Landscape-Scale Genetic Structure of Wild Rice *Zizania latifolia*: The Roles of Rivers, Mountains and Fragmentation

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Wild rice (*Zizania latifolia*) is an important genetic resource and it has important ecological functions in aquatic ecosystems as well. Here, we used landscape genetics to investigate how the landscape features, including rivers, mountains and habitat fragmentation, affect the genetic connectivity or create dispersal barriers for *Z. latifolia*. In this report, seventeen populations from the Sanjiang Plain and its surrounding areas were genotyped by ten microsatellite markers. A high genetic differentiation and genetic discontinuity were found among populations within each river investigated, suggesting that the rivers be not acting as corridors for dispersal. Meanwhile, genetic discontinuity was detected from different sides of the Lesser Khingan and its branch, the Qinghei Mountains, demonstrating that gene flow was blocked by the complex topography of mountains. The results that historical gene flow was much higher than the contemporary gene flow might infer that recent habitat fragmentation resulted in decreased gene flow. For all sampled *Z. latifolia* populations, the result of low level of genetic variation ($n_a = 2.3$, $H_E = 0.328$) and high genetic divergence ($F_{ST} = 0.405$, $D_{est} = 0.414$ and $Ø_{pt} = 0.424$) was consistent with the decreased gene flow, an inbreeding system and repeated genetic bottlenecks. A conservation strategy for protecting as many populations as possible to maximize genomic representation of the species is proposed. In addition, dredging the watercourses should be carried out to improve habitat stability and to facilitate connectivity among populations.

Keywords: landscape genetic structure, habitat fragmentation, genetic barriers, gene flow, corridor function, *Zizania latifolia*

INTRODUCTION

Dispersal is a key process to determine the fate of populations and species, and it becomes more and more important due to pressures from habitat fragmentation, land-use change and climate change (Aavik et al., 2013; Sondermann et al., 2015; Wubs et al., 2016). Likewise, species' genetic diversity can significantly contribute to their adaptability to ever-changing environments (Hughes et al., 2008). Therefore, a good understanding of gene flow patterns and genetic diversity could help effective protection of endangered species and quality management of economically or ecologically important species (Yan et al., 2016).

With the advanced molecular tools, some investigators developed a landscape genetic approach to study the effects of landscape composition, configuration and matrix quality on gene flow and spatial genetic variation (Manel et al., 2003). One of the major topics of landscape genetics is to identify potential barriers or corridors for dispersal (Guillot et al., 2005; Storfer et al., 2007). For example, rivers, which facilitate movement of individuals and genes across the landscape, are generally recognized as corridors for riparian or aquatic plants (Chen et al., 2012a,b; Yan et al., 2016). On the other hand, mountain ridges as geographical barriers may hinder gene flow and cause genetic differentiation in plants (Tsuda et al., 2010). Fragmentations in habitats may disrupt or reduce gene flow and erode the genetic variation of plants (Werth et al., 2014). In a riparian-mountain landscape, rivers or streams in mountainous areas are usually located at the bottom of valleys, and the distribution patterns of streamside or riparian plants are generally fragmented by natural (e.g., highlands) and man-made (e.g., dams and reclamation) barriers. In this situation, it is difficult to predict the genetic structure of riparian species when the competing factors (rivers, mountains and fragmentation) might work together on historical or current dispersal of seeds or vegetative propagules. The consequences of fragmentation in riparian-mountain habitats are less well understood, though previous researches are available for a few streamside tree species (Hu et al., 2010; Wei et al., 2013). In the example of the streamside tree, *Euptelea pleiospermum* Hook. f. et Thoms, mountain ridges were genetic barriers; whereas river valleys provided important corridors for dispersal (Wei et al., 2013). If growth and dispersal were considered, riparian or aquatic plants may be more dependent upon rivers. Based on current available data, there raise some questions: Can we make the same conclusion as for the tree species? Or is the corridor function of rivers reduced by fragmentations?

The Sanjiang Plain is the largest area of freshwater marsh in China. The alluvial plain is drained by the Heilong River and its main tributaries (the Songhua River and Wusuli River), and it is surrounded by mountains (the Wanda Mountains and Qinghei Mountains). However, the plain has suffered from intensive reclamation over the past 50 years (1954–2005) as shown by the facts that more than 75% of its wetlands have disappeared (Wang et al., 2011). The range of geographical and anthropogenic factors in the plain provided excellent opportunities to study the genetic consequences of human-induced fragmentation in riparian-mountain habitats. Identifying the genetic structure of riparian and aquatic plants across threatened aquatic systems is a pre-requisite for managing their ecosystem services (James et al., 2013).

Zizania latifolia Turcz., known as Manchurian wild rice, is a perennial, aquatic or paludal, monoecious grass that grows along the littoral of freshwater marshes and streams and it is native to East Asia (Chen S. et al., 2006). *Z. latifolia* is widely distributed in wetlands of the Sanjiang Plain. It is wind-pollinated to reproduce sexually by seeds or asexually by rhizomes. *Z. latifolia* is a pioneer weed species for dyke consolidation because of its high clonal reproduction; it also has a high nutrient uptake capacity which is used to purify wastewater. Its seeds or young shoots are popular foods or vegetables in China (Guo

et al., 2007). Moreover, some eminent traits in *Z. latifolia* are used for rice breeding owing to its close relationship to rice (*Oryza sativa* L.) (Chen Y. et al., 2006). And the release of genome sequences of *Z. latifolia* would greatly accelerate the progress of molecular breeding related to the species (Guo et al., 2015). Therefore, wild *Z. latifolia* populations not only have important ecological functions in aquatic ecosystems, but also are important genetic resources. Understanding genetic diversity of the valuable species would help effectively implement conservation practices for the species and rationally utilize the genetic resources (Yan et al., 2016). Abundant genetic variations with weak population structure were previously reported using ten nuclear microsatellites markers in *Z. latifolia* from lakes of the Middle-lower Yangtze Plain, a potamo-lacustrine system in central China (Chen et al., 2012a). In another report, nine *Z. latifolia* populations from the Sanjiang Plain in northeastern China were shown a low level of genetic diversity with evident population structure based on SRAP markers (Fan et al., 2016), and the results inferred that mountains and fragmentation might contribute greatly to the genetic patterns of *Z. latifolia* in the Sanjiang Plain. However, the effects of rivers, mountains and fragmentation on gene flow were not cautiously verified (Fan et al., 2016).

