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Nuclear DNA Microsatellites Reveal Genetic Variation but a Lack of Phylogeographical Structure in an Endangered Species, *Fraxinus mandshurica*, Across North-east China

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• *Background and Aims* The widely accepted paradigm that the modern genetic structure of plant species in the northern hemisphere has been largely determined by recolonization from refugia after the last glacial maximum fails to explain the presence of cold-tolerant species at intermediate latitudes. Another generally accepted paradigm is that mountain ridges act as important barriers causing genetic isolation of species, but this too has been challenged in recent studies. The aims of the work reported here were to determine the genetic diversity and distribution patterns of extant natural populations of an endangered cool temperate species, *Faxinus mandshurica*, and to examine whether these two paradigms are appropriate when applied to this species over a wide geographical scale.
• *Methods* 1435 adult individuals were sampled from 30 natural populations across the main and central range of the species, covering major mountain ranges across North-east China (NEC). Genetic variation was estimated based on nine polymorphic nuclear microsatellite loci. Phylogeographical analyses were employed using various approaches, including Bayesian clustering, spatial analysis of molecular variance, Monmonier's algorithm, neighbor-joining trees, principal co-ordinate analysis and isolation by distance.
• *Key Results* Genetic diversity within populations was relatively high, and no significant recent bottlenecks were detected in any of the populations. A significant negative correlation between intra-population genetic diversity and latitude was identified. In contrast, genetic differentiation among all the populations examined was extremely low and no clear geographic genetic structure was identified, with the exception of one distinct population.
• *Conclusions* The modern genetic structure in this species can be explained by extensive gene flow, an absence of mountains acting as barriers, and the presence of a wide refuge across NEC rather than multiple small refugia. Intra-population genetic variation along latitudes is probably associated with the systematically northward shifts of forest biomes in eastern China during the mid-Holocene. To determine important genetic patterns and identify resources for conservation, however, it will be necessary to examine differentially inherited genetic markers exposed to selection pressures (e.g. chloroplast DNA) and to investigate different generations.

Key words: *Fraxinus mandshurica*, nuclear microsatellites, latitude variation, historical migration, fossil pollen, spatial genetic structure, genetic barriers.

INTRODUCTION

The modern genetic structure of many plant species has been shaped by climatic fluctuations during the Quaternary (e.g. Comes and Kadereit, 1998; Hewitt, 2000). The identification and characterization of areas where refugia were located during the last glacial maximum [LGM, about 18000 BP (^{14}C); e.g. Willis *et al.*, 2000; Petit *et al.*, 2003] are especially relevant to population genetics since they are informative when setting priorities for conservation and management of genetic resources (e.g. Hampe and Petit, 2005). However, the simple paradigm that plant species recolonized after the LGM from several southern refugia in Europe has been challenged in several recent studies (e.g. Lascoux *et al.*, 2004; Maliouchenko *et al.*, 2007), partly because many cold-tolerant species were able to survive the LGM at intermediate latitudes. Moreover, recent findings indicate that the modern genetic diversity of plant species was shaped not only as a result of the LGM, but over multiple interglacial episodes (Heuertz *et al.*, 2006; Magri *et al.*, 2006).

Migratory movements are not only a function of geographic distance (Wright, 1943) and historical events (Petit *et al.*, 2003), but are also influenced by the presence of barriers (Dupanloup *et al.*, 2002). Specifically, genetic barriers, i.e. areas with abrupt genetic changes (Barbujani *et al.*, 1989), are increasingly considered to influence genetic differentiation and spatial structure of species at a broad geographic scale (Manni *et al.*, 2004) and/or landscape scale (Manel *et al.*, 2003). Generally, ridges of mountains or maintain ranges are assumed to act as genetic barriers, causing the isolation of genetic lineages of plant species (Taberlet *et al.*, 1998). However, some recent studies have shown that mountain ridges do not act as barriers in this way (e.g. Magri *et al.*, 2006).

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North-east China (NEC) is a megadiversity area with complex topography (China EPA, 1998; Xu *et al.*, 1999); however, there is no evidence to suggest that NEC was divided into multiple refugia during the LGM (Ren *et al.*, 1979). Unfortunately, there is limited fossil pollen data for NEC from the LGM (Yu *et al.*, 2000). In contrast, biome reconstructions, which are supported by sufficient fossil pollen data and modern pollen taxa, imply that forests expanded systemically northwards in NEC during the mid-Holocene (about 6000 BP, ^{14}C; (Yu *et al.*, 1998, 2000; Member of China Quaternary Pollen Data Base, 2000). However, to date, there are no genetic data relating to a single species that can link its current genetic distribution to the historical biomes that have been reconstructed in NEC.

