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## Phylogeographical studies of *Glycine soja*: implicating the refugium during the Quaternary glacial period and large-scale expansion after the Last Glacial Maximum

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**Abstract:** Wild soybean (*Glycine soja* Sieb. & Zucc.), the progenitor of cultivated soybean (*Glycine max* (L.) Merr.), is widely distributed in the Sino-Japanese Floristic Region (SJFR). Therefore, it is an excellent model organism to study the effect of Quaternary climatic changes on the distribution and migration of plant taxa in the SJFR. To investigate the phylogeography of *G. soja*, a total of 632 individuals representing 52 populations throughout the species distribution range were genotyped by using the sequencing data of a nuclear locus, AF105221, and a chloroplast locus, *trnQ-rps16*. We detected a total of 23 haplotypes from two loci, including five common and 18 rare haplotypes. Among them, 17 rare haplotypes were limited to Northeast Asia (Northeast China (NEC) and Korea) and the Yangzi River basin (middle and downstream of the Yangzi River (MDYR) and the eastern end of Southwest China (SWC)). Moreover, two common haplotypes were detected throughout the species distribution wide range. All the haplotypes from Japan and the Korean peninsula were also found in China. The haplotype distribution patterns of the two loci suggested that Northeast Asia (NEC and Korea) and the Yangzi River basin (MDYR and the eastern end of SWC) might have been the refugia for *G. soja* during the Quaternary glacial period, and *G. soja* expanded rapidly after the end of the Last Glacial Maximum. There was a land bridge between East China, the Korean peninsula, and Japan that permitted gene flow among populations of these regions in the ice age during the Quaternary.

**Key words:** Conservation, phylogeography, Pleistocene glaciation, population genetic structure, wild soybean

### 1. Introduction

The climatic oscillations during the Quaternary profoundly affected the genotype distribution patterns of plant taxa growing in temperate zones of the northern hemisphere (Hewitt, 2000). Fossil data and phylogeographic studies suggest that the distribution ranges of temperate plant species in Europe and North America, and especially those of trees and shrubs, changed with the latitudinal range shifts during glacial and inter-/postglacial periods. They tended to migrate towards lower latitudes in the ice age, and were then reestablished in northern regions after the end of the ice age (Petit et al., 2005). However, unlike Europe and North America, most parts of East Asia had not been covered by extensive ice sheets, and glaciers appeared only in high mountains and the northern part during the Quaternary (Zheng et al., 1998). The severe periodic climatic oscillations also profoundly affected the ecological environment and land topography of East Asia and thus the distribution and genotypic geographical patterns of plant species (Liu, 1988; Harrison et al., 2001).

For example, the large volume of accumulated ice reduced sea levels by about 120 m (Chung, 2007). The lowering of the sea level during ice ages produced land bridges in the east sea basin that linked the Japanese islands and some other islands with East China and the Korean peninsula. Those islands were then isolated from East China and the Korean peninsula again by the sea when sea levels rose during the postglacial period. Hence, gene flow between plant taxa could have happened between China, Japan, and the Korean peninsula (the CJK region) by a so-called land bridge in an ice age, and then was interrupted during the postglacial period (Dobson and Kawamura, 1998). Extreme climate events such as cold/warm spells and dry/wet periods are common in the mainland of East Asia (An, 2000). It is well known that both temperature and humidity have remarkable effects on the distribution of plant and animal taxa.

Phylogeographical studies suggested that the distribution ranges of some plant species in East Asia experienced no latitudinal expansion and multiple cryptic

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refuges may exist in different latitudes (Qiu et al., 2011). Meanwhile, plant taxa were severely affected by habitat fragmentation and population isolation in the CJK region (Li et al., 2008; Qiu et al., 2009a, 2009b; Baloch et al., 2010), North China (Tian et al., 2009; Bai et al., 2010), Southwest China (Wang et al., 2008; Liu et al., 2009), and subtropical China (Gao et al., 2007; Gong et al., 2008; Qiu et al., 2009c; Guan et al., 2010) of the Sino-Japanese Floristic Region (SJFR) during the Quaternary.

Overall, the effects of climatic oscillations on species distribution patterns are not clear in the SJFR during the Quaternary. We inferred the following four possible reasons for this: 1) the taxa for plant phylogeographic studies were not enough; 2) plant taxa were more rich in this region; 3) the heterogeneity of ecological and climatic factors, such as temperature and humidity, showed more pronounced effects on species distribution in the SJFR than Europe or North America; 4) the studied taxa in the SJFR were mainly trees or shrubs with relatively limited geographic distributions (Aizawa et al., 2007; Gao et al., 2007; Wang et al., 2009).

Wild soybean (*Glycine soja*) is the progenitor species of soybean (*G. max*) and one of the world's most important crops (Smil, 2000; Shahid et al., 2009). *G. soja* is widely distributed in East Asia, including China, Korea, Japan, and the Russian Far East, areas located between 24°N and 53°N and 97°E and 143°E, largely belonging to the SJFR. *G. soja* is an annual vine and usually grows in agricultural fields and along field margins, roadsides, riverbanks, lakesides, and marshlands at altitudes ranging from 5 to 2650 m above sea level (Lu, 2004). *G. soja* is a self-fertilized species with outcrossing rates ranging from 2.4% to 19% (Kiang et al., 1992; Fujita et al., 1997). Short-distance dispersal by the means of pod dehiscence is a major way for the expansion of *G. soja* populations, and longer distances of dispersal may occur via water or birds (Kiang et al., 1992; Choi et al., 1999; Kuroda et al., 2006).

Sequence-based studies of population structure, historical demography, and phylogeography offer greater precision and repeatability than molecular marker-based analyses, particularly if both organelle and single-copy nuclear loci are employed (Petit et al., 2005; Wang et al., 2015a). Here, we executed the phylogeographic study of 632 *G. soja* individuals representing 52 populations by using chloroplast and nuclear loci sequences. The major aim was to address the current geographical patterns of the *G. soja* genotypes and the demographical history dynamics of *G. soja* in the Quaternary, such as the possible refugium in the ice age, the gene flow route during the glacial stage, and the expansion during the postglacial period. The results would not reveal only the evolutionary history of this species but would also provide the basic information for the conservation and utilization of *G. soja*

for breeding programs. Moreover, they will contribute to our understanding of the effects of climatic oscillations on species distribution patterns in the SJFR during the Quaternary.

