Comparative phylogeography of the wild-rice genus *Zizania* (Poaceae) in eastern Asia and North America[1]

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- *Premise of the study:* Comparative phylogeography of intercontinental disjunct taxa allowed us not only to elucidate their diversification and evolution following geographic isolation, but also to understand the effect of climatic and geological histories on the evolutionary processes of closely related species. A phylogeographic analysis was conducted on the eastern Asian-North American disjunct genus *Zizania* to compare intracontinental phylogeographic patterns between different continents.
- *Methods:* Surveys were conducted of 514 individuals using three chloroplast DNA fragments and three nuclear microsatellite loci. These individuals included 246 from 45 populations of *Zizania latifolia* in eastern Asia, and the following from North America: 154 individuals from 26 populations of *Z. aquatica,* 84 individuals from 14 populations of *Z. palustris*, and 30 individuals from one population of *Z. texana*.
- *Key results:* The genetic diversity of North American *Zizania* was significantly higher than that of eastern Asian *Zizania*. High levels of genetic differentiation among populations and no signal of population expansion were detected in three widespread species. No phylogeographic structure was observed in *Z. latifolia*, and discordant patterns of cpDNA and microsatellite markers were observed in North American *Zizania*.
- *Conclusions:* Reduced variation in *Zizania latifolia* likely reflects its perennial life history, the North American origin of *Zizania*, and the relative homogeneity of aquatic environments. High levels of genetic differentiation suggest limited dispersal among populations in all *Zizania* species. The more complex patterns of diversification and evolution in North American *Zizania* may be driven by the greater impact of glaciation in North America relative to eastern Asia.

Key words: cpDNA; eastern Asia; intercontinental disjunction; microsatellite; North America; phylogeography; *Zizania*.

The plant taxa of eastern Asia and North America have been extensively studied and compared due to their similar latitudes and close phylogenetic relationships (Li, 1952; Boufford and Spongberg, 1983; Wen, 1999; Guo and Ricklefs, 2000; Wang et al., 2009). Many studies have demonstrated that the taxonomic richness of vascular plants in eastern Asia greatly exceeds that of North America at the continental scale (Guo et al., 1998; Qian and Ricklefs, 2000; Wang et al., 2009). Several historical and ecological factors are likely responsible for this bias in species abundance, such as the complex topography and reduced impact of glaciation in Asia (Wen, 1999). The bias seems also to be reflected in the genetic diversity of closely related taxa of disjunct congeners based on several previous studies (Dane et al., 2003; Nie et al., 2006; Zhao et al., 2013). This hypothesis is worthy of testing by more investigations on the disjunct taxa of eastern Asia and North America. Comparing phylogeographic patterns and genetic diversity in closely related taxa between eastern Asia and North America can clarify the diversification and evolution of the disjunct taxa following geographic isolation and help to understand the effect of climatic and geological histories on their evolutionary processes (Xiang et al., 2004; Albach et al., 2006; Wen et al., 2009).

Several comparative phylogeographic analyses of multiple species with similar geographic distributions have been conducted in Europe, North America, and elsewhere, revealing common patterns of genealogical relationships due to historical events (Soltis et al., 1997, 2006; Taberlet et al., 1998; Hewitt, 1999, 2000). However, only a few studies have compared patterns

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of phylogeographic history and genetic diversity among closely related taxa from different continents with distinct climatic and geological histories; such studies have been limited to the Arctic or to Europe and North America (Abbott and Brochmann, 2003; Albach et al., 2006; Grivet et al., 2006). Although 91 to 120 plant genera have disjunct taxa in eastern Asia and North America (Wu, 1983; Hong, 1993), these genera have been the subject of few comparative phylogeographic studies, with the exception of the *Smilax hispida* Muhl. ex Torr. (Smilacaceae) group (Zhao et al., 2013).

One taxon suitable for such an intercontinental comparison is the wild-rice genus *Zizania* L., which comprises four species distributed in disjunct populations in eastern Asia and North America. Three species, *Zizania palustris* L., *Zizania aquatica* L., and *Zizania texana* Hitchcock, are native to North America. *Zizania palustris* and *Z. aquatica* are annual species broadly distributed throughout the Great Lakes region and the Atlantic coastal plain, respectively, with some range overlap. The perennial *Z. texana* is an endangered species restricted to the upper San Marcos River in south-central Texas, isolated from all other *Zizania* taxa (Terrell et al., 1997). The eastern Asian species *Zizania latifolia* (Griseb.) Turcz. ex Stapf is a clonal perennial and widely distributed in eastern Asia (Wu et al., 2006). The phylogeny and biogeography of the genus were inferred in a recent study based on DNA sequences (Xu et al., 2010). The genus most likely originated in North America and then dispersed into eastern Asia via the Bering land bridge in the late Tertiary (Xu et al., 2010). This biogeographic hypothesis of *Zizania* is also supported by the genealogical pattern of *Z. latifolia* suggested based on the nuclear *Adh1a* gene in eastern Asia (Xu et al., 2008). However, due to limited sampling in previous studies, the phylogeography of North American *Zizania* was still unknown. This knowledge is necessary to understand the evolutionary history and diversification of *Zizania* in eastern Asia and North America.