Fraxinus mandshurica (Manchurian ash) is a wind-pollinated, wind-dispersed, dioecious, cold-tolerant tree species (Wu, 1980; Kong, 2004; Kong *et al.*, 2008). It is widely but discontinuously distributed across NEC, part of North-west China, the Russian Far East, northern Japan and North Korea. Of these areas, NEC is the species' main and central range. It is a most important broadleaved timber tree and a key species under the climax forest community in NEC (Wang, 1983). As a result of over-exploitation and deforestation (to meet the increasing need for timber for the economic development of China during the past 50 years) the species is becoming increasingly threatened. Consequently, it has been designated as an endangered species (Fu, 1992) and a national priority protected plant (Chinese Ministry of Forestry, 1999) in China. Since tree species that have suffered from widespread deforestation and over-exploitation have become the focus of conservation concerns (e.g. Newton *et al.*, 1999), numerous studies aimed at conserving existing individuals and restoring populations of *F. mandshurica* have been conducted (e.g. Wang, 2001; Xie, 2005). Assessment of the population genetics of an endangered species is essential for biological conservation (O'Brien, 1994); however, to date, no attempts have been made to understand the population genetics of *F. mandshurica* in NEC.

The general objective of this study was to obtain an understing of the genetic diversity and spatial structure of extant natural populations of *F. mandshurica* across NEC, focusing especially on the relationships between current genetic diversity and the historical geographic distribution of *F. mandshurica* in NEC. We also examined whether mountain ridges in NEC act as genetic barriers causing the isolation of genetic lineages of *F. mandshurica*. The results should assist in the conservation and management of the genetic resources of *F. mandshurica* in NEC.

MATERIALS AND METHODS

Sampling and study sites

During the mid-summer of 2006, when leaves were fully expanded, leaf samples from 1435 adult *Fraxinus mandshurica* Rupr. individuals from 30 natural populations were collected. Samples covered the species' entire current distribution in North-east China (NEC), including the major mountain ranges: the Xiaoxing'anling Mountains (X); Wandashan Mountains (W); Laoyeling Mountains (L); Zhangguangcailing Mountains (Z); and Changbaishan Mountains (C; Fig. 1). All individuals sampled within each population were widely spaced, separated by at least 30 m, to avoid collecting close relatives. Spatial co-ordinates (latitude, longitude and altitude) of each population were recorded using a hand-held GPS (e-Trex Venture, Garmin Co. Ltd.). Diameter at breast height and height of each individual, along with the forest type of each sampled stand and its dominant tree species were recorded (Table 1). All the sampled individuals were older than about 50 years, according to stand records from the Local Forestry Bureaus in NEC.

DNA extraction and genotype

All the samples were stored in silica gel at room temperature until DNA extraction. Total DNA was extracted by the modified CTAB method (Lian *et al.*, 2003). Four pairs of novel nuclear SSR loci specifically for *F. mandshurica*, i.e. fm04, fm06, fm13 and fm14 (S. Goto and C. Lian, the University of Tokyo, Japan, unpubl. res.) were originally used in this study. In addition, an attempt was made to use nuclear SSR loci from European *Fraxinus* species. Of these, only five, namely M2-30 (Brachet *et al.*, 1999), FEMSATL-4, FEMSATL-16 and FEMSATL-19 (Lefort *et al.*, 1999) and FR16 (Verdú *et al.*, 2006), were sufficiently stable and polymorphic. Therefore, nine loci were used in this study and these were classified into two mixture groups: G_1 (fm04, fm06, fm13, fm14 and FR16) and G_2 (M2-30, FEMSATL-4, FEMSATL-16 and FEMSATL-19) due to their different temperature requirements during PCR. For primer mixtures for G_1 and G_2, each pair of primers was dissolved in TE buffer with 2 μM except in the case of fm06, FEM-SATL4 and M2-30 where 4 μM was used. The PCR reaction mixtures (5·0 μL in total) contained 1·0 μL of 5 μM template DNA, 2·5 μL of Multiplex MM buffer (QIAGEN Multiplex PCR Master Mix), 1·0 μL of H_2O (RNase-free water) and 0·5 μL of primer mixture G_1 or G_2, respectively. The PCR thermal profile for G_1 was: 95 °C for 15 min, followed by 30 cycles of 94 °C for 30 s, annealing temperature of 56 °C for 90 s, and 72 °C for 60 s in sequence, with a final elongation at 60 °C for 30 min. For G_2 the profile was: 95 °C for 15 min, followed by 30 cycles of 94 °C for 30 s, annealing temperature of 52 °C for 90 s, and 72 °C for 90 s in sequence, with a final elongation at 72 °C for 10 min. The thermal profiles were achieved using a PCR thermal cycler (TAKARA PCR thermal Cycler TP-600). All PCR products were analysed and aligned using automated fluorescent scanning detection with an ABI 3100 sequencer.