## 2. Materials and methods

### 2.1. Sampling

More than 1500 wild soybean individual leaf samples were obtained from 52 populations (43, 5, and 4 populations from China, Korea, and Japan, respectively). The sampled populations were clustered into the following eight groups based on geographical distribution and climatic conditions: South China (SC), the middle and downstream of the Yangzi River (MDYR), Southwest China (SWC), Northwest China (NWC), North China (NC), Northeast China (NEC), Japan, and Korea (Table 1). These groups were largely consistent with the floristic provinces of the Eastern Asiatic region (Wu and Wu, 1998). The young leaves were kept in sealed plastic bags and dried by silica-gel. A total of 632 individuals were randomly selected from 52 populations for PCR, with an average of 4–13 individuals (mostly 12) per population, and sequenced for the final analysis.

#### 2.1.1. DNA extraction and selection of loci/genes

Total genomic DNA was extracted from silica gel-dried leaves using the CTAB method (Doyle and Doyle, 1987), dissolved in 0.1X TE buffer (10 mmol/L Tris-HCl, pH 8.0, 1 mmol/L EDTA) to a final concentration of 50–100 ng/μL for PCR. In this study, a nuclear gene locus (AF105221) and a chloroplast gene locus (*trnQ-rps16*) were selected for investigations. Previous study had shown that AF105221 is a single-copy gene in *G. soja* populations (Wang et al., 2015b) and that cpDNA locus *trnQ-rps16* evolved at a fast rate (Shaw et al., 2007).

#### 2.2. PCR amplification and sequencing

The PCR amplification was done by using a gene-specific pair of primers for AF105221 and *trnQ-rps16* as described by Van et al. (2004) and Shaw et al. (2007), and *trnQ-rps16* is a universal primer, i.e. one pair of the primer is likely to amplify nearly all angiosperm taxa (Shaw et al., 2007). PCR amplification was carried out in a volume of 50 μL containing 50–100 ng of DNA template, 0.1–0.2 μmol of each primer, 0.25 mM of each dNTP, 1.5 mM MgCl<sub>2</sub>, and 0.5 U of Taq DNA polymerase. The PCR protocol was used as follow: 94 °C for 5 min; 35 cycles of 94 °C for 30 s, 56 °C for 45 s, and 72 °C for 90 s; and a final extension at 72 °C for 10 min. The PCR products were purified with PCR Purify Kit UNIQ-10 (Sangon Biotech, China) following the manufacturer's protocol. The purified PCR products were sequenced with the 3730/ABI sequencer (Thermo Fisher Scientific, USA). PCR amplifications and sequencing were repeated twice for each product to confirm the sequence variation.

**Table 1.** The location and natural habitats of *G. soja* populations.

Geographical region	Population name	Location of sampling	Longitude (N)	Latitude (E)	Altitude (m)	Habitat
SC	Population AF	Anfu County, Jiangxi Province	27°23'17"	114°36'06"	85	Beside road
	Population JO	Jianou County, Fujian Province	27°01'28"	118°17'34"	126	Beside river
	Population HY	Hengyang County, Hunan Province	26°57'43"	112°09'12"	123	Beside river
	Population RY	Ruyuan County, Guangdong Province	24°55'12"	113°08'12"	510	Beside road
	Population QZ	Quanzhou County, Guangxi Province	25°52'21"	110°51'43"	722	Beside road
MDYR	Population WC	Wuchang District, Hubei Province	30°31'16"	114°23'44"	19	Beside road
	Population XU	Xuanwu District, Jiangsu Province	32°02'44"	118°51'39"	28	Wasteland
	Population WQZ	Duqing County, Zhejiang Province	30°33'00"	119°58'20"	15	Beside canal
	Population SC	Shucha County, Anhui Province	31°18'51"	117°07'40"	45	Beside road
SWC	Population TB	Tongbai County, Henan Province	32°22'12"	113°24'01"	33	Beside road
	Population CK	Chengkou County, Chongqing	31°58'98"	108°40'02"	805	Valley
	Population YJ	Yinjiang County, Guizhou Province	28°00'03"	108°24'36"	458	Valley
	Population GH	Guangan City, Sichuan Province	30°59'49"	104°20'55"	458	Beside river
	Population CY	Chayu County, Xizang Province	27°03'53"	100°50'03"	1685	Valley
NWC	Population NL1	Ninglang County, Yunnan Province	28°30'08"	97°00'38"	2600	Beside field
	Population NL2	Ninglang County, Yunnan Province	28°06'54"	99°51'02"	2550	Beside field
NWC	Population BX	Bingxian County, Shaanxi Province	35°02'26"	108°04'39"	835	Valley
	Population HX	Huixian County, Gansu Province	33°53'36"	105°49'33"	1126	Canal
	Population YY	Youyu County, Shanxi Province	40°08'38"	112°20'22"	1279	Beside road river
	Population LW	Lingwu County, Ningxia Province	38°08'44"	106°19'33"	1103	Canal
	Population WS	Wenshui County, Shanxi Province	37°25'02"	112°01'02"	759	Beside canal
	Population YA	Ya'an County, Shaanxi Province	36°37'29"	109°27'18"	962	Along river
	Population YL	Yulin city, Shanxi Province	38°16'50"	109°44'18"	1051	Along river
	Population LXS	Linxian County, Shanxi Province	37°56'51"	110°59'15"	948	Along river
	Population YC	Yichuan County, Shanxi Province	36°03'31"	110°11'20"	829	Along river
	Population BJ	Baoji city, Shanxi Province	34°22'04"	107°0'05"	586	Along river
NC	Population WN	Weinan city, Shaanxi Province	34°31'01"	109°30'00"	355	Along river
	Population JZ	Jizhou County, Hebei Province	37°44'31"	115°41'12"	23	Beside road
	Population DY	Dongying City, Shandong Province	37°34'25"	118°31'27"	6	Beside ditches
	Population WQT	Wuqing District, Tianjing	39°26'57"	117°14'57"	-6	Beside ditches
	Population XH	Xuanhua County, Hebei Province	40°35'34"	115°01'17"	601	Beside river
	Population QHD	Qinghuangdao city, Hebei Province	39°48'29"	119°25'54"	18	Beside river
NEC	Population YT	Yantai city, Shandong Province	37°28'65"	121°27'14"	10	Wasteland
	Population LXH	Lanxi County, Heilongjiang Province	46°13'06"	126°20'16"	139	Beside pond
	Population JH	Jiaohe County, Jinlin Province	43°48'28"	127°14'15"	126	Beside river
	Population XM	Xinming County, Liaoning Province	41°57'19"	122°51'29"	28	Beside pond
	Population MDJ	Mudanjiang city, Heilongjiang Province	44°34'07"	129°36'26"	253	Beside river
	Population BS	Baishan city, Jilin Province	42°27'00"	128°07'44"	701	Roadside
	Population KS	Keshan County, Heilongjiang Province	48°17'00"	125°29'54"	325	Beside field
	Population QQHE	Qiqihaer city, Heilongjiang Province	47°20'27"	123°56'24"	304	Beside river
	Population HG	Hegan city, Heilongjiang Province	47°12'20"	130°13'46"	213	Beside road
Japan	Population DH	Dunhua city, Jilin Province	43°22'28"	128°13'36"	495	Beside road
	Population HL	Hulin city, Heilongjiang Province	42°02'24"	128°03'16"	73	Beside field
	Population J1	Kanagawa, Japan	34°57'33"	137°08'20"	12	Wetland
	Population J2	Tokyo, Japan	34°49'41"	135°46'13"	35	Wetland
Korea	Population J3	Hirakata, Osaka, Japan	34°48'52"	135°29'02"	11	Wetland
	Population J4	Okazaki, Japan	34°57'33"	137°08'20"	37	Wetland
Korea	Population K1	Gangwon-do, South Korea	37°35'15"	128°24'33"	520	Wetland
	Population K2	Gangwon-do, South Korea	37°54'47"	128°29'56"	340	Wetland
	Population K3	Incheon, South Korea	37°27'30"	126°21'53"	11	Wetland
	Population K4	Yeongcheon-si city, South Korea	37°54'47"	128°29'56"	102	Along road
	Population K5	Moonkyeong-si city, South Korea	37°27'30"	126°21'53"	77	Along road

