genetic Diversity

After screening 84 primer pairs with 10 individuals, 32 primer pairs that produced clear bands in most samples were used for further analyses of population genetic diversity and genetic structure (Supplemental Table S1). The 32 loci were spread over the 12 chromosomes of cultivated rice. Different levels of genetic variability were detected among the 32 loci in 278 individuals from 12 populations (Supplemental Table S2). The Ae varied widely among loci and ranged from 1.05 (RM477) to 7.70 (RM567) with an average of 3.52. The He values ranged from 0.27 (RM190) to 0.87 (RM567). The genetic differentiation among three in situ and nine ex situ populations was evaluated based on F-statistic values. The Fis ranged from −0.71 (RM30) to 1 (RM184), with an average of 0.31. Most of the loci had a positive Fis, except for RM30, and the data for RM30 were excluded from analyses of genetic structure and diversity. The gene differentiation index (Fst) ranged from 0.03 to 0.49 (mean = 0.16), indicating that there was genetic variation among populations.

Population Genetic Structure and Genetic Diversity of In Situ Populations

The genetic structure of in situ populations was evaluated using a NJ tree and a PCA. The NJ tree was constructed using PowerMarker software based on Nei's standard genetic distance (Nei, 1972). In situ AJ, ZT, and ST were clearly separated in the NJ tree (Fig. 1a), with individuals from each population clustering together. In addition, in situ ZT and ST were grouped close together and were clearly separated from in situ AJ. These NJ tree results were supported by the results of the PCA analysis. In the PCA plot, the first two eigenvectors explained 24.4% of the total variation (Fig. 1b) and clearly separated the three in situ populations.

To analyze the genetic structure of the populations in more detail, we used STRUCTURE to calculate the values of genetic components and conduct a cluster analysis. The results show a peak of Evanno's ΔK at $K = 2$ (Supplemental Fig. S1). When $K = 2$, in situ populations were divided into two major groups, one (blue) containing AJ, and the other (red) containing ZT and ST (Fig. 1c). Only two admixed individuals (when the Q value of prior population is <0.8) in AJ were detected among the in situ accessions. When $K = 3$, a new inferred cluster (yellow) contained all in situ accessions from ST and some from AJ. All in situ accessions from ST were admixed individuals and were grouped in the new inferred cluster (yellow) with a probability of 0.6. There were 21 (38.89%) admixed individuals in the in situ AJ population. Although all the admixed individuals in the in situ AJ population had recent ancestry in the new inferred cluster (yellow), the probability of the 21 admixed individuals from the new inferred cluster was <0.5. When $K = 4$, another inferred cluster (green) contained mainly individuals from the in situ AJ population. Two inferred clusters (blue and green) also contained individuals from the in situ AJ population. However, the NJ tree and PCA plot showed no clear separation between the two inferred clusters (blue and green) of in situ AJ (Supplemental Fig. S2). Therefore, the STRUCTURE results combined with the NJ tree and PCA plot indicated that the optimal genetic clustering of