In this study, we examined the phylogeographic patterns of *Zizania* in eastern Asia and North America with a comprehensive sampling scheme based on three chloroplast DNA fragments and three nuclear microsatellite loci. Our specific objectives were to: (1) compare the genetic diversity of *Zizania* populations in eastern Asia with that in North America; (2) elucidate the intraspecific phylogeographic patterns of three widespread species of *Zizania*; and (3) investigate how differences in phylogeographic pattern between disjunct taxa correspond to the distinct climatic and geological histories of each continent.

MATERIALS AND METHODS

***Plant materials*—**Samples from 268 individuals were collected in the USA and Canada, representing 41 populations of all three species of North American *Zizania* (Fig. 1A). Fourteen populations of *Z. palustris*, 26 populations of *Z. aquatica*, and one population of *Z. texana* were sampled, the last of which is the only known population of the taxon. The populations in the overlapping ranges of *Z. palustris* and *Z. aquatica* were identified according to the different trait of lemmas of pistillate spikelets (Terrell et al., 1997). Samples of 246 individuals representing 45 populations of *Z. latifolia* were collected in eastern Asia; specifically in China, with the exception of one population from Japan (Fig. 1B). Our sampling covers most of the distribution range of *Z. aquatica* and *Z. latifolia* and the southern part of the range of *Z. palustris*. Six individuals per population were randomly sampled at an interval of at least 10 m to prevent collecting ramets from a single genet in the clonal *Z. latifolia*. Young, healthy leaves were collected in the field and dried with silica gel for subsequent DNA extraction. Voucher specimens were deposited in the herbarium of Wuhan University (WH) and the United States National Herbarium (US). The name, locality, and number of individuals sampled of the 86 populations are provided in Appendix S1 (see Supplemental Data with the online version of this article).

***Amplification, sequencing, and genotyping*—**Total genomic DNA was extracted from the silica-dried leaves using the DNeasy Plant Mini Kit (Qiagen, Chatsworth, California, USA). Forty-eight individuals, randomly selected from the four species, were used to screen for polymorphisms in nine newly explored cpDNA regions (Shaw et al., 2007). Two intergenic spacers (*rpl32-trnL* and *rps16-trnK*) exhibited high levels of polymorphism within these 48 individuals (Appendix S2, see Supplemental Data with the online version of this article), as did the *rps16* intron, which was the most variable region identified in a previous phylogenetic study (Xu et al., 2010). These three regions were then used to survey all 514 accessions. Polymerase chain reaction (PCR) amplifications were performed using 20 ng of genomic DNA, 5 pmol of each primer, 0.2 mM of each dNTP, 2 mM $MgCl_2$, and 0.6 U GoTaq DNA polymerase (Promega, Madison, Wisconsin, USA) in a 25 μL volume under the following conditions: 5 min at 95°C, followed by 35 cycles of 30 s at 94°C, 30 s at 50-55°C, and 90 s at 72°C; and then a final 5 min extension at 72°C. Amplifications were carried out in a PTC-225 Peltier Thermal Cycler (MJ Research, Watertown, Massachusetts, USA).

All PCR products were purified using the polyethylene glycol (PEG)/NaCl method of Kusukawa et al. (1990). Purified PCR products were sequenced using the BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, California, USA). Sequencing reactions were purified by gel filtration chromatography using Sephadex columns (Amersham Pharmacia Biotech, Piscataway, New Jersey, USA) and run on an ABI 3730xl DNA analyzer (Applied Biosystems). The program Sequencher 4.5 (GeneCodes Corporation, Ann Arbor, Michigan, USA) was used to evaluate chromatograms for base confirmation and to edit contiguous sequences. All sequences of different haplotypes were deposited into GenBank (accession numbers KP085441-KP085518).

In previous studies, 8 nuclear microsatellite loci were isolated from *Zizania texana* (Richards et al., 2004, 2007) and 16 from *Z. latifolia* (Quan et al., 2009). We conducted preliminary experiments to test whether these primers could be applied to all four *Zizania* species. Only three microsatellites (ZM4, ZM26, ZT26) were successfully amplified and used in all samples. PCR amplification protocols followed Richards et al. (2004) at locus ZT26 and Quan et al. (2009) at loci ZM4 and ZM26. PCR products were analyzed on an ABI 3730XL DNA analyzer, and genotyping was performed using GeneMapper 4.0 (Applied Biosystems).

***Data analysis*—**All cpDNA sequences were aligned with the MAFFT 6.7 software (Katoh et al., 2002) using the L-INS-i algorithm with "maxiterate" set to 1000. A haplotype network was constructed using TCS 1.18 (Clement et al., 2000), which implements statistical parsimony to connect haplotypes constrained by 95% confidence intervals. In this analysis, indels were treated as single mutation events and coded as substitutions (A or T). Nucleotide diversity (π) and haplotype diversity (Hd) were calculated with DnaSP 5.10 (Librado and Rozas, 2009). Genetic variation within and among populations was assessed by analyses of molecular variance (AMOVA) implemented in ARLEQUIN 3.0 (Excoffier et al., 2005). Two measures of population differentiation, G_{ST} and N_{ST}, were calculated using the program HAPLONST (Pons and Petit, 1996). Whereas G_{ST} makes use of haplotype frequencies, N_{ST} takes into account both haplotype frequencies and their genetic distances. An N_{ST} value significantly higher than G_{ST} typically indicates the presence of phylogeographic structure, with closely related haplotypes found in the same area more often than are less closely related haplotypes (Pons and Petit, 1996). We examined pairwise mismatch distributions to detect historical demographic expansions in *Zizania* using DnaSP. Populations at demographic equilibrium should present multimodal or random and rough distributions of pairwise differences, whereas populations that experienced sudden demographic expansion are expected to display a unimodal, smooth distribution (Slatkin and Hudson, 1991; Rogers and Harpending, 1992). We also performed Fu's F_S test to detect population growth using ARLEQUIN. This test is particularly sensitive to past population expansion, and Fu's F_S statistic is expected to have a large negative value under conditions of demographic expansion (Fu and Li, 1993).