SC = South China; MDYR = Middle and downstream of the Yangzi River; SWC = Southwest China; NWC = Northwest China; NC = North China; NEC = Northeast China.

### 2.3. Data analysis

DNA sequences were aligned using ClustalX version 2.0 (Thompson et al., 1997) and then adjusted manually by the program BioEdit 4.10 (Hall, 1999). All sequences were deposited in GenBank (accession numbers: GU594719–GU594728 and GU594681–GU594692, KX060992). For further analyses, insertion and deletion mutations were excluded from aligned sequences.

The haplotype occurrences and their frequencies were identified with the program DAMBE (Xia and Xie, 2001). Median-joining haplotype networks were constructed for the two loci using Network version 4.2.0.1 (Bandelt et al., 1999) (<http://www.fluxus-engineering.com>). The geographical distribution of the different haplotypes identified in *G. soja* was mapped using ArcMap 9.1 (<http://www.esri.com/software/arcgis/>).

The parameters of genetic diversity ( $H_s$  and  $H_T$ ) and of genetic differentiation ( $G_{ST}$  and  $N_{ST}$ ) were estimated by using the program PERMUT (available at <http://www6.bordeaux-aquitaine.inra.fr/biogeco/Production-scientifique/Logiciels/Contrib-Permut/Permut>).  $G_{ST}$  (coefficient of gene differentiation) and  $N_{ST}$  (frequencies and similarities among individuals) were compared by using a permutation test with 1000 permutations (Burban et al., 1999).

## 3. Results

### 3.1. Haplotype statistics and evolutionary network

The sequences of the AF105221 and *trnQ-rps16* loci were obtained with aligned lengths of 541 bp and 476 bp after eliminating the insertion/deletion sites from 616 and 617 individuals, respectively. We identified 11 and 9 polymorphic sites and 13 (A1–A13) and 10 (B1–B10) different haplotypes at loci AF105221 and *trnQ-rps16*, respectively (Tables 2 and 3). Among A1 to A13, A1 exhibited the highest frequency and accounted for 71.75% of total sequences of locus AF105221, followed by A3 and A2, which accounted for 9.42% and 6.17% of individuals, respectively. Five haplotypes, A5, A9, A10, A11, and A13, appeared only one time, and all other haplotypes were found to be rare haplotypes (<5%) (Table 4). A1 appeared in the central position of the evolutionary network, and eight haplotypes, including the second and third most frequent haplotypes (A3 and A2), linked directly with the A1 haplotype (Figure 1a). Among B1 to B10, two main haplotypes, B1 and B2, accounted for 34.36% and 51.38% of total sequences of locus *trnQ-rps16*, respectively. The third highest was B3, which accounted for 9.40%, and the other seven were rare haplotypes (<5%) (Table 5). B2 was found in the central position of the evolutionary network, and six haplotypes, including the second and third most frequent haplotypes, B1 and B3, exhibited a direct link with the B2 haplotype (Figure 1b).

### 3.2. Geographical distribution patterns of haplotypes

For AF105221, the dominant haplotype A1 was distributed widely in the species range, appearing in 43 populations of China and two Japanese and two Korean populations, excluding two populations of SWC, one population of NC, and four populations of NEC. B1 was the dominant haplotype (Freq. > 50%) in all populations, and it was the only haplotype found in 19 populations. The A2 haplotype was distributed in five populations across four geoecological regions (i.e. one population of SWC, one population of MDYR, two populations of SC, and one population of Korea). A3, the second highest frequent haplotype, was mainly distributed (37.93%) in five populations of NEC and was also found in four populations of MDYR, one population of NC, two populations of NWC, one population of the Korean region, and two populations of the Japanese region. Among the rare haplotypes of locus AF105221, A4 was distributed mainly in NEC, but was also found in MDYR, Korea, and Japan in one or two populations with low frequency. A7 was distributed across four geoecological regions but just appeared in one or two populations with very low frequency. Other rare haplotypes, such as A5, A6, A8, A9, A10, A11, and A13, just appeared in one population and belonged to different geographical regions. A11 was the dominant haplotype but was limited to the Korean region (Figure 2a; Table 4).

For *trnQ-rps16*, B1 was distributed mainly in SC, SWC, MDYR, and the Korean region, and it was also found in other geoecological regions. B1 occupied the whole SC area alone and dominated the SWC and MDYR regions. B2 was distributed mainly in NEC, NC, NWC, and SWC and it was the dominant haplotype in NEC, NC, and NWC. B3 was detected in the MDYR and Japanese regions. Interestingly, the shore of the Bohai Sea, Jiaodong peninsula, was also occupied by B3. Other rare haplotypes were limited to one geoecological region or a population. NEC had the highest haplotype diversity, and six haplotypes, including two common haplotypes, B2 and B3, and four rare haplotypes, B7–B10, were limited to three populations of this region. The geoecological groups with the second highest haplotype diversity were MDYR and SWC, and four haplotypes were detected in each region. We found three common haplotypes (B1, B2, and B3) and one rare haplotype (B4) in MDYR, which was limited to one population. There were two common haplotypes (B1 and B2) and two rare haplotypes (B5 and B6) in SWC, and each rare haplotype was limited to one population and located in the eastern end of this region, near the MDYR region. NWC, NC, Korea, and Japan shared two common haplotypes (Figure 2b; Table 5).