Simple sequence repeat (SSR, or microsatellite) markers are ideal for genetic studies of population structures due to their codominance and high reproducibility. Here, we used population genetic analyses of microsatellite markers to investigate the roles of mountains, rivers and fragmentation on dispersal /population structure of *Z. latifolia* from the Sanjiang Plain and its surrounding area. The questions we would like to address are: (i) within one river, has the direction of water flow dominated the level of gene flow: water flow or habitat fragmentation? (ii) have the mountains blocked the gene exchange and where is the geographical location of the genetic discontinuities? (iii) has the fragmentation resulted in the decrease of gene flow, and what are the levels of historical and contemporary gene flows among populations? Additionally, high level of genetic diversity in *Z. latifolia* is expected in this study due to the nature of hyper-variability of the SSR markers. Our study would give more insights into the genetic structure and gene flow of the aquatic plant *Z. latifolia* from critically fragmented habitats, and should be beneficial for developing appropriate restoration strategies for the threatened aquatic systems.

MATERIALS AND METHODS

Investigation Area and Sample Collection

The Sanjiang Plain is a river basin illuviated by the Heilong River, Songhua River and Wusuli River, and it is located in Northeast China. Seventeen *Z. latifolia* populations (named ZL1 to ZL17) were collected from the Sanjiang Plain and its neighboring areas (**Table 1**; **Figure 1**; Appendix 1 in Supplementary Material). Populations ZL1-4 were from the Nenjiang River basin, the largest tributary of Songhua River. Populations ZL5-9, ZL10-13, and ZL14-17 were along the Heilong River, Wusuli River and Songhua River, respectively. For each population, 20-24 individuals were sampled separately at intervals of at least 10 m

TABLE 1 | Location, habitats and summary measures of clonal diversity and genetic variation for each population of *Zizania latifolia*.

Population	Habitats	Affiliation to rivers	N	G	D	n_a	n_e	H_O	H_E	F_{IS}
ZL1	Reservoir	Nenjiang River	24	11	0.844	1.8	1.4	0.282	0.242	−0.119[NS]
ZL2	Pond	Nenjiang River	22	11	0.896	1.8	1.6	0.227	0.256	0.160[*]
ZL3	Pond	Nenjiang River	24	18	0.942	2.3	1.6	0.211	0.277	0.263[***]
ZL4	Marsh	Nenjiang River	21	21	1.000	2.5	1.4	0.248	0.251	0.039[NS]
ZL5	Pond	Heilong River	15	15	1.000	2.3	1.7	0.340	0.348	0.059[NS]
ZL6	Pond	Heilong River	24	17	0.964	2.1	1.5	0.235	0.300	0.244[**]
ZL7	Marsh	Heilong River	23	20	0.988	2.1	1.6	0.270	0.309	0.150[*]
ZL8	Marsh	Heilong River	24	24	1.000	2.4	1.6	0.208	0.326	0.378[***]
ZL9	Stream	Heilong River	24	24	1.000	2.8	1.9	0.413	0.397	−0.018[NS]
ZL10	Pond	Wusuli River	24	17	0.967	2.3	2.0	0.347	0.401	0.164[*]
ZL11	Pond	Wusuli River	22	21	0.996	2.4	1.7	0.338	0.347	0.050[NS]
ZL12	Pond	Wusuli River	12	8	0.848	1.6	1.4	0.275	0.203	−0.294[*]
ZL13	Marsh	Wusuli River	22	22	1.000	2.4	2.0	0.350	0.406	0.161[**]
ZL14	Stream	Songhua River	20	7	0.642	2.1	1.6	0.486	0.310	−0.511[***]
ZL15	Pond	Songhua River	24	24	1.000	2.4	1.6	0.329	0.315	−0.023[NS]
ZL16	Stream	Songhua River	24	23	0.996	2.5	1.8	0.339	0.425	0.222[***]
ZL17	Marsh	Songhua River	24	23	0.996	3.1	2.2	0.535	0.471	−0.114[*]
Mean			21.9	18.0	0.946	2.3	1.7	0.320	0.328	0.048

N, number of individual plants; G, the number of genotypes; D, Simpson's diversity index; n_a, observed alleles number; n_e, effective allele number; H_O, observed heterozygosity; H_E, expected heterozygosity; F_{IS}, inbreeding coefficient.
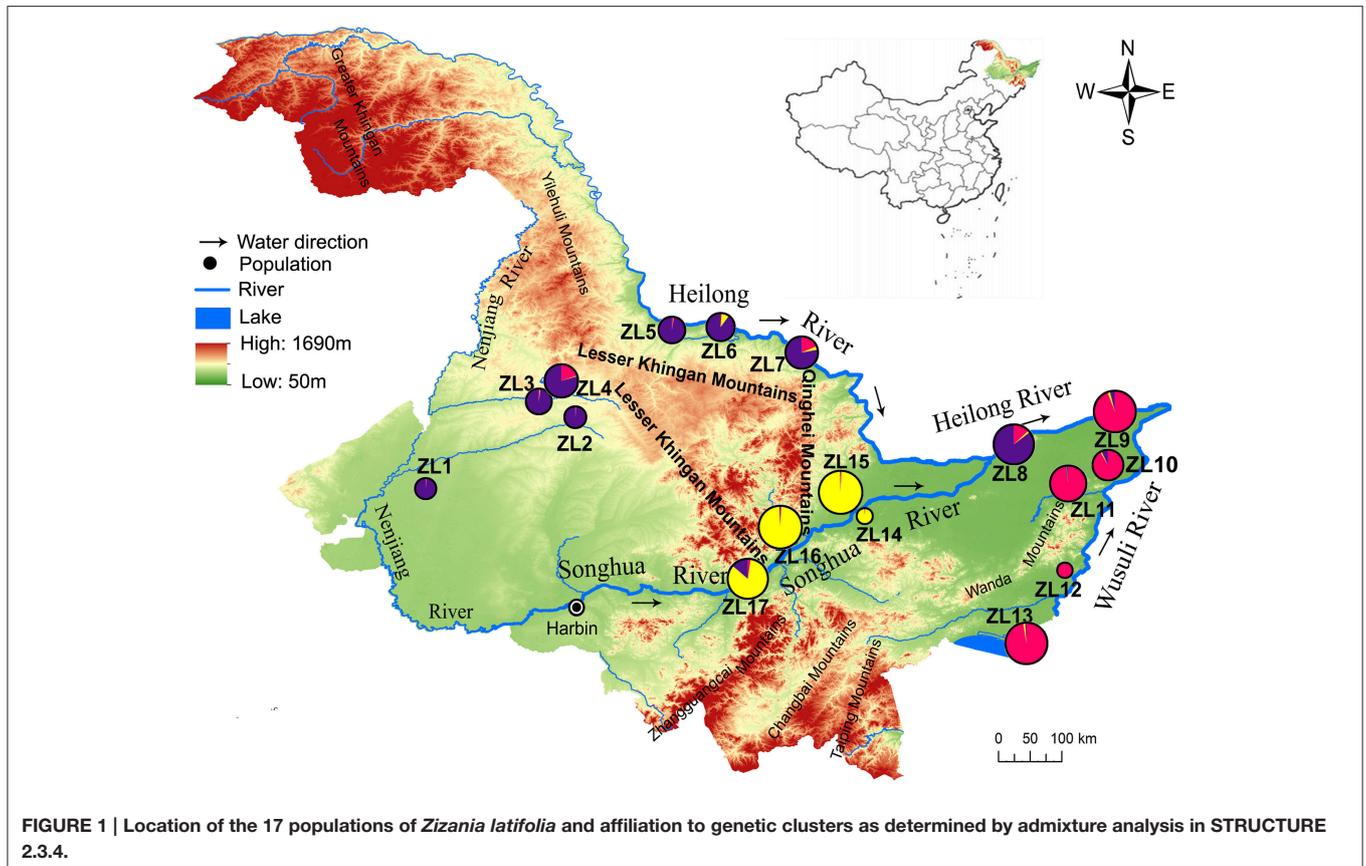*[*]P < 0.05; [**]P < 0.01; [***]P < 0.001; [NS]not significant.*