The microsatellite loci were used to calculate two measures of genetic diversity for each species using FSTAT 2.9.3 (Goudet, 1995), i.e., the mean number of alleles per locus (N_A) and the expected heterozygosity (H_E). These measures were not calculated at the population level due to small sample sizes. The genotype number of each population was calculated using GenoDive (Meirmans and Van Tienderen, 2004). AMOVA was used to estimate the assignment of genetic variation within and among populations. The Bayesian clustering approach

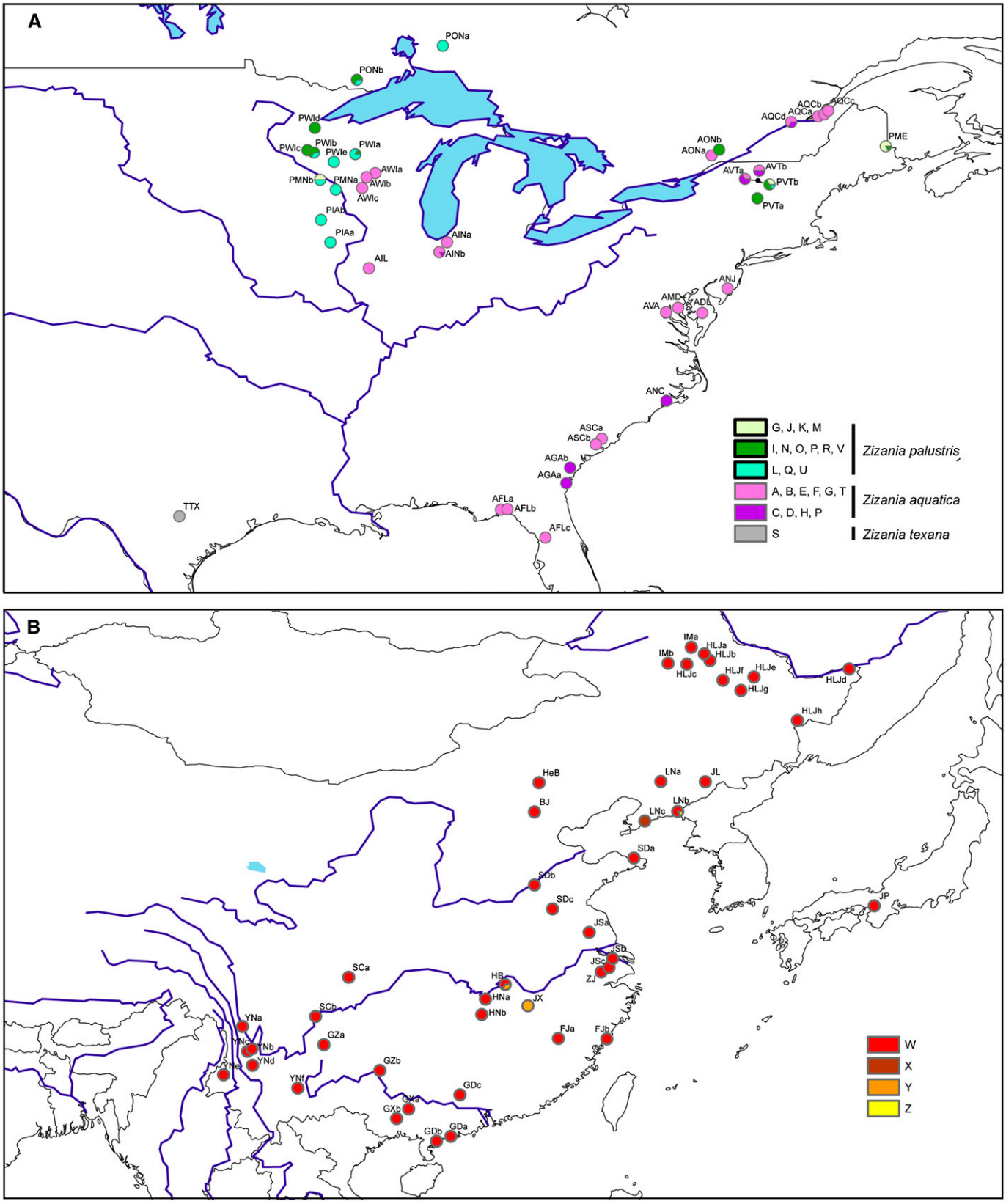

Fig. 1. Sampling localities of 86 *Zizania* populations and the geographic distribution of chloroplast haplotypes. (A) 41 populations from North America. Three subgroups of haplotypes from *Z. palustris* and two subgroups of haplotypes from *Z. aquatica* are presented in different colors (see Fig. 2 for subgroup information). (B) 45 populations of *Z. latifolia* in eastern Asia. Four haplotypes are presented in different colors.

implemented in STRUCTURE 2.0 (Falush et al., 2003) was used to detect population structure. Twenty independent runs were performed for each K value (K = 1 to 10), with a burn-in period of 20 000 iterations and 100 000 Markov chain Monte Carlo (MCMC) iterations under the admixture model. The best-fit number of clusters was determined based on the ΔK method (Evanno et al., 2005).