### 3.3. Genetic diversity of geographical structure

We detected approximately 2.15 and 2.7 times higher total genetic diversity,  $H_T$ , than average diversity within

**Table 2.** Nucleotide substitutions detected at AF105221 locus in *G. soja* and their accession numbers.

Haplotype	22*	34	44	63	166	208	221	224	243	413	500	Accession number
A1	A	A	C	T	C	A	G	G	T	A	A	GU594681
A2	A	A	C	T	C	A	A	G	T	A	A	GU594682
A3	A	A	C	T	C	A	G	G	C	A	A	GU594683
A4	A	A	C	C	C	A	G	G	T	G	A	GU594684
A5	A	A	C	T	C	G	G	G	T	A	A	GU594685
A6	A	A	A	T	C	A	G	G	T	A	A	GU594686
A7	A	G	C	C	C	A	G	G	T	G	A	GU594687
A8	T	A	C	T	C	A	G	G	T	A	A	GU594688
A9	A	A	C	T	C	A	G	G	T	G	A	GU594689
A10	A	A	C	T	C	A	G	T	T	A	A	GU594690
A11	A	G	C	T	C	A	A	G	T	A	A	GU594691
A12	A	A	C	T	T	T	A	A	G	A	A	GU594692
A13	A	A	C	T	T	T	A	A	G	A	T	KX060992

\*Numbers indicate the order of polymorphic sites in gene segment.

**Table 3.** Nucleotide substitutions detected at *trnQ-rps16* locus in *G. soja* and their accession numbers.

Haplotype	4*	124	203	236	237	287	313	375	383	Accession number
B1	A	T	A	A	T	T	T	C	G	GU594719
B2	A	T	A	A	T	G	T	C	G	GU594720
B3	A	T	A	A	T	G	T	A	G	GU594721
B4	C	T	A	A	T	G	T	A	G	GU594722
B5	A	T	A	A	T	T	T	C	A	GU594723
B6	A	G	A	A	T	G	T	C	G	GU594724
B7	A	T	A	T	T	T	T	C	A	GU594725
B8	A	T	A	A	G	G	T	C	G	GU594726
B9	A	T	C	A	T	G	T	C	G	GU594727
B10	A	T	A	A	T	G	G	C	G	GU594728

\*Numbers indicate the order of polymorphic sites in gene segment.

populations ( $H_s$ ) across populations for the AF105221 and *trnQ-rps16* loci. However, there was a nonsignificant difference between  $G_{ST}$  and  $N_{ST}$  for both loci, although  $N_{ST}$  was higher than  $G_{ST}$  (Table 6), which suggested the presence of a relatively weak phylogeographic structure within the species.

#### 4. Discussion

##### 4.1. The possible refugia of *G. soja* during the ice age

Phylogeographic studies suggested that plant taxa did not experience long-distance dispersal southward to northward during the interglacial cycle of the Quaternary

glacial period and the Last Glacial Maximum (LGM) in the East Asia region, and these plant taxa were believed to survive in multiple cryptic refugia near their natural habitats during the interglacial cycle (Qiu et al., 2011). Similar behavior was noticed in *G. soja*. Here, we demonstrated that there were at least two refugia for *G. soja*: one refugium was in Northeast Asia, including the NEC region and the Korean peninsula, and another was in the Yangtze River basin, including the MDYR region and the eastern region of SWC. The reasons for this conclusion are as follows: two characters, such as high haplotype diversity and appearance of unique haplotypes, were regarded as

**Table 4.** Number of haplotypes per population identified at AF105221 locus.

Population	A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	A11	A12	A13	Total
SC group	55	4	0	0	0	0	0	0	0	0	0	0	0	59
G1_AF	11	1	0	0	0	0	0	0	0	0	0	0	0	12
G1_JO	12	0	0	0	0	0	0	0	0	0	0	0	0	12
G1_HY	8	3	0	0	0	0	0	0	0	0	0	0	0	11
G1_RY	12	0	0	0	0	0	0	0	0	0	0	0	0	12
G1_QZ	12	0	0	0	0	0	0	0	0	0	0	0	0	12
MDYR group	45	3	9	3	0	0	0	0	0	0	0	0	0	60
G2_WC	8	3	1	0	0	0	0	0	0	0	0	0	0	12
G2_XU	7	0	5	0	0	0	0	0	0	0	0	0	0	12
G2_WQZ	12	0	0	0	0	0	0	0	0	0	0	0	0	12
G2_SC	8	0	1	3	0	0	0	0	0	0	0	0	0	12
G2_TB	10	0	2	0	0	0	0	0	0	0	0	0	0	12
SWC group	50	10	0	0	1	10	0	0	0	0	0	0	0	71
G3_CK	2	10	0	0	0	0	0	0	0	0	0	0	0	12
G3_YJ	11	0	0	0	1	0	0	0	0	0	0	0	0	12
G3_GH	1	0	0	0	0	10	0	0	0	0	0	0	0	11
G3_CY	12	0	0	0	0	0	0	0	0	0	0	0	0	12
G3_NL1	12	0	0	0	0	0	0	0	0	0	0	0	0	12
G3_NL2	12	0	0	0	0	0	0	0	0	0	0	0	0	12
NWC group	127	0	3	0	0	0	1	0	0	0	0	0	0	131
G4_BX	12	0	0	0	0	0	0	0	0	0	0	0	0	12
G4_HX	11	0	1	0	0	0	0	0	0	0	0	0	0	12
G4_YY	12	0	0	0	0	0	0	0	0	0	0	0	0	12
G4_LW	12	0	0	0	0	0	0	0	0	0	0	0	0	12
G4_WS	10	0	2	0	0	0	0	0	0	0	0	0	0	12
G4_YA	11	0	0	0	0	0	1	0	0	0	0	0	0	12
G4_YL	11	0	0	0	0	0	0	0	0	0	0	0	0	11
G4_LXS	12	0	0	0	0	0	0	0	0	0	0	0	0	12
G4_YC	12	0	0	0	0	0	0	0	0	0	0	0	0	12
G4_BJ	12	0	0	0	0	0	0	0	0	0	0	0	0	12
G4_WN	12	0	0	0	0	0	0	0	0	0	0	0	0	12
NC group	66	0	12	0	0	0	1	4	0	0	0	0	0	83
G5_JZ	12	0	0	0	0	0	0	0	0	0	0	0	0	12
G5_DY	12	0	0	0	0	0	0	0	0	0	0	0	0	12
G5-WQT	8	0	0	0	0	0	0	4	0	0	0	0	0	12
G5_XH	11	0	0	0	0	0	0	0	0	0	0	0	0	11
G5_QHD	11	0	0	0	0	0	1	0	0	0	0	0	0	12
G5_YT	0	0	12	0	0	0	0	0	0	0	0	0	0	12
G5_JN	12	0	0	0	0	0	0	0	0	0	0	0	0	12
NEC group	73	0	22	19	0	0	3	0	1	1	0	0	0	119
G6_LXH	7	0	2	3	0	0	0	0	0	0	0	0	0	12
G6_JH	9	0	3	0	0	0	0	0	0	0	0	0	0	12

Table 4. (Continued).