FIGURE 1 | Location of the 17 populations of *Zizania latifolia* and affiliation to genetic clusters as determined by admixture analysis in STRUCTURE 2.3.4.

to reduce the chance of collecting samples from the same clone. In populations ZL5 and ZL12, only 15 and 12 individuals were sampled because of the small population area. All in all, young leaves from 373 individuals were collected and immediately dried in silica gel.

DNA Extraction and Genotyping Using Microsatellite Markers

Total genomic DNA was isolated from 0.3 to 0.5 g dried leaves using a modified 2% CTAB protocol (Doyle and Doyle, 1987). Twenty-two SSR markers, including 16 markers developed for *Z. latifolia* (Quan et al., 2009) and six for *Z. texana* Hitchc. (Richards et al., 2007), were screened in PCR amplification on 20 random individuals. Ten markers which produced polymorphism and clear banding patterns were selected for further analysis. Microsatellite genotyping was performed at loci ZM4, ZM5, ZM13, ZM16, ZM24, ZM25, ZM26, ZM36, ZM40, and ZM44 (Quan et al., 2009). The PCR amplifications were performed in a final volume of 10 μL containing 10 mM Tris–HCl (pH 8.4), 50 mM $(NH_4)_2SO_4$, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 μM each primer, 1 unit Taq polymerase (Fermentas, Vilnius, Lithuania), and 20 ng of genomic DNA. The amplification procedure was performed as described by Quan et al. (2009). Amplified products were separated on a 6% denatured polyacrylamide gel. A 25bp DNA ladder (Promega, Madison, WI, USA) was used to identify alleles. The fragments were visualized by silver staining. All samples were amplified twice, except those samples with different profiles which were run a third time.

Data Analysis

Clonal Diversity and Genetic Variations in Populations

We used GenoType/GenoDive (Meirmans and van Tienderen, 2004) to identify the clonal structure, and samples were assigned to the same multilocus genotypes and were considered to be clonemates if their SSR profiles were identical (threshold 0). With the software, the clonal diversity within populations was calculated, including (1) the number of genotypes G; (2) Simpson's diversity index D. Repeating genotypes within populations were excluded from all further genetic analyses.

For each population, genetic diversity was estimated across all loci using the observed number of alleles (n_a), effective number of alleles (n_e), the expected and observed heterozygosity (H_E and H_O) using GenAlEx 6.2 (Peakall and Smouse, 2006). With the same software, genetic polymorphism for each microsatellite locus was assessed by calculating the total number of alleles (A, allelic diversity), H_E and H_O. FSTAT 2.9.3 (Goudet, 2001) was used to calculate the fixation index (F_{IS}) and genetic differentiation among populations (F_{ST}; Weir and Cockerham, 1984). Populations with heterozygote deficiency were further analyzed with INEst 2.0 (Chybicki and Burczyk, 2009) utilizing a Bayesian approach for estimating both null alleles and inbreeding simultaneously (Campagne et al., 2012). Two parameters were used for the model comparison (n: null alleles; f: inbreeding). Three models (n, f, and nf) were run with 50,000 burn-in and 500,000 cycles for each population. The model with the lowest Deviance Information Criterion (DIC) outperforms the other model and was selected as the best fit for this

population. The deviation was also tested from the Hardy-Weinberg equilibrium and linkage disequilibrium (LD) using FSTAT 2.9.3. The significant values for LD were corrected for multiple comparisons by Bonferroni correction (Rice, 1989).

The program Bottleneck 1.2.02 was used to test for deviation from mutation-drift equilibrium which is expected if populations have experienced a recent demographic decline (Cornuet and Luikart, 1996; Piry et al., 1999). This test is provided for three mutational models: the infinite alleles model (IAM); the stepwise mutation model (SMM); and a combination of those two extreme hypotheses, the two phase model (TPM). In the TPM, the proportion of IAM and SMM was set with default values (70% of the mutations were assumed to occur under IAM and 30% under SMM). For each mutational model, 5,000 replicates were performed using Wilcoxon signed-rank test.

Genetic Differentiation, Isolation by Distance and Tests for Accumulation Effect

The population genetic divergence was measured by the standard method of the estimation of F_{ST} (Weir and Cockerham, 1984). Pairwise *F*-statistics (Weir and Cockerham, 1984) between populations were calculated using FSTAT 2.9.3 with 1,000 permutations for their significance. Because F_{ST} is dependent upon within-population diversity, an alternative measure of genetic differentiation D_{est} was calculated using SMOGD (Jost, 2008; Crawford, 2010). Genetic variation within and among populations was further partitioned by analysis of molecular variance (AMOVA) using Arlequin3.5 (Excoffier and Lischer, 2010).

The isolation by distance was tested (IBD; Wright, 1943) by plotting pairwise estimates of genetic distance ($[F_{ST}/(1-F_{ST})]$; $[D_{est}/(1-D_{est})]$) against the corresponding geographical distance (km) between populations. A Mantel test (Mantel, 1967) with 5,000 matrix randomizations was used to assess the significance of correlations between genetic distances and geographical distances using the software TFPGA (Miller, 1997).

In order to examine whether the water flow dominated the gene flow of populations along rivers, the accumulation of genetic diversity was tested in downstream populations. The SPSS 16.0 (SPSS, 2008) was used to calculate the associations between genetic parameter (H_E) and the positions of populations along Heilong River, Songhua River and Wusuli River, respectively (expressed in kilometers from the furthest upstream population).