RESULTS

***Sequence variation and haplotype network*—**Because considerable differences were observed in the sequence data of the three cpDNA fragments (*rps16*, *rpl32-trnL*, and *rps16-trnK*) between eastern Asian and North American *Zizania* taxa, sequence variation are given separately. In *Zizania* in North America, the total length of aligned sequences was 2156 bp. There were 24 polymorphic sites, 13 of which were base substitutions and 11 were 3-5 bp indels (Appendix S3, see Supplemental Data with the online version of this article). In eastern Asian *Zizania*, the total length of aligned sequences was 2150 bp. There were only 4 polymorphic sites, three of which were base substitutions and one was 5 bp indel (Appendix S3).

Based on these polymorphic sites, a total of 26 haplotypes were identified for the 514 *Zizania* individuals examined. Only 4 haplotypes were found among eastern Asian *Zizania*, with the remaining 22 detected among North American *Zizania*. In North America, the endangered *Z. texana* possessed a single haplotype (S). The two widespread species in North America, *Z. aquatica* and *Z. palustris*, had 10 and 13 haplotypes, respectively, two of which (G and P) were shared between them (Appendix S1).

A haplotype network was constructed based on the three cpDNA fragments (Fig. 2). The haplotype networks in North American *Zizania* and eastern Asian *Zizania* were separate at the 95% connection limit and thus discussed separately. In North American *Zizania*, the haplotype network was divided into two distinct groups (Fig. 2A). The first consisted of two subgroups, one including five haplotypes (A, B, E, F, and T) belonging to *Z. aquatica*, and the other including three haplotypes (J, K, and M) belonging to *Z. palustris* and one (G) shared by these two species. In the first group, haplotype A was very common and occurred in 21 populations; other haplotypes occurred only in one or two populations. The second group also consisted of two subgroups, one including three haplotypes (C, D, and H) from *Z. aquatica* and three (L, U, and Q) from *Z. palustris*, and the other including five haplotypes (I, N, O, R, and V) from *Z. palustris*, one (S) from *Z. texana* and one (P) shared by *Z. palustris* and *Z. aquatica*. In this second group, the most common haplotype (L) occurred in eight populations, haplotype P occurred in three populations, and the remaining haplotypes occurred in only one or two populations (Fig. 2A). The haplotype network of eastern Asian *Zizania* is very simple, i.e., four haplotypes in a line, with two (W and X) located at internal nodes. Haplotype W was the most common, occurring in 43 of the 45 populations of *Z. latifolia*; the remaining haplotypes (X, Y, and Z) were detected in only one or two populations (Fig. 2B).

***Genetic diversity*—**Of the 45 populations of eastern Asian *Zizania*, 42 were monomorphic, consisting of a single haplotype. The remaining three populations (HNa, HB, and LNb) consisted of two haplotypes, with haplotype diversity (Hd) ranging from 0.333 to 1.0 and nucleotide diversity (π) ranging from 0.00025 to 0.00093 (Appendix S1). Among the 26 populations of *Z. aquatica*, six consisted of two haplotypes, with Hd ranging from 0.333 to 0.600 and π ranging from 0.00016 to 0.00249. Among the 14 populations of *Z. palustris*, six were polymorphic and consisted of two or three haplotypes, with Hd