G6_XM	12	0	0	0	0	0	0	0	0	0	0	0	0	12
G6_MDJ	5	0	6	1	0	0	0	0	0	0	0	0	0	12
G6_BS	4	0	3	1	0	0	3	0	0	0	0	0	0	11
G6_KS	9	0	0	2	0	0	0	0	1	0	0	0	0	12
G6_QQHE	10	0	0	2	0	0	0	0	0	0	0	0	0	12
G6_HG	3	0	5	4	0	0	0	0	0	0	0	0	0	12
G6_DH	3	0	3	6	0	0	0	0	0	0	0	0	0	12
G6_HL	11	0	0	0	0	0	0	0	0	1	0	0	0	12
Japan group	11	18	10	3	0	0	5	0	0	0	1	0	0	48
J1	2	0	8	2	0	0	0	0	0	0	0	0	0	12
J2	9	0	2	1	0	0	0	0	0	0	0	0	0	12
J3	0	12	0	0	0	0	0	0	0	0	0	0	0	12
J4	0	6	0	0	0	0	5	0	0	0	1	0	0	12
Korea group	15	3	2	2	0	0	0	0	0	0	0	22	1	45
K1	0	0	0	0	0	0	0	0	0	0	0	10	0	10
K2	0	0	0	0	0	0	0	0	0	0	0	12	0	12
K3	11	0	0	0	0	0	0	0	0	0	0	0	0	11
K4	0	0	2	2	0	0	0	0	0	0	0	0	0	4
K5	4	3	0	0	0	0	0	0	0	0	0	0	1	8
Total	442	38	58	27	1	10	10	4	1	1	1	22	1	616
Frequency (%)	71.75	6.17	9.42	4.38	0.16	1.62	1.62	0.65	0.16	0.16	0.16	3.57	0.16	

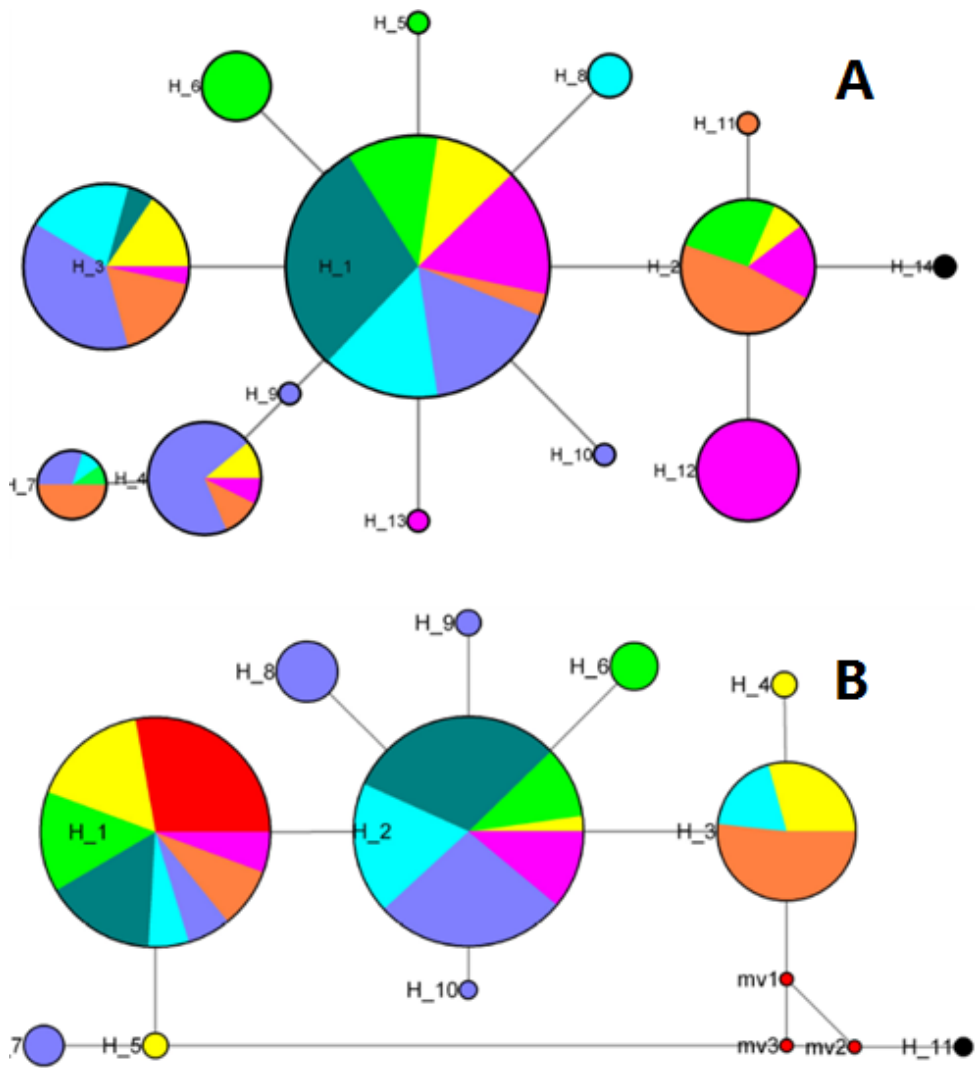
SC = South China; MDYR = Middle and downstream of the Yangzi River; SWC = Southwest China; NWC = Northwest China; NC = North China; NEC = Northeast China.

the indication of refugia of species in the ice age (Avice, 1994; Hewitt, 1996; Schmitt, 2007; Schrimpf et al., 2014). The highest haplotype diversity for two loci was found in these two regions. Moreover, most unique haplotypes were also limited to these two regions. In Northeast Asia, a total of 23 different haplotypes were detected in the Yangtze River basin. Of these 23 haplotypes, 10 were distinguished with locus *trnQ-rps16*, except three common haplotypes, and the NEC region and Yangtze River basin possessed four and three unique haplotypes, respectively. Among 13 haplotypes detected by locus AF105221, four and two rare haplotypes were limited to Northeast Asia and the Yangtze River basin, respectively. Interestingly, two unique haplotypes were found in the Japanese region, which indicates that Japan may have been one of the refugia of *G. soja* during LGM.