Population Cluster and Genetic Discontinuity among Populations

To illustrate the genetic relationships among populations, we performed principal coordinate analysis (PCoA) based on Nei's genetic distance (Nei et al., 1983) in GenAlEx 6.2. Nonhierarchical Bayesian clustering was performed to explore the genetic groups within the samples, using the program STRUCTURE 2.3.4 (Pritchard et al., 2000). The program was used with a burn-in of 100,000 replicates, followed by 500,000 replicates of the Markov chain Monte Carlo (MCMC) simulation, with the admixture model and the assumption of correlated allele frequencies among populations. Ten iterations were performed for each value of *K* from 1 to 19. The output was interpreted

with Structure Harvester (Earl and vonHoldt, 2012) using the methods of Pritchard et al. (2000) and Evanno et al. (2005) and visualized by DISTRUCT v. 1.1 (Rosenberg, 2004). Using Arlequin 3.5 (Excoffier and Lischer, 2010), AMOVA was conducted to calculate variation within and among clusters identified by STRUCTURE.

In order to visualize spatial patterns of population structure and to locate discontinuities among populations, we used GENELAND (Guillot et al., 2005) and BARRIER (Manni et al., 2004). GENELAND analysis was run with 10 replicates setting the number of groups *K* between 1 and 19. MCMC resampling was set at 100,000 repetitions, 100 thinning and 200 burn-in period. The best result was chosen based on the highest average posterior probability. The analysis was performed under uncorrelated frequency models. Monmonier's maximum difference algorithm with BARRIER 2.2 was used to determine the main genetic discontinuities among populations (Manni et al., 2004) based on the matrix of F_{ST} (Weir and Cockerham, 1984) for each of the 10 loci separately. Only barriers supported by at least half of the loci (i.e., ≥5 loci) were allowed as "consensus barriers" (Yan et al., 2016), namely main barriers.

Historical and Contemporary Gene Flows and Migration-Drift Equilibrium

The historical (M_{his}) and contemporary (M_{con}) gene flows were estimated using MIGRATE (Beerli, 2008) and BAYESASS (Wilson and Rannala, 2003), respectively. MIGRATE uses coalescent theory to estimate asymmetric gene flow M (m/μ) between populations over a long period of time (within the past $4N_e$ generations), where N_e is the effective population size, μ is the mutation rate and m is the migration rate (Beerli and Felsenstein, 2001). Here we used the Bayesian method with the Brownian motion mutation model for the microsatellite loci. The default settings of MIGRATE were used except uniform prior distributions (θ: minimum = 0, maximum = 10, delta = 1; M: minimum = 0, maximum = 400, delta = 40). Because MIGRATE needs great amount of calculation, 17 populations is beyond the computational capabilities of computers. To perform the software smoothly, the 17 populations were divided into groups based on their genetic clusters identified by STRUCTURE. BAYESASS uses assignment tests in a Bayesian framework and MCMC sampling to estimate the migration rates among populations over the last several generations (Wilson and Rannala, 2003). Each MCMC run was performed with 1×10^6 iterations after a burn-in of 1×10^5 and with a sampling frequency of 1,000. In order to make the accepted number of changes equal 30–40% of the total number of iterations (Rannala, 2011), the delta values were adjusted and set (−m = 0.15, −a = 0.50, −f = 0.75). Ten runs were performed with a different initial seed, and the one with the lowest deviance used for further analysis (Faubet et al., 2007).

GENECLASS2.0 (Piry et al., 2004) was used to perform the assignment test with the Bayesian method (Rannala and Mountain, 1997) and to detect the first-generation migrants among populations with the frequency-based method (Paetkau et al., 1995). The assignment test was used to assign or exclude the possible origins of individuals in reference populations, with

1,000,000 simulations and an alpha level of 0.01. The first-generation migrants among populations were conducted with Monte Carlo resampling of 1,000 individuals and a threshold of 0.01, which identified the immigrant individuals that were not born in the population.

We tested the relative likelihood of migration-drift equilibrium (gene flow vs. drift model) using the program 2MOD (Ciofi et al., 1999). A simulation with 1×10^5 iterations was carried out, and the first 10% of the output was discarded to avoid bias resulting from the starting values.

RESULTS

Defining Genotypes and Estimating Genetic Diversity

From the 373 genotyped individuals, 306 multilocus genotypes were identified, and no genotype was shared among populations. In six populations, ZL4, ZL5, ZL8, ZL9, ZL13, and ZL15, every individual was of a unique genotype. For the other populations, the Simpson's clonal diversity index (*D*) ranged from 0.642 (ZL14) to 0.996 (ZL11, ZL16, and ZL17) (**Table 1**). A total of 50 alleles were identified, ranging from 2 (ZM24) to 10 (ZM40 and ZM44), with an average of 5.0 alleles per locus (Table S1). The observed allele number within populations ranged from 1.6 (ZL12) to 3.1 (ZL17) (**Table 1**). The mean observed heterozygosity (H_O) and expected heterozygosity (H_E) per population were 0.320 and 0.328, respectively (**Table 1**). The smallest and the highest H_E values were observed in the ZL12 and ZL17 populations, which were growing along the Wusuli River and Songhua River, respectively. The average value of the fixation index varied greatly among populations (range: −0.511 to 0.378; mean: 0.048; **Table 1**), suggesting that *Z. latifolia* had a mixed breeding systems, including both inbreeding and outbreeding.

Of 340 locus-population combinations, 41, 19, and 9 combinations (12.1, 5.6, and 2.6%) showed significant deviation from Hardy-Weinberg equilibrium at $P = 0.05$, 0.01, and 0.001 respectively. Although 56 of 765 locus pairs (7.3%) were significantly out of genotypic disequilibrium at the $P = 0.05$ level, none of the locus pairs was in significant genotypic disequilibrium after the Bonferroni-type correction, which showed that loci were physically unlinked and statistically independent. Eleven populations showed a positive fixation index, of which eight were significant. Further analysis with INEst showed that the heterozygote deficiency in the five populations (ZL2, ZL3, ZL6, ZL7, and ZL8) was caused by inbreeding, whereas the heterozygote deficiency in the three other populations (ZL10, ZL13, and ZL16) was caused by the null allele model (Table S2).

Genetic Differentiation, Isolation by Distance and Tests for Accumulation Effect

The genetic differentiation across all populations, estimated by F_{ST} and D_{est}, were 0.405 and 0.414, respectively (Table S1). The matrix of the pairwise population differentiation estimated by F_{ST} and D_{est} was shown in Table S3. All of the 136 pairwise comparisons of F_{ST} were significant ($P < 0.05$) for genetic

differentiation between populations after correction for multiple tests. For all the values of pairwise D_{est}, the lower 95% confidence interval estimated by bootstrapping did not go below zero for any of pairwise statistics, indicating significant population differentiation. Levels of pairwise population differentiation were high, ranging between 0.137 and 0.626 (mean F_{ST} ± SEM: 0.418 ± 0.107). Consistent with these results, values of Jost's D_{est} were comparable (0.025–0.592) among all populations (mean D_{est} ± SEM: 0.231 ± 0.111). The AMOVA showed high overall population differentiation ($Ø_{pt}$ = 0.424, P = 0.000). Of the total genetic variations partitioned in the 17 *Z. latifolia* populations, 42% were attributed to the differences among populations (df = 16, P < 0.0001), whereas 58% were attributed to the differences among individuals (df = 595, P < 0.0001) (**Table 2**).