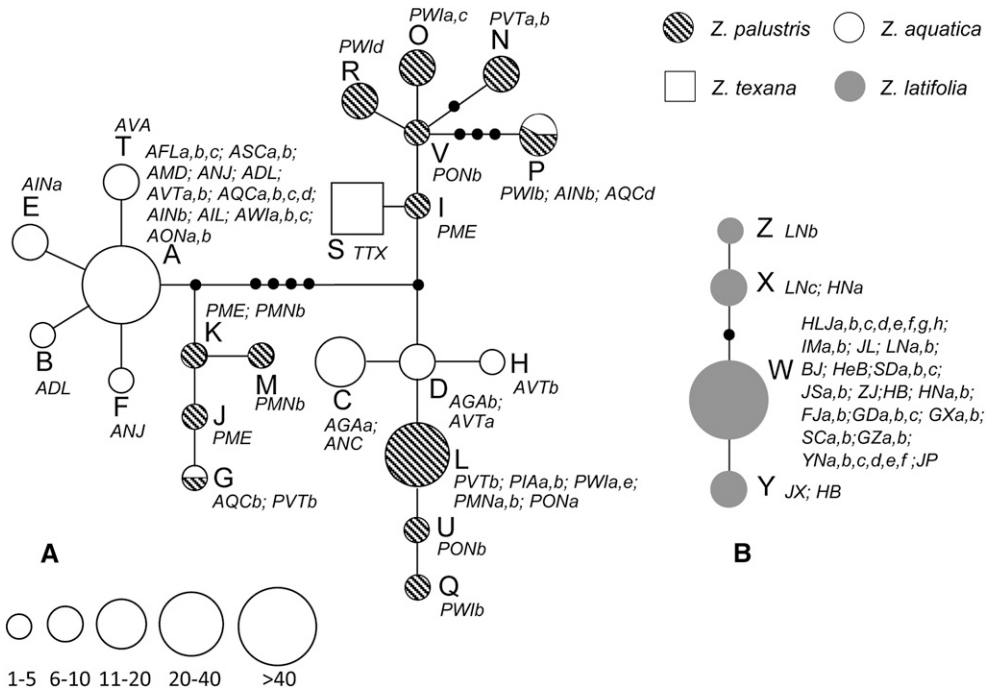

Fig. 2. Chloroplast haplotype network of *Zizania*. Circles represent different haplotypes (A–Z), with size proportional to relative frequency. Black dots represent inferred interior nodes that were absent from the samples. Population names are italicized and indicated next to the haplotypes. (A) North American *Zizania*. (B) Eastern Asian *Zizania*.

ranging from 0.333 to 0.733 and π ranging from 0.00075 to 0.00343. The single population of *Z. texana* consisted of a single haplotype with no polymorphism (Appendix S1). Nearly half of the *Z. latifolia* populations (21) consisted of a single genotype, approximately one-third of *Z. aquatica* populations (8) were monomorphic, and the lone *Z. palustris* population consisted of a single genotype (Appendix S1).

At the species level, the haplotype number, haplotype diversity, and nucleotide diversity of *Zizania latifolia* were 4, 0.125, and 0.00009, respectively, all much lower than the corresponding measures in both *Z. aquatica* (10, 0.461, and 0.00120, respectively) and *Z. palustris* (13, 0.760, and 0.00211, respectively) (Table 1). Therefore, at the continental level, the genetic diversity of North American *Zizania* (22, 0.787, and 0.00245, respectively) is much higher than that of eastern Asian *Zizania* (Table 1). *Zizania palustris* possessed the highest genetic diversity, with N_A (mean number of alleles per locus) = 17 and H_E (expected heterozygosity) = 0.630, whereas *Z. texana* had the lowest genetic diversity, with N_A = 4 and H_E = 0.121 (Table 1). Similarly, the genetic diversity of North American *Zizania* (N_A = 17, H_E = 0.606) is much higher than that of eastern Asian *Zizania* (N_A = 9, H_E = 0.371) (Table 1). We also calculated values of π and H_E for three geographic groups in *Z. aquatica*, consisting of six populations from the Great Lakes region (AWIa, b, c, AINa, b, and AIL) (π = 0.00013 and H_E = 0.368), eight populations from a northern coastal area that was glaciated during the last glacial maximum (LGM) (AVTa, b, PONa, b, and AQCa, b, c, d) (π = 0.00073 and H_E = 0.195), and 12 from a southern coastal, unglaciated area (π = 0.00083 and H_E = 0.318). π and H_E were also calculated for two geographic groups of *Z. palustris*, consisting of three coastal populations (PVTa, b, and PME) (π = 0.00098 and H_E = 0.480) and 11 populations from the Great Lakes region (π = 0.00090 and H_E = 0.521).

***Population genetic structure*—**In each of three widespread *Zizania* species, AMOVAs revealed a high level of genetic differentiation in cpDNA fragments among the sampled populations. The F_{ST} values of *Z. aquatica*, *Z. palustris*, and *Z. latifolia* were 0.710, 0.661, and 0.760, respectively (Table 2). The difference between N_{ST} and G_{ST} was not significant ($P > 0.05$) in *Z. aquatica* (0.717 and 0.746), *Z. palustris* (0.666 and 0.688) or *Z. latifolia* (0.798 and 0.823), indicating that phylogeographic structure in haplotype variation is absent in these three widespread species. The mismatch distribution for each of the three species was clearly inconsistent with the bell-shape curve expected for populations under conditions of demographic expansion (Fig. 3). In addition, no significant negative value of Fu's F_S statistic for *Z. aquatica* (0.85, $P = 0.68$), *Z. palustris* (0.73, $P = 0.65$), or *Z. latifolia* (−2.13, $P = 0.16$) was obtained. These two findings suggest that recent population expansion is unlikely in any of the three species.

AMOVAs of nuclear microsatellite loci revealed significant variation among populations of the three widespread *Zizania* species. The F_{ST} values of *Z. aquatica*, *Z. palustris*, and *Z. latifolia* were 0.607, 0.465, and 0.598, respectively (Table 2), indicating a high level of genetic differentiation among the sampled populations. The STRUCTURE analysis suggested K = 2 as the optimal number of clusters based on the calculation of ΔK in eastern Asian *Zizania* (Appendix S4, see Supplemental Data with the online version of this article). However, the assignments of many individuals were equivocal, and populations in neighboring regions were not genetically similar, indicating that no geographical structure was present (Fig. 4A). For North American *Zizania,* K = 2 was also the optimal solution (Appendix S4). One cluster comprised 26 populations of *Z. aquatica*. The second included 14 populations of *Z. palustris* and the single population of *Z. texana* (Fig. 4B), suggesting a close relationship between these two species. A few individuals of *Z. aquatica* possessed a high percentage of genetic components from *Z. palustris* (Fig. 4B), indicating a low level of gene flow between the two species. STRUCTURE analysis was also performed in North American *Zizania* with K = 3 to test whether genetic clusters corresponded to the three species. However, *Z. texana* did not separate from *Z. palustris* as an independent genetic cluster (Appendix S5, see Supplemental Data with the online version of this article). Further STRUCTURE analyses were performed in *Z. aquatica* and *Z. palustris*, and K = 2 was the optimal solution for these two species (Appendix S4). In *Z. aquatica*, the assignments of individuals from some populations were equivocal, and populations from the Great Lakes region (AWIa, b, c, AINa, b, and AIL) were genetically specific and clustered with several coastal populations (AGAb and AVTa, b) (Fig. 4C). In *Z. palustris*, all three coastal populations (PVTa, b, and PME) clustered with two populations from the Great Lakes region (PONa and PWIc) (Fig. 4D).