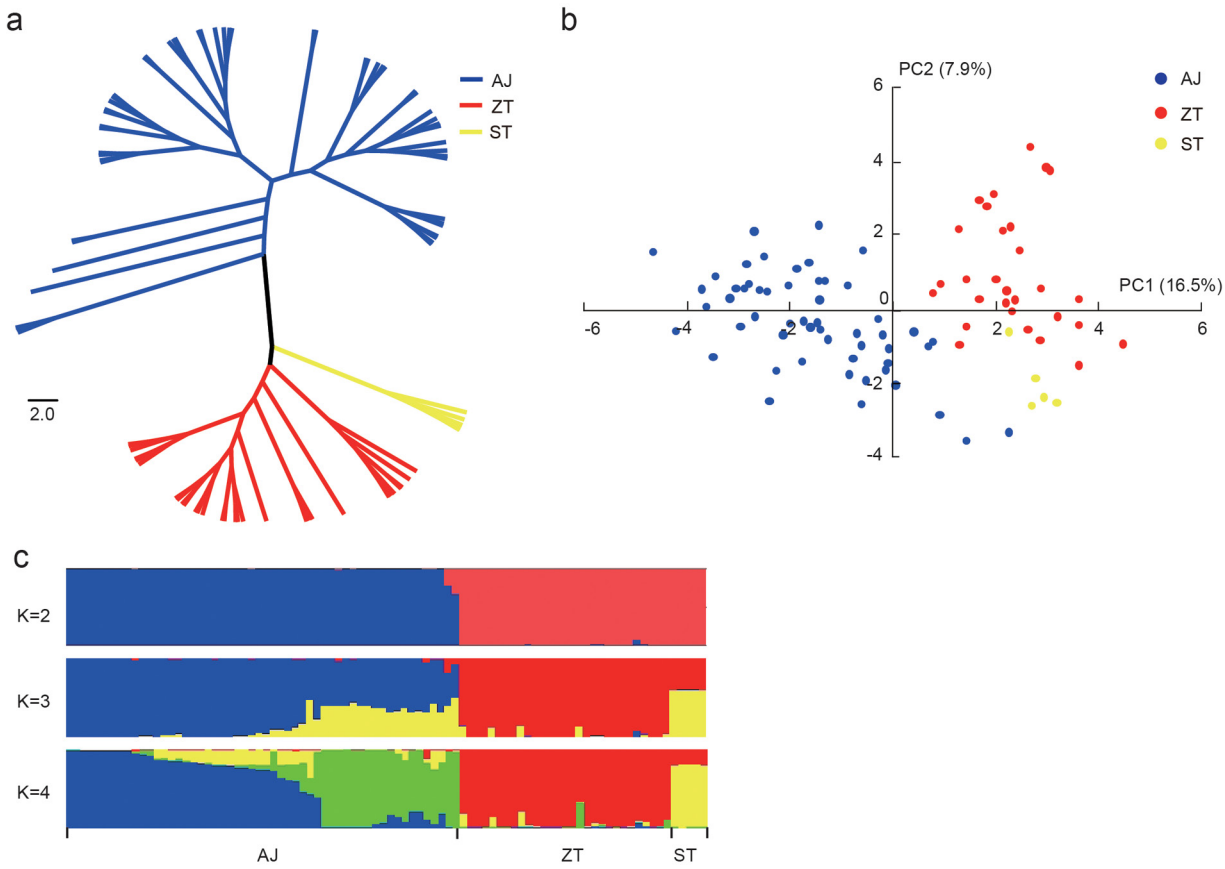

Fig. 1. Population structure analysis of three in situ populations. (a) Phylogenetic tree based on the neighbor-jointing method; AJ, Anjiashan; ZT, Zhangtang; ST, Shuitaoshu. (b) Principal component analysis (PCA). (c) STRUCTURE analysis based on the model-based clustering method.

in situ populations was at $K = 3$. The results also demonstrated that the genetic clustering of in situ populations was related to their geographical distribution.

Population genetic differentiation was determined by an AMOVA analysis (Table 1), which revealed 21.84% variation among three in situ populations. Genetic diversity parameters for all the 32 microsatellite loci for the three in situ populations are shown in Table 2. The in situ ST population showed the lowest level of polymorphism (Ppl = 59.38%, He = 0.28), and in situ AJ showed the highest level of polymorphism (Ppl = 100%) and a high He value (0.57). In situ ZT also showed a high level of polymorphism

Table 1. Analysis of molecular variance results of three in situ populations and nine ex situ populations.

Source of variation	df	Sum of squares	Variance components	Variation	P_{FST}†
				%	
Three in situ populations					
Among populations	2	229.988	2.36608	21.84	<0.001
Within populations	173	1464.688	8.46641	78.16	<0.001
Total	175	1694.676	10.83249		
Nine ex situ populations					
Among populations	8	189.856	0.39013	4.33	<0.001
Within populations	371	3200.152	8.62575	95.67	<0.001
Total	379	3390.008	9.01588		
Three in situ populations and three corresponding ex situ populations					
Among populations	5	440.636	1.24855	12.68	<0.001
Within populations	400	3439.608	8.59902	87.32	<0.001
Total	405	3880.244	9.84757		
Three in situ populations and nine ex situ populations					
Among populations	11	580.203	1.00878	10.44	<0.001
Within populations	554	4707.544	8.65357	89.56	<0.001
Total	555	5287.747	9.66235		

† P_{FST}, the P value of the gene differentiation index (Fst).

Table 2. Genetic diversity of in situ and ex situ populations based on microsatellite loci.

Population‡	Parameter†						
	Sample size	Ae	Ppl	Ho	He	*I*	Fis
			%				
In situ AJ	54	2.87	100.00	0.40	0.57	1.12	2.29
In situ ZT	29	2.60	96.88	0.36	0.53	1.02	0.30
In situ ST	5	1.55	59.38	0.13	0.28	0.42	0.50
Total for in situ	88	3.31	100.00	0.37	0.62	1.28	0.33
Ex situ AJ	58	3.28	100.00	0.34	0.58	1.21	0.40
Ex situ ZT	36	2.93	96.88	0.38	0.55	1.13	0.29
Ex situ ST	21	2.93	96.88	0.34	0.54	1.06	0.36
Total for ex situ AJ, ZT, and ST	115	3.29	100.00	0.35	0.58	1.24	0.36
Ex situ KX	12	2.77	93.75	0.40	0.55	1.05	0.25
Ex situ LC	7	2.46	93.75	0.35	0.53	0.90	0.28
Ex situ DT	9	2.82	90.62	0.38	0.57	1.03	0.28
Ex situ TX	6	2.63	84.38	0.46	0.57	0.96	0.11
Ex situ TS	35	3.05	96.88	0.32	0.58	1.17	0.43
Ex situ TL	6	2.40	87.5	0.36	0.54	0.89	0.26
Total for ex situ	190	3.38	100.00	0.36	0.58	1.27	0.30

† Ae, mean number of alleles per locus; Ppl, percentage of polymorphic loci; Ho, observed heterozygosity; He, expected heterozygosity; *I*, Shannon–Weaver information index; Fis, fixation index.

‡ AJ, Anjiashan; ZT, Zhangtang; ST, Shuitaoshu; KX, Kanxialong; LC, Linchang; DT, Dongtang; TX, Dongtangxia; TS, Dongtangshang; TL, Dongtangliangbian.

(Ppl = 96.88%, He = 0.53), which was slightly lower than that of in situ AJ. We calculated the genetic diversity of in situ groups as a whole. There was a high level of genetic diversity in the in situ group: the values of Ae, Ppl, Ho, He, and *I*, were 3.31, 100%, 0.37, 0.62, and 1.28, respectively.

Population Genetic Structure and Genetic Diversity of Ex Situ Populations

The genetic structure of the ex situ populations was analyzed by a NJ tree and a PCA. The NJ tree grouped the nine ex situ populations into three genetic clusters (Fig. 2a). However, the accessions from the same population did not group together. Instead, accessions from the nine populations were distributed among all three clusters. In the PCA plot, the first two eigenvectors explained 22.82% of the total variation (Fig. 2b). The PCA analysis also revealed no clear population partitioning and demonstrated the complicated genetic structure of the ex situ populations.