Although only a small proportion of the genetic variance was explained by geography, small but significant positive correlation was detected between genetic differentiation and geographical distances among the 17 populations (Mantel test for F_{ST}: r^2 = 0.038, P = 0.012; D_{est}: r^2 = 0.022, P = 0.043) (Figure S1). No significant positive association was found between the genetic diversity indices (H_E) and the distance of the downstream populations from the population furthest upstream for the three rivers (for the Heilong River, r = 0.595, P = 0.290; for the Wusuli River, r = 0.092, P = 0.908; for the Songhua River, r = −0.968, P = 0.032).

TABLE 2 | Analysis of molecular variance (AMOVA) for 306 individuals.

Source of variation	df	Sum of squares	Variance components	Percentage of variation	$Ø_{pt}$	p
AMONG AND WITHIN THE 17 POPULATIONS						
Among populations	16	767.783	1.292	42%		
Within populations	595	1,045.562	1.757	58%	0.424	0.000
AMONG AND WITHIN THE THREE CLUSTERS (K = 3)						
Among clusters	2	260.662	0.647	20%		
Within clusters	609	1,552.683	2.550	80%	0.202	0.000

$Ø_{pt}$, genetic differentiation; populations are grouped according to their genetic clusters as determined by STRUCTURE.
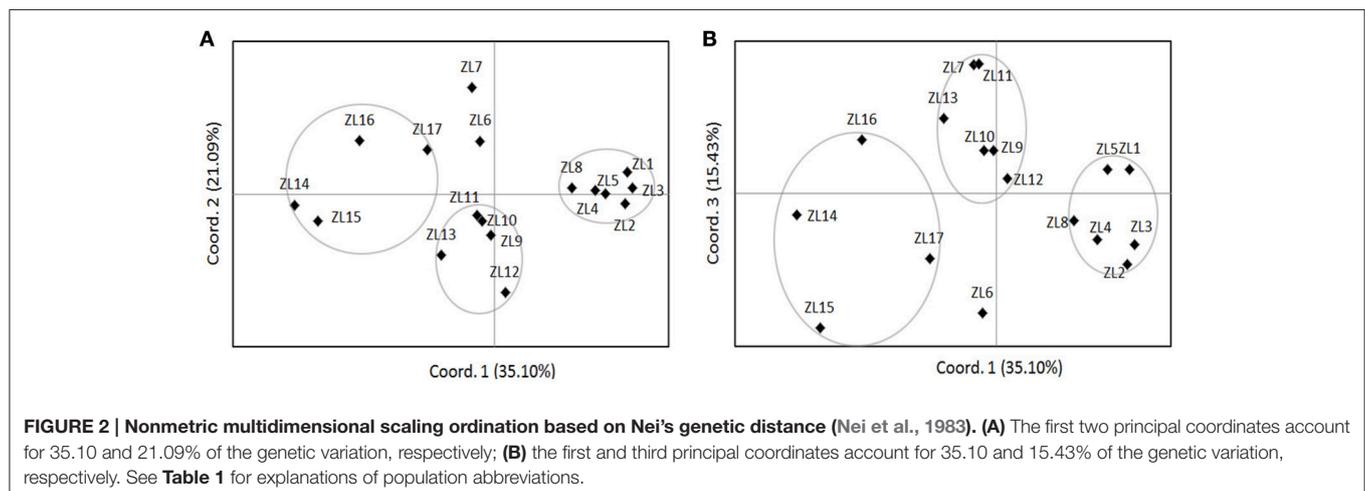
Population Cluster and Genetic Discontinuity among Populations

The Bayesian clustering implemented in STRUCTURE revealed an optimum $ΔK$ at K = 3 (**Figure 1**; Figures S2A,B). The three clusters of individuals clearly separated these collecting locations into stable gene pools (Figure S2C): populations ZL1-8, ZL9-13, and ZL14-17. The proportion of membership of the pre-defined populations in each of the three clusters ranged from 0.658 to 0.986 (Table S4). The AMOVA showed that 20% of the total molecular variations were attributed to inter-cluster differentiation (**Table 2**). When considering genetic structure based on the PCoA performed on allelic frequencies, a similar cluster pattern was found except for populations ZL6 and ZL7 (**Figure 2**). PCoA explained 71.63% of the total gene variations, on the basis of the first (35.10%), the second (21.09%) and the third dimension (15.43%).

GENELAND identified 13 genetic clusters (Figure S3). Each cluster corresponded to a sampling site with the exception of the three clusters (ZL1-3, ZL12-13, and ZL14-15). The results indicated that genetic discontinuities existed between any adjacent populations except for within the three clusters. The main genetic discontinuities were further identified using Barrier 2.2 based on pairwise F_{ST} values (**Figure 3**). Five main barriers, including barriers between ZL2 and ZL17, ZL2 and ZL16, ZL6 and ZL16, ZL7 and ZL16, and ZL7 and ZL15, integrated to form one continuous break, located in the east section of Lesser Khingan and its branch Qinghei Mountains. Additionally, three main breaks were exhibited among populations along the three rivers, i.e., between ZL6 and ZL7 along the Heilong River, between ZL15 and ZL16 along the Songhua River, and between ZL11 and ZL12 along the Wusuli River.

Gene Flow and Genetic Bottlenecks

MIGRATE revealed high level of historical gene flow among the *Z. latifolia* populations (**Table 3**). M_{his} ranged from 2.340 to 8.443 for cluster ZL1-8, from 1.568 to 11.231 for cluster ZL9-13, and from 1.206 to 10.165 from the Songhua River, respectively. On the contrary, multiple runs of BAYESASS yielded relatively low contemporary gene flow among the populations. The values of

FIGURE 2 | Nonmetric multidimensional scaling ordination based on Nei's genetic distance (Nei et al., 1983). (A) The first two principal coordinates account for 35.10 and 21.09% of the genetic variation, respectively; **(B)** the first and third principal coordinates account for 35.10 and 15.43% of the genetic variation, respectively. See **Table 1** for explanations of population abbreviations.