Why was one of the refugia located in Central China and another one in NEC, when these regions are far away from each other? We speculate that the situation was not only caused by the climate change in East Asia in the Quaternary, but also by the biological characteristics of wild soybean. *G. soja* is widely distributed in East Asia and its distributional range is between 24°N and

53°N, characterized by three climatic zones, including subtropical, warm temperate, and cold temperate zones. There is at least a 20 °C difference in the average annual temperature between southern (Heyuan, Guangdong Province: 19.9 °C) and northern (Mohe, Heilongjiang Province: 0 °C) regions of China (China Meteorological Data Network, <http://data.cma.gov.cn/>). These data show that *G. soja* has a very wide temperature range and photoperiodic adaptability, and strong tolerance to low temperatures. However, the ecological niches of *G. soja* populations are always located near places where water is in abundance, such as rivers, ponds, ditch edges, and watered fields, which ensures a sufficient quantity of moisture required for seed germination, because the seed coat of wild soybean is dense and hard, and it needs high moisture contents for germination. Thus, *G. soja* is resistant to cold but not to drought, at least during the seed germination stage. In spite of NEC and the Korean peninsula being situated at high altitudes, this area could still have been the refugium of *G. soja* during the glacial period in the Quaternary because this region is located on the shore of the West Pacific, and we deduce that the moisture was enough for growth of *G. soja*. In the Quaternary, the most





**Figure 1.** Median-joining haplotype networks of *G. soja* haplotypes: a) AF105221, b) *trnQ-rps16*.

notable climate feature was periodic warm/wet and cold/dry spells (Lu et al., 2013). In the cold stage, called an ice age, annual average temperature dropped about 10 °C, but not evenly in different East Asian regions (Shi, 2002). Meanwhile, annual precipitation was also greatly reduced in the ice age (Shi, 1989). A previous study showed that the NC and NWC regions were very arid and became desert and grasslands (Yu et al., 2000). Therefore, these two regions should not have been suitable habitats for *G. soja* in the ice age; in fact, in the LGM, because of sea regression, the Bohai Sea and the East China Sea changed to land, and all of NC was situated away from the sea, not the shore of the sea like today. The haplotypes detected by two loci in these two regions were rather common haplotypes, which widely appeared in the *G. soja* populations and represent a recent migration event happening after the last glacial period. Although we sampled 16 populations from regions

of NC and NWC, only one unique haplotype was detected from NC with AF105221. The extreme dry temperate climate might have eliminated wild soybean from most of the habitats of these two regions. These two regions shared haplotypes with the regions of NEC and MDYR, suggesting that the migration of wild soybean may have happened from NEC and MDYR after the LGM. As for the MDYR region and its contiguous zone, the east end of SWC, they still had adequate moisture to supply the needs of seed germination even in the condition of a dry climate, because the Yangzi River had plenty of water during the ice age, and this makes the MDYR region and its contiguous zone be one of the refugia for *G. soja*.

#### 4.2. Gene flow of *G. soja* by land bridge among the refugia during ice age

The CJK region was separated by the East China Sea with higher sea level during inter-/postglacial times.

**Table 5.** Number of haplotypes per population identified at *trnQ-rps16* locus.

Population	B1	B2	B3	B4	B5	B6	B7	B8	B9	B10	Total
SC group	59	0	0	0	0	0	0	0	0	0	59
G1_AF	12	0	0	0	0	0	0	0	0	0	12
G1_JO	12	0	0	0	0	0	0	0	0	0	12
G1_HY	11	0	0	0	0	0	0	0	0	0	11
G1_RY	12	0	0	0	0	0	0	0	0	0	12
G1_QZ	12	0	0	0	0	0	0	0	0	0	12
MDYR group	35	6	17	2	0	0	0	0	0	0	60
G2_WC	5	1	6	0	0	0	0	0	0	0	12
G2_XU	10	2	0	0	0	0	0	0	0	0	12
G2_WQZ	9	0	3	0	0	0	0	0	0	0	12
G2_SC	5	0	5	2	0	0	0	0	0	0	12
G2_TB	6	3	3	0	0	0	0	0	0	0	12
SWC group	30	32	0	0	2	7	0	0	0	0	71
G3_CK	0	10	0	0	2	0	0	0	0	0	12
G3_YJ	0	5	0	0	0	7	0	0	0	0	12
G3_GH	0	11	0	0	0	0	0	0	0	0	11
G3_CY	12	0	0	0	0	0	0	0	0	0	12
G3_NL1	11	1	0	0	0	0	0	0	0	0	12
G3_NL2	7	5	0	0	0	0	0	0	0	0	12
NWC group	33	98	0	0	0	0	0	0	0	0	131
G4_BX	4	8	0	0	0	0	0	0	0	0	12
G4_HX	5	7	0	0	0	0	0	0	0	0	12
G4_YY	0	12	0	0	0	0	0	0	0	0	12
G4_LW	0	12	0	0	0	0	0	0	0	0	12
G4_WS	0	12	0	0	0	0	0	0	0	0	12
G4_YA	3	9	0	0	0	0	0	0	0	0	12
G4_YL	1	10	0	0	0	0	0	0	0	0	11
G4_LXS	3	9	0	0	0	0	0	0	0	0	12
G4_YC	0	12	0	0	0	0	0	0	0	0	12
G4_BJ	5	7	0	0	0	0	0	0	0	0	12
G4_WN	12	0	0	0	0	0	0	0	0	0	12
NC group	12	59	11	0	0	0	0	0	0	0	82
G5_JZ	6	6	0	0	0	0	0	0	0	0	12
G5_DY	0	12	0	0	0	0	0	0	0	0	12
G5-WQT	0	12	0	0	0	0	0	0	0	0	12
G5_XH	6	5	0	0	0	0	0	0	0	0	11
G5_QHD	0	12	0	0	0	0	0	0	0	0	12
G5_YT	0	0	11	0	0	0	0	0	0	0	11
G5_JN	0	12	0	0	0	0	0	0	0	0	12
NEC group	13	87	0	0	0	0	5	11	2	1	119
G6_LXH	6	4	0	0	0	0	2	0	0	0	12
G6_JH	0	12	0	0	0	0	0	0	0	0	12

Table 5. (Continued).