DISCUSSION

***Comparison of genetic diversity*—**Compared to North America, the more heterogeneous ecological habitats and smaller impact of Quaternary glaciations in eastern Asia make the region an important center of survival, speciation, and evolution, yielding greater species diversity (Wen, 1999; Guo and Ricklefs, 2000; Qian and Ricklefs, 2004). The same trend has been observed in comparisons of interspecies genetic diversity between some disjunct sister species or closely related species with wide distributions. In *Castanea* (Fagaceae), the genetic diversity of the Chinese chestnut was found to be nearly twice that of the American chestnut (Dane et al., 2003). In *Phryma* (Phrymaceae), higher intracontinental variation was detected in eastern Asian populations (Nie et al., 2006). In the *Smilax hispida*

TABLE 1. Results from analysis of genetic diversity of four *Zizania* species: Sample size (n), haplotype diversity (Hd) and nucleotide diversity (π) of cpDNA data, and mean number of alleles per locus (N_A) and expected heterozygosity (H_E) of nuclear microsatellite data.

		cpDNA			Microsatellite	
Species	n	Haplotype number	Hd	π	N_A	H_E
Zizania aquatica	154	10	0.461	0.00120	4.33	0.347
Zizania palustris	84	13	0.760	0.00211	5.67	0.630
Zizania texana	30	1	0	0	1.33	0.121
North American *Zizania*	268	22	0.787	0.00245	7.0	0.606
Zizania latifolia (eastern Asian *Zizania*)	244	4	0.125	0.00009	3.0	0.371

TABLE 2. Percentages from analyses of molecular variance (AMOVA) of cpDNA and nuclear microsatellite datasets of three widespread *Zizania* species.

		cpDNA		Microsatellite	
Species	Source of variation	Percentage of variation	Fixation index	Percentage of variation	Fixation index
Zizania aquatica	Among populations	70.97%	F_{ST} = 0.710*	60.73%	F_{ST} = 0.607*
	Within populations	29.03%		39.27%	
Zizania palustris	Among populations	66.06%	F_{ST} = 0.661*	46.54%	F_{ST} = 0.465*
	Within populations	33.94%		53.46%	
Zizania latifolia	Among populations	75.95%	F_{ST} = 0.760*	59.88%	F_{ST} = 0.598*
	Within populations	24.05%		40.12%	

Note: * $P < 0.001$.

group, greater haplotype diversity was found in the eastern Asian clade (Zhao et al., 2013). However, in the current study of *Zizania*, the genetic diversity of North American populations was much higher (cpDNA: $\pi = 0.00245$, nuclear microsatellite: $H_E = 0.606$) than that of eastern Asian populations ($\pi = 0.00009$, $H_E = 0.371$). This difference may reflect the different life histories of *Zizania* species. Two widespread North American wild-rice species, *Z. aquatica* and *Z. palustris*, are nonclonal annuals, whereas the eastern Asian species *Z. latifolia* is a perennial that exhibits clonal growth, which is reflected in the high percentage of monomorphic populations (Appendix S1). Annual plants often show elevated rates of molecular evolution relative to their close perennial relatives (Gaut et al., 1997, Smith and Donoghue, 2008). For example, in *Sidalcea* (Malvaceae), Andreasen and Baldwin (2001) found that annual species had significantly higher rates of molecular evolution than perennials and, thus, higher levels of genetic variation. A second possible explanation for the observed greater genetic diversity in the North American *Zizania* populations is that wild-rice originated in North America and then dispersed into eastern Asia (Xu et al., 2010). Most lineages of wild-rice ancestors likely remained in their continent of origin, and only a few ancestral lineages gave rise to *Z. latifolia*. In the current study, 26 cpDNA haplotypes were revealed in wild-rice, 22 of which were found in North America and only four in eastern Asia. A similar pattern has also been observed at the nuclear *Adh1a* locus, i.e., 63 haplotypes were detected in North American *Zizania*, whereas only 10 haplotypes were observed in eastern Asian *Zizania* (Xu et al., 2010). In addition, the relative homogeneity of aquatic environments may weaken the advantage of the ecological heterogeneity of eastern Asia, potentially explaining the discordance in the patterns of genetic diversity between aquatic and terrestrial plants. This hypothesis requires testing in future studies of aquatic plants and their terrestrial counterparts from the two continents.