FIGURE 3 | Discontinuities in gene flow suggested by BARRIER using consensus barriers. Barriers to gene flow are indicated by solid red lines, and the number of loci supported barriers is in green. The blue lines represent hypothetical population boundaries.
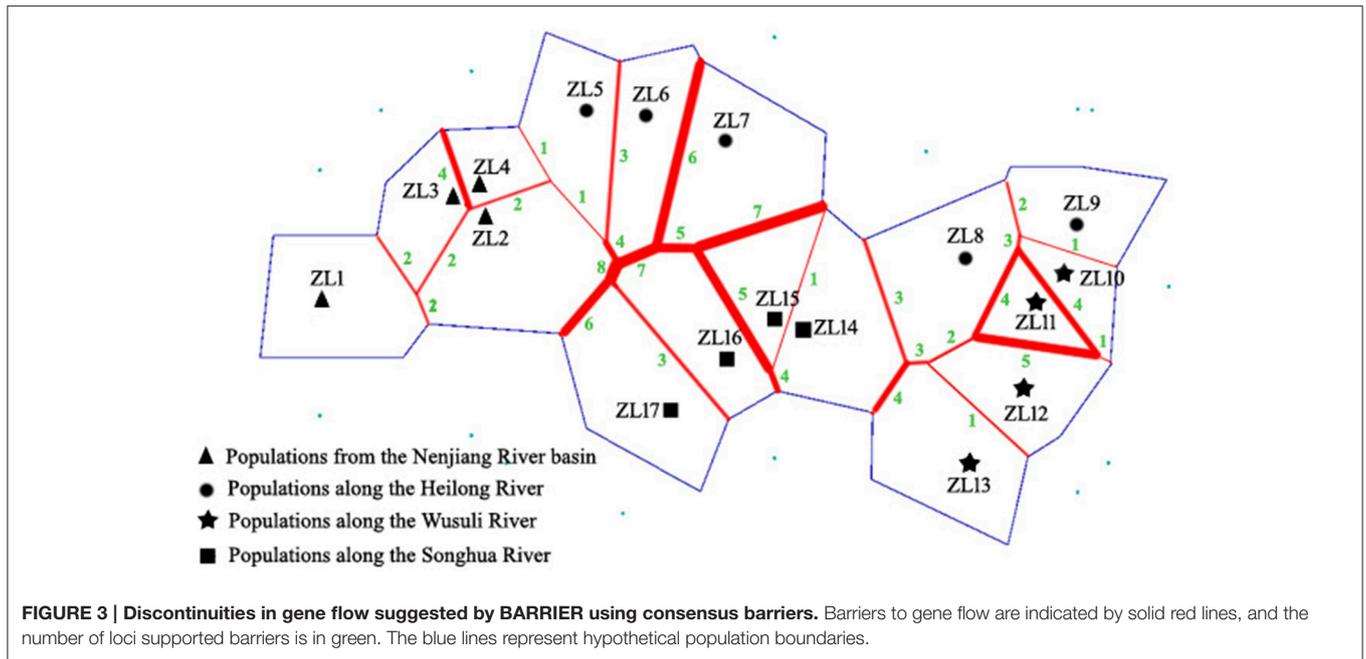
TABLE 3 | Likelihood estimates of MIGRATE of long-term and BAYESASS of recent gene flow among 17 populations of *Zizania latifolia*.

(a) Estimates of long-term (left) and recent (right) gene flow among the eight populations ZL1–ZL8

	ZL1	ZL2	ZL3	ZL4	ZL5	ZL6	ZL7	ZL8		ZL1	ZL2	ZL3	ZL4	ZL5	ZL6	ZL7	ZL8
ZL1		8.443	3.110	2.569	2.950	3.129	2.866	2.965	ZL1		0.251	0.236	0.239	0.221	0.224	0.240	0.286
ZL2	3.336		3.046	2.340	3.017	2.580	2.366	3.347	ZL2	0.287		1.924	0.246	0.276	0.226	0.238	0.348
ZL3	3.861	7.022		2.854	3.918	2.575	2.400	3.444	ZL3	0.199	0.215		0.777	0.233	0.228	0.238	0.257
ZL4	2.554	6.489	3.000		3.965	3.345	3.270	2.678	ZL4	0.191	0.194	0.230		0.224	0.224	0.236	0.252
ZL5	4.071	4.077	2.781	2.771		2.777	2.578	2.987	ZL5	0.196	0.199	0.257	0.256		0.226	0.238	0.264
ZL6	3.988	7.659	2.596	3.640	5.828		2.718	3.098	ZL6	0.194	0.198	0.227	0.242	0.219		0.238	0.250
ZL7	3.744	6.093	2.833	3.247	2.968	3.342		2.392	ZL7	0.193	0.193	0.230	0.237	0.228	0.228		0.250
ZL8	6.344	8.338	3.131	2.528	4.391	2.481	3.395		ZL8	0.222	0.251	0.230	0.246	0.230	0.228	0.242	

(b) Estimates of long-term (left) and recent (right) gene flow among the five populations ZL9–ZL13

	ZL9	ZL10	ZL11	ZL12	ZL13		ZL9	ZL10	ZL11	ZL12	ZL13
ZL9		6.897	4.104	5.421	4.999	ZL9		1.931	0.307	0.206	0.293
ZL10	3.418		3.953	5.047	3.654	ZL10	0.350		0.288	0.208	0.288
ZL11	3.306	3.582		2.497	2.745	ZL11	0.370	0.260		0.209	0.306
ZL12	1.688	3.065	1.568		2.202	ZL12	0.372	0.262	0.380		0.409
ZL13	2.227	3.120	2.043	11.231		ZL13	0.317	0.264	0.382	0.258	

(c) Estimates of long-term (left) and recent (right) gene flow among four populations along the Songhua River

	ZL14	ZL15	ZL16	ZL17		ZL14	ZL15	ZL16	ZL17
ZL14		10.165	5.865	5.181	ZL14		1.469	0.688	0.679
ZL15	2.120		5.155	4.312	ZL15	0.121		0.276	0.276
ZL16	4.534	1.206		4.798	ZL16	0.087	0.298		0.396
ZL17	2.108	4.766	4.342		ZL17	0.104	0.322	0.336	

(Populations in rows are giving migrants to populations in columns; populations are grouped according to their genetic clusters as determined by STRUCTURE).

M_{con} were 1.924 from ZL3 to ZL2, 1.931 from ZL10 to ZL9 and 1.469 from ZL15 to ZL14, but the others ranged from 0.087 from ZL14 to ZL16 to 0.777 from ZL4 to ZL3, suggesting that gene flow was greatly reduced during recent habitat fragmentation. Bi-directional gene flows were found in all data sets. The M_{his} is predominantly downstream along the Songhua River except the pairwise between ZL15 and ZL17.