G6_XM	3	9	0	0	0	0	0	0	0	0	12
G6_MDJ	0	11	0	0	0	0	0	1	0	0	12
G6_BS	4	5	0	0	0	0	0	3	0	0	12
G6_KS	0	9	0	0	0	0	0	3	0	0	12
G6_QQHE	0	10	0	0	0	0	0	0	2	0	12
G6_HG	0	12	0	0	0	0	0	0	0	0	12
G6_DH	0	3	0	0	0	0	3	4	0	1	11
G6_HL	0	12	0	0	0	0	0	0	0	0	12
Japan group	18	0	30	0	0	0	0	0	0	0	48
J1	4	0	8	0	0	0	0	0	0	0	12
J2	2	0	10	0	0	0	0	0	0	0	12
J3	12	0	0	0	0	0	0	0	0	0	12
J4	0	0	12	0	0	0	0	0	0	0	12
Korea group	12	35	0	0	0	0	0	0	0	0	47
K1	0	10	0	0	0	0	0	0	0	0	10
K2	0	12	0	0	0	0	0	0	0	0	12
K3	11	0	0	0	0	0	0	0	0	0	11
K4	1	3	0	0	0	0	0	0	0	0	4
K5	0	10	0	0	0	0	0	0	0	0	10
Total	212	317	58	2	2	7	5	11	2	1	617
Frequency	34.36	51.38	9.40	0.32	0.32	1.13	0.81	1.78	0.32	0.16	

SC = South China; MDYR = Middle and downstream of the Yangzi River; SWC = Southwest China; NWC = Northwest China; NC = North China; NEC = Northeast China.

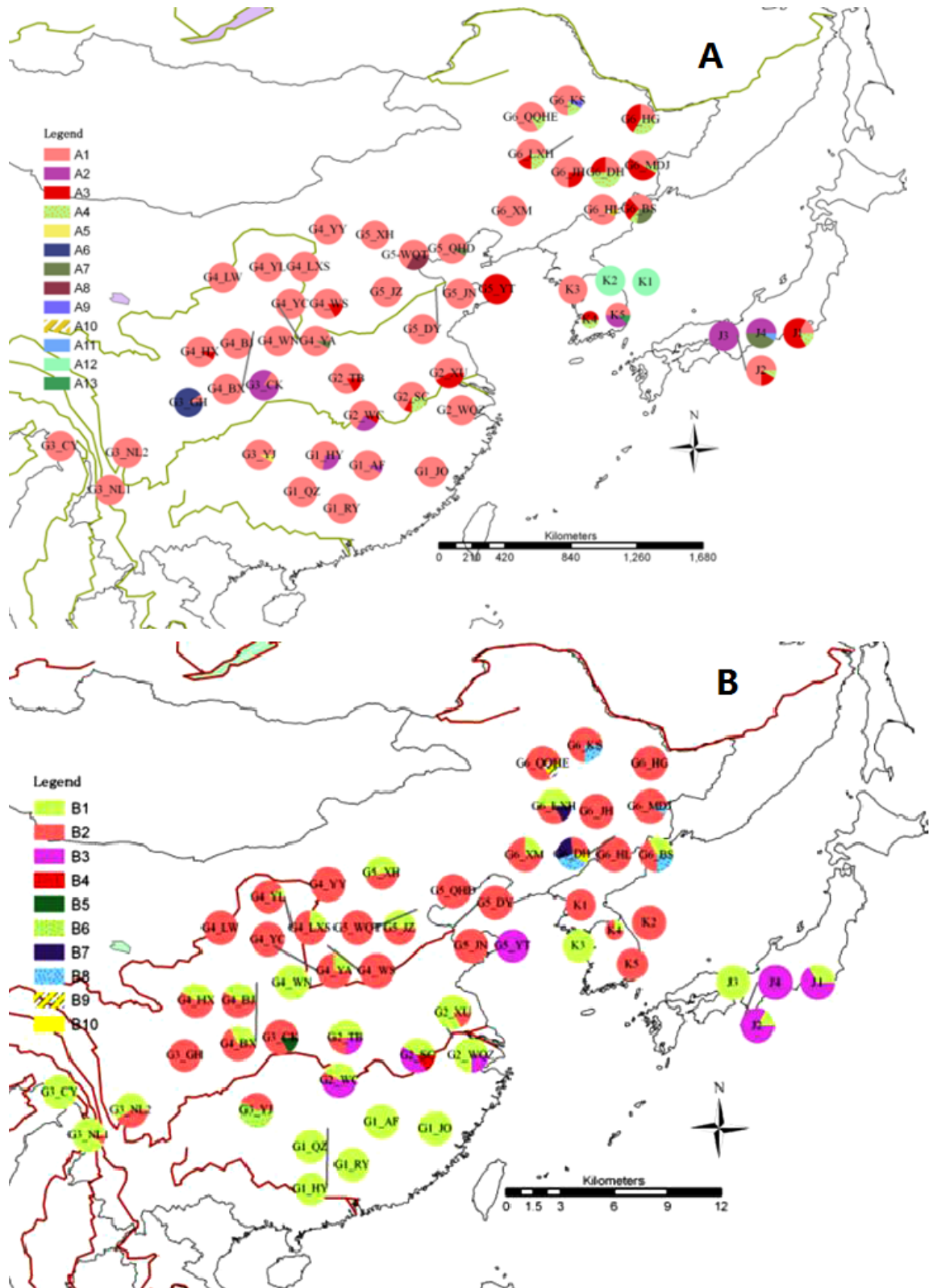
However, these regions had been connected by the East China Sea basin when sea level fell by c. 85–130/140 m during the glacial period (Millien-Parra and Jaeger, 1999; Qian and Ricklefs, 2001), especially during the LGM (Shi, 2002). The land bridge of the East China Sea served as a ‘dispersal corridor’ (Simpson, 1940; Lomolino et al., 2006), allowing intermittent migration of most warm-temperate deciduous forest-restricted plant species from the Asian mainland into Japan (or vice versa), and/or periodic secondary contact and gene flow among formerly isolated populations, possibly up to the last shelf submergence (c. 16,000–7,000 years BP) (Chung, 2007). This land bridge has been suggested to be covered by temperate deciduous forest during glacial intervals (Harrison et al., 2001). Here, we inferred that refugia of *G. soja*, such as Northeast Asia (NEC and the Korean peninsula), Japan, and the Yangzi River basin, were connected by the land bridge of the East Hai Sea, and gene flows among these regions were also detected. In the present study, eight haplotypes appeared in four Japanese populations, and two common haplotypes, B1 and A1, were found in the populations of all other ecogeological regions, while only one unique haplotype (A11) was detected with both loci. This revealed recent

migration during the LGM, and two common haplotypes, B3 and A3, with a mid-level of frequency, showed similar distribution patterns in populations of Japan, the east of NC, and the MDYR region, which suggested that an older migration event happened by the land bridge between Japan and the East Asian mainland. Wild soybean is widely distributed across Japan and more populations being sampled would depict a clear picture of gene flow in wild soybean between Japan and the East Asian mainland.