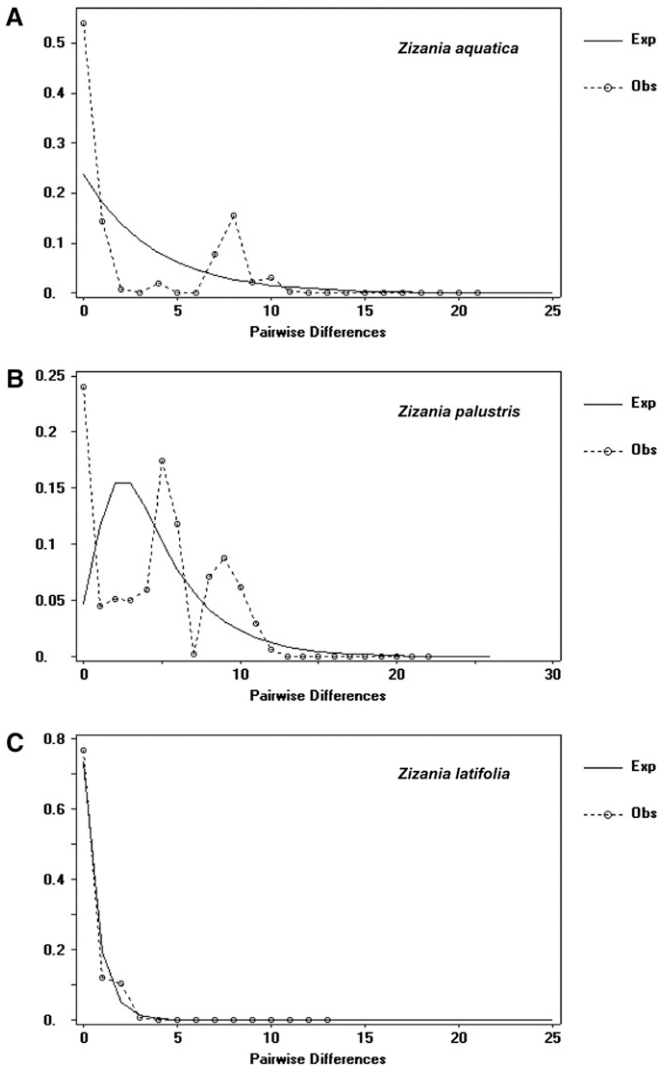

Fig. 3. Mismatch distribution for three widespread *Zizania* species showing the observed (*dotted line*) and expected (*solid line*) pairwise nucleotide site differences. (A) *Z. aquatica*. (B) *Z. palustris*. (C) *Z. latifolia*.

Comparisons of genetic diversity among the three widespread species revealed that the diversity based on cpDNA was highest in *Zizania palustris* ($\pi = 0.00211$), intermediate in *Z. aquatica* (0.00211), and lowest in *Z. latifolia* (0.00009), whereas the diversity based on nuclear microsatellites was highest in *Z. palustris* ($H_E = 0.630$), intermediate in *Z. latifolia* (0.371), and lowest in *Z. aquatica* (0.347). This discordance may be due to differences between the genetic markers. The allele in microsatellite loci was determined by the length of amplicon, a criterion different from the base variation used in DNA sequences. Thus, the genetic diversity of *Z. aquatica* may be underestimated at microsatellite loci due to homoplasy. This possibility seems likely because a previous study based on nuclear *Adh1a* sequences also revealed the same order of genetic diversity in *Zizania* (Xu et al., 2010) as that indicated by the cpDNA fragments studied here.

***Comparison of population genetic structure*—**An important feature of all three widespread *Zizania* species is the high level of genetic differentiation among populations (the minimum F_{ST} is 0.465) based on both cpDNA and microsatellite

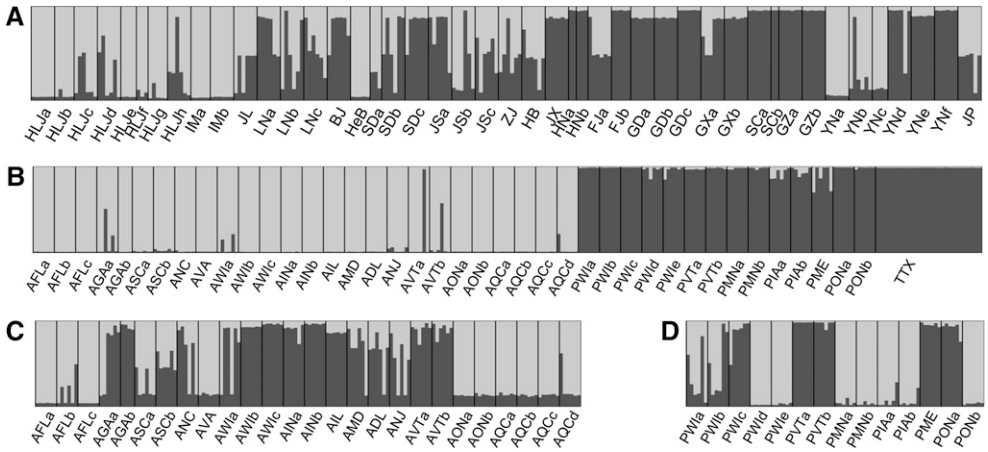

Fig. 4. Genetic clusters identified by STRUCTURE based on microsatellite data with each bar representing the proportional membership assignments of an individual according to the population codes shown. (A) Eastern Asian *Zizania*. (B) North American *Zizania*. (C) *Z. aquatica*. (D) *Z. palustris*.

markers, indicating a low rate of gene flow among populations. Similar results were observed in *Z. latifolia* using nuclear *Adh1a* sequences (Xu et al., 2008) and *Z. palustris* using isozyme markers (Lu et al., 2005). The aquatic habitat of *Zizania* is discrete and patchy. Dispersal of seeds by waterfowl is unknown in *Zizania*, and the seeds are unlikely to disperse via water currents between very spatially isolated populations. In wind-pollinated species, pollen can be carried long distances, but most effective pollination occurs at a local scale (Willson, 1983). In another *Zizani*a species, *Z. texana*, most pollen was found to disperse within 1.5 m of the source plant, with an average wind speed of 1.5 m/s (Oxley et al., 2008). Therefore, limited gene flow among populations likely explains the high levels of population differentiation in *Zizania*.