An assignment test calculated by GENECLASS showed that individuals from their population of origin were 27.6% or higher in all the populations, and 18.4% or fewer of the individuals were assigned to their nearest origin population. Additionally, 1.3% or fewer of the individuals was assigned to the other populations. Some 47.7% or more of the individuals were not assigned to any of the populations sampled (Figure S4). Seven first-generation immigrants were detected, of which two had migrated up from the downstream populations and four had migrated down from upstream populations (Table S5).

Due to the low number of genotypes of some populations, tests for signatures of recent bottlenecks were only conducted on 15 of the 17 populations. Nine populations deviated significantly from mutation-drift equilibrium under the infinite allele model, and five populations displayed significant deviation under the two phase model (**Table 4**). The microsatellites used in *Z. latifolia* are either of dinucleotide perfect repeats or of imperfect repeats, both of which may tend toward the IAM (Cornuet and Luikart, 1996). So the results under IAM give more accurate estimates, suggesting most populations (nine of 15 populations) have experienced recent bottlenecks. In the 2MOD analysis, the likelihoods of the gene flow model and pure drift models were 0 and 1, respectively, i.e., none of the simulations supported the gene flow model.

DISCUSSION

Clonal Diversity and Genetic Variation

Clonal diversity for *Z. latifolia* was remarkably high ($D = 0.946$) compared with the mean values ($D = 0.62$) summarized by Ellstrand and Roose (1987), which may be partly attributed to the large sampling interval within populations (at least 10 m). Additionally, all populations comprised multiple genotypes, indicating that seeds or sexual reproduction largely contributed to the population construction of *Z. latifolia*. No multilocus genotype was shared among populations, suggesting that a clonal strategy mainly occur in the same population and clonal ramets cannot disperse among populations over the large geographic scale studied here.

In the present study, *Z. latifolia* from the Sanjiang Plain appeared to possess low intra-population microsatellite variation ($n_a = 2.3$, $H_E = 0.328$) compared to *Z. texana* from Texas in America ($n_a = 11.7$, $H_E = 0.662$, Richards et al., 2007; $n_a = 4.4$–6.3, $H_E = 0.53$–0.65, Wilson et al., 2017) and *Z. latifolia* from the Yangtze River in central China ($n_a = 5.0$, $H_E = 0.610$; Chen et al., 2012a). A lower level of genetic variation ($H_E = 0.156$ based on SRAP markers) was found in nine *Z. latifolia* populations in the same area (Fan et al., 2016). The present low genetic variation may result from the genetic bottlenecks experienced by the populations. The test for mutation-drift equilibrium

TABLE 4 | Test for mutation-drift equilibrium in *Zizania latifolia*.

Population	Mutation-drift test		
	IAM	**TPM**	**SMM**
ZL1	0.191	0.320	0.527
ZL2	0.039*	0.039*	0.055
ZL3	0.273	0.371	0.527
ZL4	0.752	0.875	0.918
ZL5	0.019*	0.082	0.248
ZL6	0.125	0.248	0.410
ZL7	0.004**	0.012*	0.055
ZL8	0.102	0.326	0.545
ZL9	0.007**	0.082	0.285
ZL10	0.002**	0.002**	0.002**
ZL11	0.024*	0.248	0.455
ZL12	–	–	–
ZL13	0.004**	0.004**	0.008**
ZL14	–	–	–
ZL15	0.246	0.423	0.722
ZL16	0.005**	0.016*	0.097
ZL17	0.012*	0.097	0.348

Probabilities from Wilcoxon signed-rank tests for heterozygosity excess using the program Bottleneck.
*P < 0.05; **P < 0.01. IAM, infinite allele model; SMM, stepwise mutation model; TPM, two phases model.

under IAM suggested that most *Z. latifolia* populations (nine of 15 populations) have experienced recent bottlenecks. *Z. latifolia* is shallow-rooted and has recalcitrant seeds (Berjak and Pammenter, 2008), and these specific traits may make the species poor at handling drought. Therefore, the ephemeral habitats combined with drastic anthropogenic activities can easily cause genetic bottlenecks in *Z. latifolia* populations.

Furthermore, inbreeding systems partly explain the low genetic variation in some populations. The Bayesian analysis of INEST suggested that inbreeding, rather than null alleles, resulted in heterozygote deficiency in five populations (ZL2, ZL3, ZL6, ZL7, and ZL8). We inferred that the fragmented habitats increased the biparental inbreeding behavior (mating among relatives) within *Z. latifolia* populations. Contrastingly, a high degree of inbreeding in the reproductive system ($F_{IS} = 0.793$) was reported in the *Z. latifolia* populations from the Sanjiang Plain based on SRAP markers (Fan et al., 2016). Given that the two marker systems (SSRs and SRAPs) produce different genetic profiles, the results from the two markers may not be strictly comparable. But this study and Fan et al. (2016) both showed that *Z. latifolia* in the plain has exhibited inbreeding. On the contrary, *Z. latifolia* populations from the Yangtze River showed predominant outcrossing reproductive system ($F_{IS} = -0.200$–0.019; Chen et al., 2012a). The different results could be ascribed to the habitat types: most *Z. latifolia* populations in the Sanjiang Plain occur in the sides of ponds and streams, which easily lead to mating events among close relatives or intra-clones; while *Z. latifolia* from the Yangtze River grows along the littorals of lakes and forms large populations (Chen et al., 2012a), and this makes

the populations stand a good chance to receive more migrant pollen and act as outcrossing.

Roles of Rivers, Mountains and Fragmentation

Both aquatic and riparian plant communities are strongly influenced by water-mediated dispersal (Merritt et al., 2010; Nilsson et al., 2010; Fraaije et al., 2015), and river habitats are long-recognized corridors for riparian or aquatic plants (Tero et al., 2003; Fér and Hroudova, 2008; Wei et al., 2013). However, this was not the case in the present study. A high genetic differentiation was observed among populations within each river. The GENELAND analysis detected that genetic discontinuities existed between most adjacent populations. The barrier analysis further confirmed the main genetic break within each river, i.e., ZL6 vs. ZL7, ZL11 vs. ZL12, and ZL15 vs. ZL16 along the Heilong River, Wusuli River and Songhua River, respectively. The scenarios suggested that there was little exchange among the corresponding populations, which showed that the rivers were not acting as corridors. Additionally, the downstream accumulation of genetic diversity caused by water-mediated dispersal was not found along the three rivers, which might be explained by a number of factors. Firstly, upstream dispersal of seeds and plant fragments: both historical and contemporary gene flow for *Z. latifolia* were bi-directional within in the three rivers detected by MIGRATE and BAYESASS. Secondly, various degrees genetic bottlenecks experienced by populations within rivers: the genetic bottlenecks in most *Z. latifolia* populations would eliminate the accumulation trends in downstream when downstream populations suffered more severe bottlenecks than those of upstream populations. Thirdly, the influence of unsampled populations: the assignment test using GENECLASS showed that about 50% of the individuals were not assigned to any of the populations sampled. So the influence of unsampled (or disappeared) populations needs to be considered. In order to detect dispersal events among populations efficiently, more populations along rivers should be sampled, as suggested by Wubs et al. (2016).