We used STRUCTURE to further infer the population genetic structure of the nine ex situ populations (Fig. 2c). The results showed peaks of Evanno's ΔK at $K = 2$ and 4 (Supplemental Fig. S3) and indicated that there were two classifications of the nine ex situ populations at $K = 2$ and 4. When $K = 2$, there were 80 (42.1%) admixed individuals within ex situ populations. However, two genetic clusters were not apparent among the nine ex situ populations. The ex situ populations did not belong to one inferred genetic cluster, and admixed individuals were distributed among almost all of the ex situ populations. When $K = 3$, a new inferred cluster (yellow) consisted of mainly ex situ ZT and ST. There were 95 (50%) admixed individuals within ex situ populations at $K = 3$. When $K = 4$, another new inferred cluster (green) contained mainly the ex situ ZT population, and there were 80 (42.1%) admixed individuals within the ex situ populations. Overall, the STRUCTURE results indicated that there were no clear distinguishable genetic clusters among the nine ex situ populations, consistent with the results of the NJ tree and PCA plot. The results also showed that the numbers of admixed individuals were much higher in ex situ populations than in in situ populations.

The AMOVA analysis revealed 4.33% ($P < 0.001$) variation among the nine ex situ populations, which was much lower than the variation among the three in situ populations (21.84%, Table 1). This low level of variation among the nine ex situ populations explained why accessions from each population failed to cluster together. Genetic diversity parameters were calculated for the nine ex situ populations (Table 2). The values varied among populations, with Ae ranging from 2.40 (ex situ TL) to 3.28 (ex situ AJ), Ho ranging from 0.32 (ex situ TS) to 0.46 (ex situ TX), He ranging from 0.53 (ex situ LC) to 0.58 (ex situ AJ, ex situ TS), and *I* ranging from 0.89 (ex situ TL) to 1.21 (ex situ AJ). Ex situ AJ showed the highest level of polymorphism (Ppl = 100%, He = 0.58) among the ex situ populations. The genetic diversity of three ex situ population groups (AJ, ZT, and ST) and nine ex situ population groups were also calculated (Table 2). These two groups both showed a high level of polymorphism (Ppl = 100%, He = 0.58), and had similar genetic diversity.

Population Genetic Structure and Genetic Diversity of In Situ and Ex Situ Populations

To evaluate the genetic variation between the ex situ and the in situ populations as a whole, we analyzed the population structure of the combined accessions of three in situ