In the current study, different phylogeographic patterns were revealed between eastern Asian and North American *Zizania*. In eastern Asia, four cpDNA haplotypes were observed in *Z. latifolia*, with one widespread and the other three restricted to one or two populations. No obvious phylogeographic structure was observed. A similar pattern has been observed in another aquatic plant, *Sagittaria trifolia* L. (Alismataceae), with two cpDNA haplotypes throughout China and the remaining one limited to individual populations (Chen et al., 2008). However, a recent phylogeographic analysis of *Z. latifolia* based on nuclear *Adh1a* sequences showed that levels of genetic diversity gradually declined from north to south (Xu et al., 2008). This trend is supported by our finding that most of polymorphic populations, based on three microsatellite loci, were from the northern part of China (Appendix S1). This result needs to be confirmed with more microsatellite loci and a larger size of individual *Z. latifolia* populations. In North America, the divergence of *Zizania* is estimated at 0.71 mya (Xu et al., 2010) or 1.1 mya (Walker, 2011), suggesting that the North American *Zizania* began to diverge in the early or middle Pleistocene. The relatively recent diversification in North America is consistent with the unilateral interspecific crossability barrier between *Z. aquatica* and *Z. palustris* (Duvall and Biesboer, 1988) and the fact that haplotypes of *Z. aquatica* and *Z. palustris* are not all most closely related to each other within taxa as expected (Fig. 2A). This pattern is likely due to incomplete lineage sorting, as two distinct genetic clusters corresponding to *Z. aquatica* and *Z. palustris* were identified based on microsatellite data (Fig. 4B), and the discordance between the cpDNA and microsatellite data are likely due to the different evolutionary rates of the chloroplast and nuclear genomes. During the last glacial maximum, the Laurentide ice sheet covered the northern part of North America, and its southern margin extended south of the Great Lakes region (Dyke and Prest, 1987). Therefore, species have undergone postglacial range expansions from ice-age refugia in the southern region, and there is a general pattern of reduced variation in northern populations relative to southern populations (Avise, 2000; Soltis et al., 2006). This pattern has been revealed in two species of aquatic plants, *Sagittaria latifolia* Willd. (Dorken and Barrett, 2004) and *Podostemum ceratophyllum* Michx. (Podostemaceae) (Fehrmann et al., 2012). However, no such pattern was apparent in North American *Zizania* in the current study. No evidence of recent population expansion was detected in *Z. aquatica* or *Z. palustris* based on mismatch distributions and neutrality tests; therefore, we speculate that the effects of the LGM on these two species was not severe and that little genetic diversity was lost. In *Z. aquatica*, the southern coastal area had more haplotypes and greater nucleotide diversity than either the northern coastal area or the Great Lakes region, consistent with the general pattern mentioned above. However, the Great Lakes region had the highest expected heterozygosity, although the populations grouped into one cluster at the microsatellite loci, suggesting it was unlikely to have been colonized from the southern coastal area. In addition, the inconsistent patterns between the cpDNA and microsatellite data indicate a complex evolutionary history in *Z. aquatica* and that further investigations are warranted. In *Z. palustris*, the majority of its extant range was covered by the Laurentide ice sheet; therefore, its distribution during the LGM should be far south of its extant range, possibly even as far as central Texas, where *Z. texana* grows. A close relationship between *Z. palustris* and *Z. texana* was revealed by our cpDNA and microsatellite data as well as previous studies (Duvall, 1987; Horne and Kahn, 1997; Xu et al., 2010), supporting the hypothesis that *Z. texana* is a relict population that was isolated from ancestral *Z. palustris* during the northward postglacial colonization. The Great Lakes region and the coastal area share haplotypes from three subgroups of *Z. palustris* (Fig. 1A) and similar levels of nucleotide diversity and expected heterozygosity, suggesting that no bias existed between these two glaciated

regions during the northward postglacial colonization from the refugia. More extensive samples are needed to test whether the general pattern described above is apparent in *Z. palustris*. Relative to eastern Asian *Zizania*, the complex evolutionary history of North American *Zizania* was likely caused by greater latitude shifts in their distribution driven by the greater impact of glaciations in North America.

In conclusion, our study reveals higher genetic diversity and more complicated phylogeographic pattern in North America than those in eastern Asia in the *Zizania* genus, which is contrast to a priori expectation of eastern Asian bias based on previous studies (Dane et al., 2003; Nie et al., 2006; Zhao et al., 2013). As one of a few case studies, our study also provides insight into the diversification and evolution of the eastern Asian-North American disjunct taxa in both continents. Phylogeographic analyses of other taxa that exhibit disjunct distributions in eastern Asia and North America are needed, including trees, terrestrial herbs, and other aquatic plants, to deepen our understanding of this intercontinental disjunction.

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