A mountain ridge is the predominant factor causing genetic barrier for mountain plants (Tsuda et al., 2010; Wei et al., 2013). In the present study, the main genetic breaks detected by BARRIER 2.2 were distributed in the places where the east section of Lesser Khingan and its branch Qinghei Mountains are located, suggesting that the mountains greatly hinder the gene exchange among populations on both sides of the mountains. It is interesting to note that no main genetic discontinuity was found between the populations distributed in the north (ZL2 and ZL4) and south (ZL5 and ZL6) sides of the west section of the Lesser Khingan. This suggested that, compared with the east section of the mountains, the west section was of relatively weak blocking effects on gene flow. This difference might be related to the topography and terrain in the different section of the mountains. The east section of Lesser Khingan and its branch Qinghei Mountains are made up of rugged ridges, deep valleys and covered by dense forests, which would efficiently block the gene exchange on different sides of the mountains. On the other hand, the west section of the Lesser Khingan has lower slopes, and

some places have been reclaimed into farmlands, including dry lands and paddy fields. The irrigation ditches and extant ponds embedded in the farmlands would provide a "transition" for dispersal.

Fragmentation decreases habitat size and increases isolation between habitats and populations. These physical changes are generally expected to reduce gene flow and increase inter-population genetic divergence of plant populations (Young et al., 1996). However, some species can adapt to habitat destruction, such as tropical tree *Swietenia humilis* Zuccarini. which increases pollen flow to counteract fragmentation (White et al., 2002). The wetlands in the Sanjiang Plain and its surrounding areas have suffered from over reclamation for decades and have been separated into small wetland mosaic sandwiched between farmlands (Wang et al., 2011). Our results suggest that the recent habitat fragmentation in the plain is accompanied with a greatly decreased gene flow among *Z. latifolia* populations. Two results lead to the conclusion: firstly, a much higher historical gene flow was detected compared with contemporary gene flow; secondly, only seven first-generation immigrants were detected among populations. Although the wind-pollinated distance for *Z. latifolia* is unavailable, it has been reported to be limited to 6–9 km for the wild rice *O. rufipogon* Griff. (Gao, 2004). The pollen-mediated dispersal for *Z. latifolia* seems to be effective only at local scale with the smallest distance of 26 km among populations (between ZL3 and ZL4). Therefore, the magnitude of genetic differentiation among *Z. latifolia* populations is almost solely determined by the ability to disperse seeds or propagules. However, the fragmented nature of wetlands in this area has resulted in the hydrological disconnectivity between ponds or marshes and the arteries of rivers, which could have hindered the dispersal via seeds or propagules of the plant. Although episodic floods can result in temporary hydrological connectivity and facilitate the gene exchange, as suggested in *O. rufipogon* populations (Wang et al., 2008), high values of F_{ST} and D_{est} among pairs of populations along the same river indicate that episodic floods were not an effective path of dispersal for *Z. latifolia*.

Genetic Structure

There was a high level of inter-population differentiation in *Z. latifolia* populations ($F_{ST} = 0.405$, $D_{est} = 0.414$, $\emptyset_{pt} = 0.424$). This is comparable to the genetic differentiation that was recently reported for nine populations of *Z. latifolia* ($F_{ST} = 0.299$ based on 344 SRAP markers) and eight populations of *Nymphaea tetragona* Georgi ($F_{ST} = 0.371$ based on 289 ISSR markers) in the same habitat and area (Fan et al., 2016; Chen et al., in press). The high genetic divergence of *Z. latifolia* may be explained by decreased gene flow, the inbreeding systems in some populations and repeated genetic bottlenecks in the critically fragmented habitats. Furthermore, when gene flow rate is greatly limited, population genetic differentiation will increase due to genetic drift (Slatkin, 1977; Hutchison and Templeton, 1999). In the present study, the 2MOD analysis suggested that genetic drift may have an important impact on the present genetic patterns. Additionally, the evident population differentiation may be associated with different environmental adaptations of

the populations (Hufford and Mazer, 2003; Iacolina et al., 2016), and common-garden experiments should be carried out to test this assumption.

Interestingly, although the high genetic differentiation and genetic breaks among populations within each river allowed us to reject the corridor function of rivers, the Bayesian clustering using STRUCTURE revealed that most populations along the three rivers clustered together according to their river system origins, which was supported by the results of PCoA.

The AMOVA further showed that 20% of the total molecular variation was attributed to the differences among the three genetic clusters identified by STRUCTURE. At the same time, the significant positive correlation between genetic and geographic distances was detected in *Z. latifolia*, indicating that the IBD pattern influenced population differentiation. Given the populations from the same river are located close to each other, the clustering results of STRUCTURE and PCoA were mainly explained by the effects of IBD instead of hydraulic dispersal.

Implications for Management

Genetic variation is important for a species to maintain its evolutionary potential to cope with ever-changing environments. The genetic information obtained in this study can be used to propose appropriate management strategies for the *Z. latifolia* wild populations in the critically fragmented flood plain, the Sanjiang Plain.

Currently, low genetic variation and reduced gene flow are found in the *Z. latifolia* populations from the Sanjiang Plain and its surrounding areas, indicating that the habitat fragmentation has already negatively impacted the genetic diversity of the plant. Genetic diversity needs many generations (at least 10, but often >100 generations) of fragmentation to be severely affected (Mona et al., 2014). Consequently, the adverse impact of fragmentation on genetic diversity will probably become more evident in the future given the long-lived life history of *Z. latifolia*, which highlights the need for conservation efforts of the species. The result that more than 40% of microsatellite variation

is partitioned among *Z. latifolia* populations is instructive for adopting a plan to protect as many populations as possible. The populations with a high level of genetic diversity, such as ZL9, ZL14, and ZL17, should be given the top priority for both *in situ* and *ex situ* conservation. In this study, genetic discontinuities were found among most populations, even populations from the same river, suggesting that the rivers be not acting as corridors for dispersal. Therefore, dredging the watercourses between the isolated habitats and the arteries of the rivers should be carried out, which will improve the stability of wetlands habitats and facilitate connectivity among populations.

AUTHOR CONTRIBUTIONS

YC collected the samples, designed the experiments, and wrote the manuscript; Yang Liu performed the SSR experiments and checked English grammar; XF performed the experiments and conducted data analysis; WL collected the samples; Yanling Liu contributed to the supervision of the work and wrote the manuscript. All authors read and approved the final version of manuscript. The authors declare no conflict of interest.

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SUPPLEMENTARY MATERIAL

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