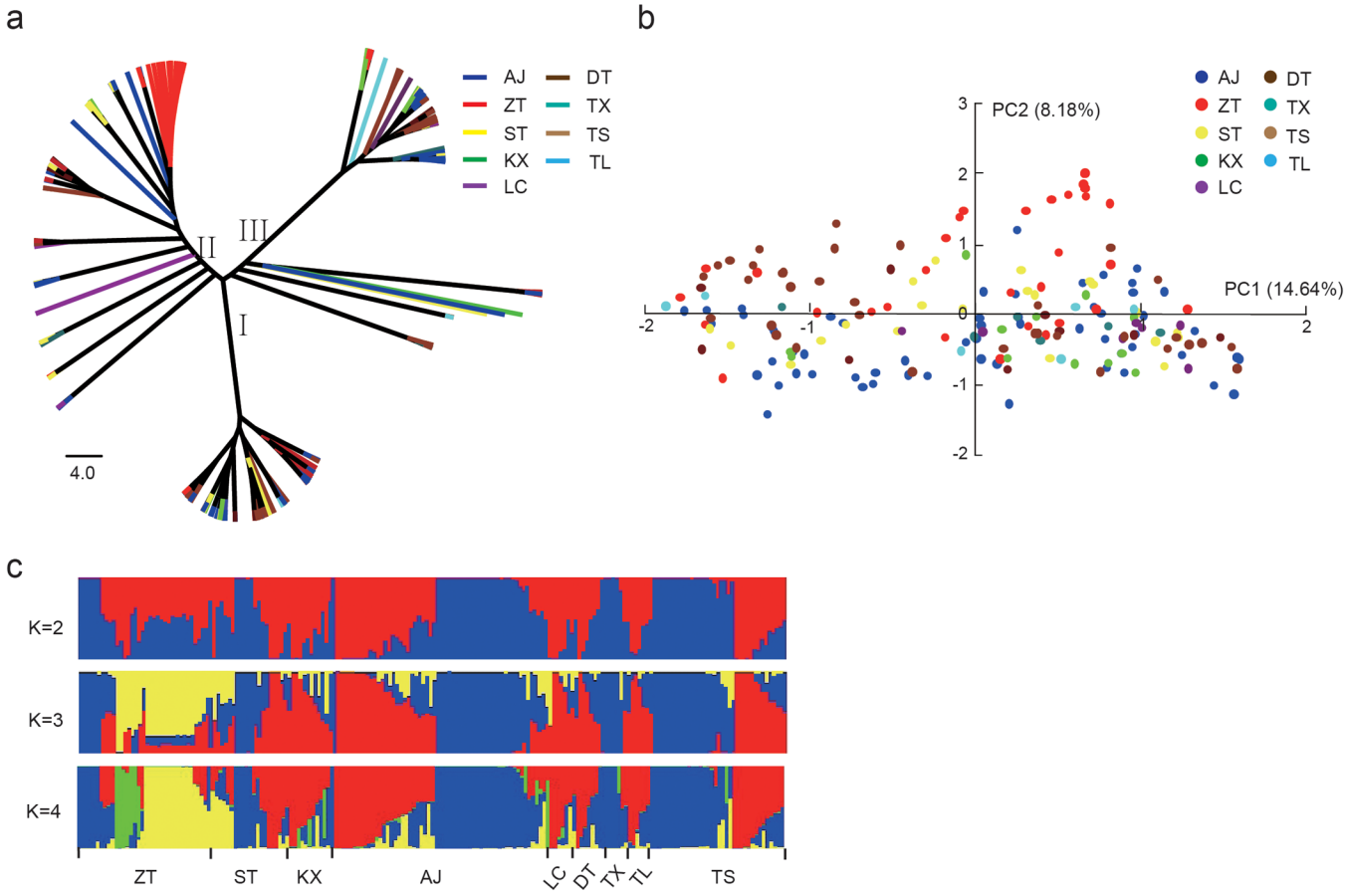

Fig. 2. Population structure analysis of nine ex situ populations. (a) Phylogenetic tree based on the neighbor-jointing method; AJ, Anjiashan; DT, Dongtang; ZT, Zhangtang; TX, Dongtangxia; ST, Shuitaoshu; TS, Dongtangshang; KX, Kanxialong; TL, Dongtangliangbian; LC, Linchang. (b) Principal component analysis (PCA). (c) STRUCTURE analysis based on the model-based clustering method.

and nine ex situ populations. As shown in the NJ tree, the accessions of in situ and ex situ populations formed three clusters (Fig. 3a). The first cluster (I) contained 71 ex situ accessions and all accessions of in situ ST. The second cluster (II) contained two ex situ accessions and all accessions of in situ ZT. The third cluster (III) contained the remaining ex situ accessions and 53 accessions of in situ AJ. Two of the in situ populations, ZT and ST, grouped together. However, accessions from in situ AJ did not group together but were distributed with ex situ accessions. The NJ tree also indicated that the ex situ populations did not contain any in situ ZT accessions. In the PCA plot, the first two eigenvectors explained 19.65% of the total variation (Fig. 3b). The PCA plot supported the NJ tree results.

We used STRUCTURE to confirm the population structure of the combined accessions (Fig. 3c). When $K = 2$, there were 90 (32.37%) admixed individuals, of which 74 (38.95%) were from the ex situ populations, 13 (24.07%) were from in situ AJ, and three were from in situ ZT. When $K = 3$, a new inferred cluster (red) contained mainly in situ ZT, and all in situ ST accessions had >0.3 probability from the new inferred cluster. When $K = 3$, there were 106 admixed individuals (38.13%), of which 81 (42.63%) were from the ex situ populations, 18 (33.33%) were from in situ AJ, two (6.89%) were from in situ ZT, and five (100%) were from in situ ST. When $K = 4$, another new inferred cluster (yellow) contained mainly ex situ accessions and in situ AJ. There were 131 admixed individuals (38.13%), of which 87 (42.63%) were from the ex situ populations, 37 (68.52%) were from in situ AJ (33.33%), two (6.89%) were from in situ ZT, and five (100%) were from in situ ST. As shown in the plots, the genetic structure at $K = 4$ was similar to that at $K = 3$, but there were more admixed individuals at $K = 4$. Therefore, the optimal genetic structure was at $K = 3$. Together, the results showed that all in situ ZT accessions clustered together and were separate from the ex situ accessions, but all in situ AJ accessions were distributed with ex situ accessions, consistent with the NJ tree and PCA plot. In addition, the in situ ZT population contained almost no admixed individuals, indicating that it is a recent population.

We constructed a NJ tree of three in situ populations and the three corresponding ex situ populations (Supplemental Fig. S4). The NJ tree did not separate the in situ populations and the ex situ populations. Except for in situ ZT and ST, the accessions from other populations were grouped together, and only in situ ZT was