Phylogeographic studies on the genus *Croonia* (Li et al., 2008), *Kirengeshoma palmata* (Qiu et al., 2009b), and *Platycrater arguta* (Qiu et al., 2009c) suggested that deep allopatric-vicariant differentiation of isolated lineages appeared in the CJK region (Qiu et al., 2011). In spite of one unique haplotype from Korea, most of the Korean wild soybean haplotypes were similar to those of East China, and this revealed that the gene flow among the three regions occurred frequently and not so long ago.

#### 4.3. Population expansion of *G. soja* in the Holocene after the Last Glacial Maximum

Among 23 haplotypes detected by AF105221 and *trnQ-rps16* loci in *G. soja* populations, three haplotypes, A1, B1, and B2, appeared in more than half of the total samples



**Figure 2.** Frequencies and geographical distribution of *G. soja* haplotypes: a) AF105221, b) *trnQ-rps16*. Numbers at the end of Chinese population names indicate the region from which they derived: G1–G6 represent SC, MDYR, SWC, NWC, NC, and NEC regions, respectively.

**Table 6.** Genetic differentiation in the *G. soja* populations detected by using nuclear and chloroplast loci.

Locus	$H_s$	$H_T$	$G_{ST}$	$N_{ST}$
<i>trnQ-rps16</i>	0.238 (0.0362)	0.614 (0.0341)	0.613 (0.0562)	0.564 (0.0638)
AF105221	0.229 (0.0359)	0.491 (0.0641)	0.534 (0.0680)	0.589 (0.1093)

$H_s$ , Gene diversity within populations;  $H_T$ , Total gene diversity;  $G_{ST}$ , Coefficient of gene differentiation;  $N_{ST}$ , Frequencies and similarities among haplotypes. The values in parentheses represent standard errors.

and in almost all populations. For example, haplotype A1 was distributed in almost the whole species range. B1 and B2 were also distributed widely; B1 was just found to be absent in NC, and B2 in SC and Japan. These results showed the presence of a large-scale migration and expansion in *G. soja* populations in the recent warm Holocene after the LGM. In general, an expanding population would show a star-like haplotype network (Harpending, 1994). In this study, haplotype network analysis of both AF10522A and *trnQ-rps16* loci revealed a star-like haplotype network (Figure 1), and this result suggested that *G. soja* is an expanding population.

Wild soybean of the modern age has two isolated distribution areas: one in Ninglang County in the northeastern Yunnan Province and another one in Chayu County of the southeastern Tibet Province; the former is also the highest distribution habitat with an altitude of 2600 m. These two regions are separated by over 450 km of distance from the wild soybean's main distribution. Two populations sampled from Ninglang County and one population from Chayu County contain one to two haplotypes, but without a unique haplotype, which suggests the recent colonization of wild soybean in these regions. The possible reason for this is that humans transferred these wild soybeans through the ancient "Tea Horse Road", a trade road that lasted for thousands of years and connected Yunnan, Sichuan, and Tibet provinces of China with Burma, Nepal, and India. Horses were used as the major transportation source, and wild soybean is a good source of forage for horses. At the same time, sites of wild soybean populations were present along the ancient Tea Horse Road in Ninglang and Chayu counties. To offer the best forage to their horses and to complete the hard journey, perhaps ancient caravans brought the seeds of *G. soja* and planted them along the roadside of important posts. If our hypothesis is true, this is the first report of plant transfer along the ancient Tea Horse Road by human beings.

#### 4.4. Implications for conservation

Preservation of genetic diversity and evolutionary potential, and prevention of inbreeding depressions, are key goals in conservation (Frankel and Soulé, 1981; Hufford and Mazer, 2003; Rauch and Bar-Yam, 2005;

Wang et al., 2015a; Baloch et al., 2016). Wild soybean has undergone considerable habitat fragmentation and population extinction due to human activity in the recent past (Li et al., 2005), and our field investigations failed to find populations at many sites where wild soybean had been reported previously. For example, wild soybean was widely distributed along the Fenhe River in Shanxi Province and different lines have distinguished characteristics: one variety had a 100-grain weight as high as 14.7 g; another had gray hairs, white flowers, and obvious stems; and another had pale green, yellow, and brown colored seeds. These previously recorded *G. soja* populations disappeared after the expansion of cities and conversion of land for urban and farming purposes (Li et al., 2005). Our field investigations also revealed that wild soybean is usually found in small populations in these regions. The regions containing the MDYR and NEC groups deserve special conservation considerations because these two regions have the most abundant genetic diversity and the largest number of private alleles. The MDYR region is located in the most economically developed area of China, and the frequent industrial and agricultural activities and the expansion of cities are threatening the natural habitats of *G. soja*. The NEC region, the most important agricultural area in China, has seen heavy fragmentation and loss of *G. soja* habitat, resulting in reductions and/or extinctions of distinctive wild populations from this region (Li et al., 2005). Group SWC is another region worthy of concern, where we detected a high number of private alleles, although only a limited number of samples were included in this study from SWC. The populations of NL in Yunnan Province and CY in Xizang Province are isolated from the main *G. soja* distribution area by more than 400 km in linear distance, and wild soybean from population CY is an important breeding resource because of its high protein contents.

#### 4.5. Conclusion

We used two loci to investigate the distribution patterns and possible refugia of wild soybean and the results revealed that the climatic oscillations during the Quaternary profoundly affected the geographical distribution patterns of *G. soja*. The haplotype distribution

patterns suggested that Northeast Asia (NEC and Korea) and the Yangzi River basin (MDYR and east end of SWC) might have been the refugia for *G. soja* during the Quaternary glacial period, and *G. soja* expanded rapidly after the LGM. There was a land bridge among East China, the Korean peninsula, and Japan that permitted gene flow among populations of these regions in the ice age during the Quaternary. We inferred that NEC and MDYR are the key regions for the protection of *G. soja* germplasm.

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