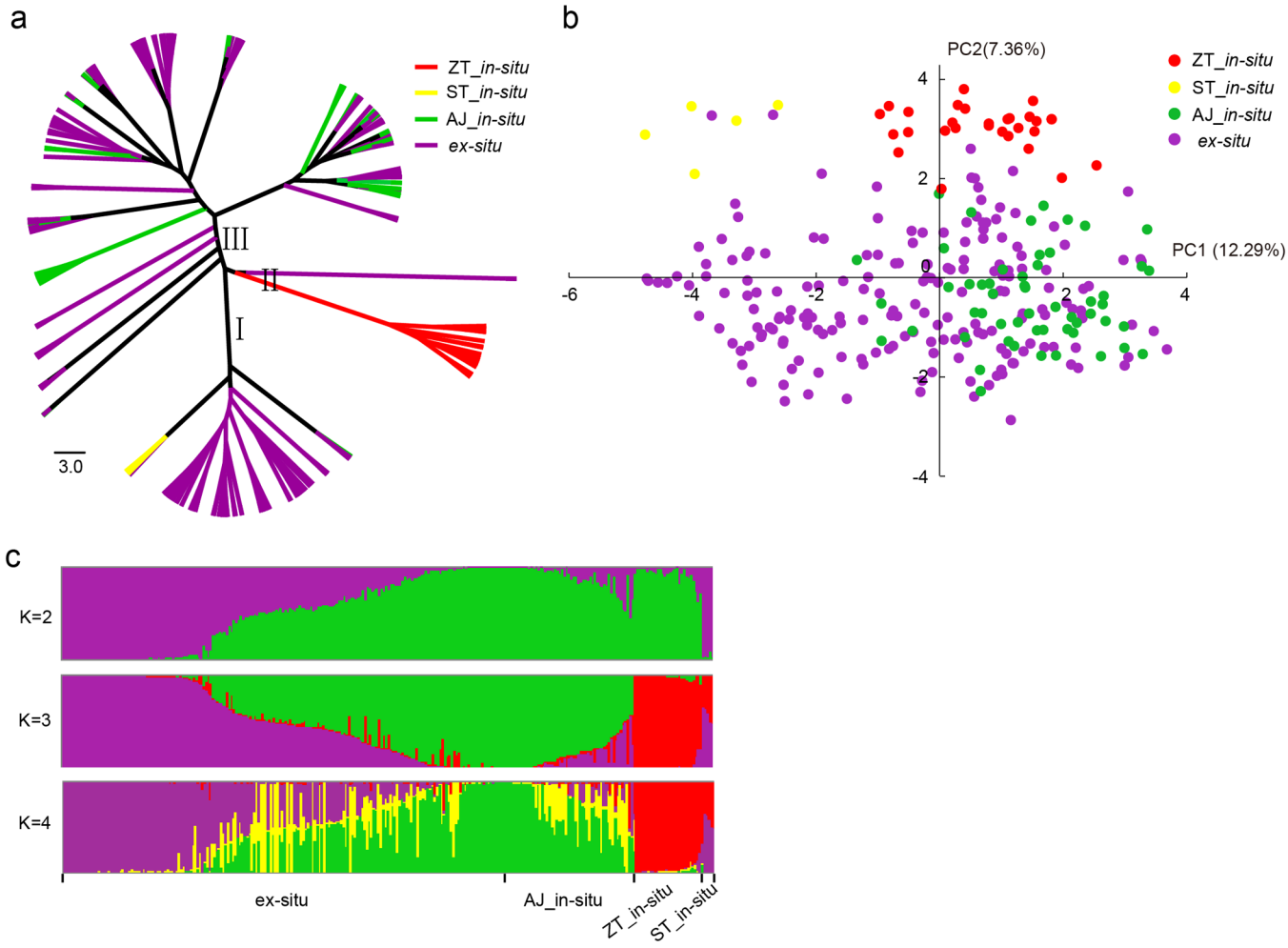

Fig. 3. Population structure analysis of three in situ populations and nine ex situ populations. (a) Phylogenetic tree based on the neighbor-jointing method; AJ, Anjiashan; ZT, Zhangtang; ST, Shuitaoshu. (b) Principal component analysis (PCA). (c) STRUCTURE analysis based on the model-based clustering method.

clearly separated from the three ex situ populations. The AMOVA analysis revealed 10.44% ($P < 0.001$) variation among the three in situ and nine ex situ populations and 12.68% ($P < 0.001$) variation among the three in situ and their three corresponding ex situ populations, which all showed greater variation within than among populations. Comparing the genetic diversity between the three in situ populations and the three corresponding ex situ populations, the level of genetic diversity was slightly higher for accessions from the three in situ populations (Ae = 3.31, He = 0.62) than for accessions from the three corresponding ex situ populations (Ae = 3.29, He = 0.58).

DISCUSSION

Significant Difference in Genetic Structure between In Situ and Ex Situ Populations of DXWR

As pointed out by previous study about three in situ and nine ex situ populations of DXWR, accessions of two in situ populations (AJ and ZT) and all ex situ populations did not cluster together, respectively, and there was good separation of the three in situ populations from three corresponding ex situ populations (Xie et al., 2010). In present study, we analyzed 278 accessions from in situ (88) and ex situ (190) populations of DXWR using multiple approaches and found that there were significant differences between in situ and ex situ populations. The in situ populations clustered into three groups, AJ, ZT, and ST (Fig. 1), indicating that their genetic clustering was related to their geographical distribution. The AMOVA analysis showed highly significant genetic differentiation among the three geographical populations (Fst = 0.22, $P < 0.001$). The genetic structure of in situ populations demonstrated that there was reproductive isolation among these populations in their original habitat. However, the genetic structure of the ex situ populations was more complicated. Accessions of each ex situ population did not cluster together, respectively (Fig. 2), which was also indicated by Xie et al. (2010). Individuals from the nine ex situ populations were mixed together with a low level of variation (4.33%) among populations (Table 1). In addition, the NJ tree of three in situ populations and three

corresponding ex situ populations indicated that there was not a separation between in situ and ex situ populations (Supplemental Fig. S4), which was not consistent with the cluster results of these six populations in Xie et al. (2010); however, we hold that the in situ populations and their corresponding ex situ populations should not have a clearly separation but have similar genetic relationships. The NJ tree of all ex situ and in situ accessions showed that ex situ accessions did not contain any accessions from the in situ ZT population (Fig. 3) from the perspective of kinship. We also found that the numbers of admixed individuals were much higher in ex situ populations than in in situ populations. These results indicated that the ex situ population genetic structure has changed greatly.

The significant difference in the genetic structure between ex situ and in situ populations was possibly resulted from the irregular collection and conservation processes. At the time of collection, there was limited information on the growth and breeding characteristics of common wild rice, as well as the limits of scientific and technological development at that time, and the sample collection and germplasm preservation techniques for ex situ conservation may not have been optimal. Our study found that the phylogenetic relationships of ex situ accessions were consistent with only one in situ population. It appears that the samples collected for ex situ conservation were not representative of the level of natural diversity. How to select the best sampling sites and the optimum number of samples are common problems for conservation biologists and germplasm resource experts.

In our results, spatial or physical isolation and local adaptation might be major factors contributing to the population divisions within this species. We detected significant genetic differences among the three geographical groups, which highlights the importance of in situ and ex situ conservation of these three groups. The strategies to collect germplasm from geographically separated areas to maximize genetic resources for ex situ collections were also substantiated by our results. Sampling from many sites and maintaining ex situ collections of those accessions may be the best strategies to preserve the germplasm resources of DXWR (Votava et al., 2002). Also, a high proportion of admixed accessions was present in ex situ populations. There may be human and/or technical errors during the renewal and conservation of plants in the ex situ conservation garden, resulting in genetic drift causing genetic introgression for the ex situ conservation populations (Lawrence et al., 2002). Dongxiang wild rice is a perennial and outcrossing plant that can be propagated sexually or asexually, so each accession of DXWR has been harvested and replanted each year. After several generations, small populations often show reduced genetic variability because of inbreeding caused by reduced gene flow between populations and genetic drift (Leimu et al., 2006). This could explain the change in population genetic structure over multiple propagation cycles and changes in the habitat. In the natural ecological environment, genetic variation in wild plants can occur during interaction with the environment. Genetic variation plays an essential and decisive role in population genetic structure. Certainly, the ecological environment differs between in situ and ex situ conserved populations and environmental changes could lead to a loss or gain of variability, thus leading to changes in the genetic structure of the populations.

Conservation of DXWR

The wild rice species DXWR represents a very precious rice germplasm resource (Zhang et al., 2006; Xie et al., 2010), and this important genepool should be conserved. In recent years, a large number of populations of this species have faced extinction in human-dominated ecosystems (Gao et al., 2000). The nine populations of DXWR in isolated areas in 1978 had decreased to three by 2000 (Zhong et al., 2003). Because of this dramatic reduction, the unique genepool is now endangered, and it is imperative to conserve it for the effective use and protection of the remaining genetic resources (Xie et al., 2010).

The SSR data obtained in this study indicated that the ex situ accessions (Ae = 3.31, Ppl = 100%, He = 0.62), as a whole, had a similar high level of diversity as in situ accessions (Ae = 3.38, Ppl = 100%, He = 0.58). In the current study, all populations in the in situ and ex situ groups had positive Fis values. A positive Fis value indicates that there is a deficiency of heterozygous offspring within a population (Zhou et al., 2003; Stoeckel et al., 2006). The Fis, also known as the population inbreeding coefficient, indicates whether the natural population deviates from the ideal population based on the Hardy–Weinberg expectation (Wright, 1965). Zhou et al. (2003) also detected positive Fis values in many *O. rufipogon* populations and concluded that the deficiency of heterozygosity may be caused by inbreeding. Both ex situ and in situ populations of DXWR, and especially subpopulations, should be conserved to maintain the extant genetic diversity of this species. For in situ conservation, among the three surviving populations, the in situ ST population showed the lowest genetic diversity (Ae = 1.55, Ppl = 59.38%, He = 0.28). The ability of in situ populations to maintain their inherent genetic diversity is related to their natural ecological environment (Hughes et al., 2008). The ZT populations grow in marsh areas where there is a sufficient water supply, whereas the ST population grows in a seasonal drought environment corresponding to the flowering time of wild rice (Xie et al., 2010). The decrease in the genetic diversity of in situ ST may be related to changes in its ecological environment. The in situ ST population has only four accessions, and it is likely that the small population size has contributed to the low level of genetic diversity.

When there are rapid changes in the in situ environment, ex situ conservation is better than none, as the offspring can be used to supplement in situ populations with low recruitment. Therefore, to protect DXWR, the combination of these two conservation strategies is necessary. According to the results of this study, the following points should be considered for ex situ conservation: first, there were no accessions from the in situ ZT population among the ex situ accessions. Therefore, the ZT population should be sampled again based on the population sampling principle (Xie et al., 2001), and the collected individuals should be planted in the ex situ conservation garden. This would ensure that completely distinct genotypes from a population are preserved and would better represent the extant genetic diversity of DXWR. Additional recommendations for ex situ conservation are related to the transplanting and renewal processes. The collected samples should be numbered individually to ensure their preservation. Also, because of the growing and breeding characteristics of DXWR, each plant should be grown in a separate pot to ensure strict reproductive isolation. We also recommend ex situ cultivation in a near-natural environment, allowing for generation overlap, as proposed in previous studies (Volis and Blecher, 2010; Lauterbach et al., 2012). For ex situ conservation, the main goal is to protect populations from extinction and to safeguard representative material for reinforcement programs to establish a remote wild population (Lauterbach et al., 2012). However, consistent with the findings of Xie et al. (2010), our results showed that the nine ex situ populations of DXWR have not maintained their genetic identities, as reflected by the change in the genetic structure of ex situ populations due to the effects of genetic drift and gene flow between individuals. Therefore, our results highlight the importance of in situ conservation and emphasize that special emphasis should be given to in situ conservation of DXWR.

Conflict of Interest

The authors declare that there is no conflict of interest.

Supplemental Material Available

Supplemental material for this article is available online.

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References

Applied Biosystems. 2006. AmpFlSTR® Yfiler™: PCR amplification kit. User's manual. Applied Biosystems, Foster City, CA. http://projects.nfstc.org/workshops/resources/literature/Amplification/yfiler-users-manual.pdf (accessed 24 July 2017).

Belkhir, K., V. Castric, and F. Bonhomme. 2002. IDENTIX, a software to test for relatedness in a population using permutation methods. Mol. Ecol. Notes 2:611–614. doi:10.1046/j.1471-8286.2002.00273.x

Bradbury, P.J., Z. Zhang, D.E. Kroon, T.M. Casstevens, Y. Ramdoss, and E.S. Buckler. 2007. TASSEL: Software for association mapping of complex traits in diverse samples. Bioinformatics 23:2633–2635. doi:10.1093/bioinformatics/btm308

Enßlin, A., T.M. Sandner, and D. Matthies. 2011. Consequences of ex situ cultivation of plants: Genetic diversity, fitness and adaptation of the monocarpic *Cynoglossum officinale* L. in botanic gardens. Biol. Conserv. 144:272–278. doi:10.1016/j.biocon.2010.09.001

Evanno, G., S. Regnaut, and J. Goudet. 2005. Detecting the number of clusters of individuals using the software STRUCTURE: A simulation study. Mol. Ecol. 14:2611–2620. doi:10.1111/j.1365-294X.2005.02553.x

Excoffier, L., G. Laval, and S. Schneider. 2005. Arlequin (version 3.0): An integrated software package for population genetics data analysis. Evol. Bioinform. Online 1:47–50.

Falush, D., M. Stephens, and J.K. Pritchard. 2003. Inference of population structure using multilocus genotype data: Linked loci and correlated allele frequencies. Genetics 164:1567–1587.

Gao, L.Z., W. Chen, W.Z. Jiang, S. Ge, D.Y. Hong, and X.K. Wang. 2000. Genetic erosion in northern marginal population of the common wild rice *Oryza rufipogon* Griff, and its conservation, revealed by the change of population genetic structure. Hereditas 133:47–53. doi:10.1111/j.1601-5223.2000.00047.x

Ge, H.M., and M. Ren. 2011. DataTrans1.0: A software for microsatellite data processing based on Excel Macro. Mol. Plant Breed. 9:1359–1365.

Goodall-Copestake, W.P., M.L. Hollingsworth, P.M. Hollingsworth, G.I. Jenkins, and E. Collin. 2005. Molecular markers and ex situ conservation of the European elms (*Ulmus* spp.). Biol. Conserv. 122:537–546. doi:10.1016/j.biocon.2004.09.011

Henry, P., D. Miquelle, T. Sugimoto, D.R. McCullough, A. Caccone, and M.A. Russello. 2009. In situ population structure and ex situ representation of the endangered Amur tiger. Mol. Ecol. 18:3173–3184. doi:10.1111/j.1365-294X.2009.04266.x

Hughes, A.R., B.D. Inouye, M.T.J. Johnson, N. Underwood, and M. Vellend. 2008. Ecological consequences of genetic diversity. Ecol. Lett. 11:609–623. doi:10.1111/j.1461-0248.2008.01179.x

Jakobsson, M., and N.A. Rosenberg. 2007. CLUMPP: A cluster matching and permutation program for dealing with label switching and multimodality in analysis of population structure. Bioinformatics 23:1801–1806. doi:10.1093/bioinformatics/btm233

Kovach, M.J., M.T. Sweeney, and S.R. McCouch. 2007. New insights into the history of rice domestication. Trends Genet. 23:578–587. doi:10.1016/j.tig.2007.08.012

Krauss, S.L., B. Dixon, and K.W. Dixon. 2002. Rapid genetic decline in a translocated population of the endangered plant *Grevillea scapigera*. Conserv. Biol. 16:986–994. doi:10.1046/j.1523-1739.2002.01105.x

Lauterbach, D., M. Burkart, and B. Gemeinholzer. 2012. Rapid genetic differentiation between ex situ and their in situ source populations: An example of the endangered *Silene otites* (Caryophyllaceae). Bot. J. Linn. Soc. 168:64–75. doi:10.1111/j.1095-8339.2011.01185.x

Lawrence, J.G., G.F. Hatfull, and R.W. Hendrix. 2002. Imbroglios of viral taxonomy: Genetic exchange and failings of phenetic approaches. J. Bacteriol. 184:4891–4905. doi:10.1128/JB.184.17.4891-4905.2002

Leimu, R., P.I.A. Mutikainen, J. Koricheva, and M. Fischer. 2006. How general are positive relationships between plant population size, fitness and genetic variation? J. Ecol. 94:942–952. doi:10.1111/j.1365-2745.2006.01150.x

Li, Q., Z. Xu, and T. He. 2002. Ex situ genetic conservation of endangered *Vatica guangxiensis* (Dipterocarpaceae) in China. Biol. Conserv. 106:151–156. doi:10.1016/S0006-3207(01)00240-3

Liu, K., and S.V. Muse. 2005. PowerMarker: An integrated analysis environment for genetic marker analysis. Bioinformatics 21:2128–2129. doi:10.1093/bioinformatics/bti282

Mao, D.H., L. Yu, D.Z. Chen, L.Y. Li, Y.X. Zhu, Y.Q. Xiao et al. 2015. Multiple cold resistance loci confer the high cold tolerance adaptation of Dongxiang wild rice (*Oryza rufipogon*) to its high-latitude habitat. Theor. Appl. Genet. 128:1359–1371. doi:10.1007/s00122-015-2511-3

Namoff, S., C.E. Husby, J. Francisco-Ortega, L.R. Noblick, C.E. Lewis, and M.P. Griffith. 2010. How well does a botanical garden collection of a rare palm capture the genetic variation in a wild population? Biol. Conserv. 143:1110–1117. doi:10.1016/j.biocon.2010.02.004

Nei M. 1972. Genetic distance between populations. The American Naturalist. 106:283–292. doi:10.1086/282771

Nevo, E. 1998. Genetic diversity in wild cereals: Regional and local studies and their bearing on conservation ex situ and in situ. Genet. Resour. Crop Evol. 45:355–370. doi:10.1023/A:1008689304103

Parzies, H.K., W. Spoor, and R.A. Ennos. 2000. Genetic diversity of barley landrace accessions (*Hordeum vulgare* ssp *vulgare*) conserved for different lengths of time in *ex situ* gene banks. Heredity 84:476–486. doi:10.1046/j.1365-2540.2000.00705.x

Rambaut A. 2012. FigTree v1.4. Molecular evolution, phylogenetics and epidemiology. Univ. of Edinburgh, Inst. of Evol. Biol., Edinburgh, UK.

Rucińska, A., and J. Puchalski. 2011. Comparative molecular studies on the genetic diversity of an ex situ garden collection and its source population of the critically endangered polish endemic plant *Cochlearia polonica* E. Fröhlich. Biodivers. Conserv. 20:401–413. doi:10.1007/s10531-010-9965-z

Simberloff, D. 1988. The contribution of population and community biology to conservation science. Annu. Rev. Ecol. Syst. 19:473–511. doi:10.1146/annurev.es.19.110188.002353

Soleri, D., and S.E. Smith. 1995. Morphological and phenological comparisons of two Hopi maize varieties conserved in situ and ex situ. Econ. Bot. 49:56–77. doi:10.1007/BF02862278

Stoeckel, S., J. Grange, J.F. Fernandez-Manjarres, I. Bilger, N. Frascaria-Lacoste, and S. Mariette. 2006. Heterozygote excess in a self-incompatible and partially clonal forest tree species: *Prunus avium* L. Mol. Ecol. 15:2109–2118. doi:10.1111/j.1365-294X.2006.02926.x

Sun, C.Q., X.K. Wang, Z.C. Li, A. Yoshimura, and N. Iwata. 2001. Comparison of the genetic diversity of common wild rice (*Oryza rufipogon* Griff.) and cultivated rice (*O. sativa* L.) using RFLP markers. Theor. Appl. Genet. 102:157–162. doi:10.1007/s001220051631

Tel-zur, N., S. Abbo, D. Myslabodski, and Y. Mizrahi. 1999. Modified CTAB procedure for DNA isolation from epiphytic cacti of the genera *Hylocereus* and *Selenicereus* (Cactaceae). Plant Mol. Biol. Report. 17:249–254. doi:10.1023/A:1007656315275

Volis, S., and M. Blecher. 2010. Quasi in situ: A bridge between ex situ and in situ conservation of plants. Biodivers. Conserv. 19:2441–2454. doi:10.1007/s10531-010-9849-2

Votava, E.J., G.P. Nabhan, and P.W. Bosland. 2002. Genetic diversity and similarity revealed via molecular analysis among and within an in situ population and ex situ accessions of chiltepin (*Capsicum annuum* var. *glabriusculum*). Conserv. Genet. 3:123–129. doi:10.1023/A:1015216504565

Wang, C.H., X.M. Zheng, Q. Xu, X.P. Yuan, L. Huang, H.F. Zhou et al. 2014. Genetic diversity and classification of *Oryza sativa* with emphasis on Chinese rice germplasm. Heredity 112:489–496. doi:10.1038/hdy.2013.130

Wright, S. 1965. The interpretation of population structure by *F*-statistics with special regard to systems of mating. Evolution 19:395–420. doi:10.1111/j.1558-5646.1965.tb01731.x

Xie, J., H.A. Agrama, D. Kong, J. Zhuang, B. Hu, Y. Wan et al. 2010. Genetic diversity associated with conservation of endangered Dongxiang wild rice (*Oryza rufipogon*). Genet. Resour. Crop Evol. 57:597–609. doi:10.1007/s10722-009-9498-z

Xie, Z.W., Y.Q. Lu, S. Ge, D.Y. Hong, and F.Z. Li. 2001. Clonality in wild rice (*Oryza rufipogon*, Poaceae) and its implications for conservation management. Am. J. Bot. 88:1058–1064. doi:10.2307/2657088

Yang, Q.W., L.Q. Yu, W.X. Zhang, D.Z. Chen, J.X. Shi, J.F. Ren et al. 2005. Comparative studies on genetic diversities between *in-situ* and *ex-situ* conserved germplasm of *oryza rufipogon*. Zhongguo Nongye Kexue (Beijing, China) 38:1073–1079. doi:10.3321/j.issn:0578-1752.2005.06.001

Yeh, F.C., R. Yang, T.J. Boyle, Z. Ye, and J.M. Xiyan. 2000. PopGene32, Microsoft Windows-based freeware for population genetic analysis, version 1.32. Univ. of Alberta, Edmonton, AB.

Zhang, X., S. Zhou, Y. Fu, Z. Su, X. Wang, and C. Sun. 2006. Identification of a drought tolerant introgression line derived from Dongxiang common wild rice (*O. rufipogon* Griff.). Plant Mol. Biol. 62:247–259. doi:10.1007/s11103-006-9018-x

Zhong, P.N., Y.J. Huang, and D.Z. Chen. 2003. Conservation of Dongxiang wild rice (*Oryza rufipogon* Griff.) genetic resources and their utilization in breeding. Jiangxi Nongye Daxue Xuebao 25:12–16. doi:10.3969/j.issn.1000-2286.2003.01.004

Zhou, H.F., Z.W. Xie, and S. Ge. 2003. Microsatellite analysis of genetic diversity and population genetic structure of a wild rice (*Oryza rufipogon* Griff.) in China. Theor. Appl. Genet. 107:332–339. doi:10.1007/s00122-003-1251-y