Data analysis

Genetic diversity within populations. Allelic diversity statistics, i.e. the total number of detected alleles (N_A), the range of allele sizes (*RAS*), the allelic richness based on a

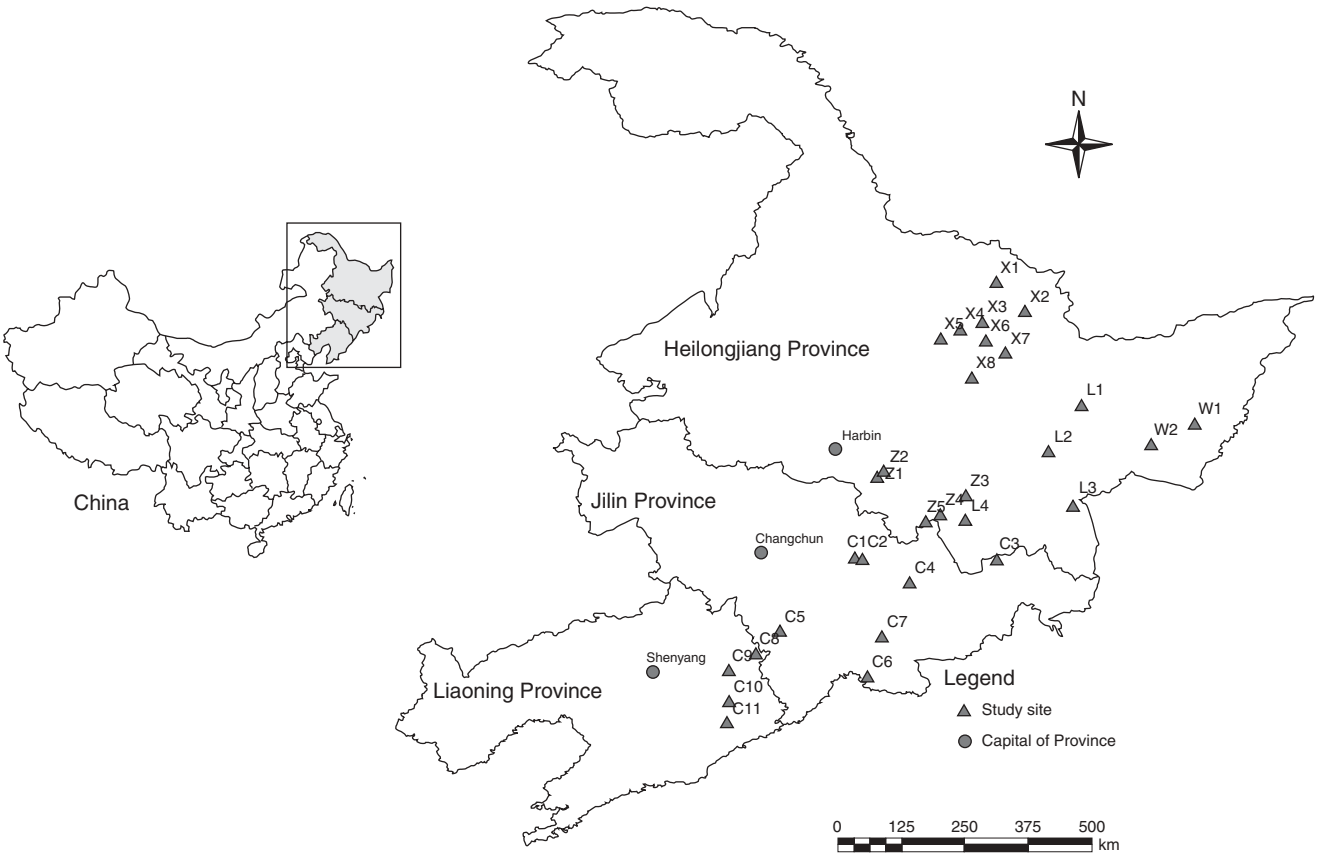

FIG. 1. Geographic locations of the 30 natural populations of *Fraxinus mandshurica*, focusing on major five mountain ranges across three Provinces in North-east China.

minimum population size of 43 diploid individuals (86 gene copies; A_S), the average of expected heterozygosity (H_e), the total gene diversity (H_T) and Wright's inbreeding coefficient (F_{IS}), were calculated for all individuals at each locus and multilocus estimate using the FSTAT 2·9·3·2 software (FSTAT; Goudet, 2001).

Within each population, the significance of deviation from Hardy–Weinberg equilibrium (HWE) at each locus and multilocus estimate was tested based on 5400 randomizations at the nominal level (5 %). In addition, tests of linkage disequilibrium (LD) for pairwise-loci within each population and all the populations combined were examined by applying an adjusted sequential Bonferroni correction (Rice, 1989), based on 21 600 permutations at the nominal level (5 %). Both tests were performed using the FSTAT software.

In order to understand whether genetic variation within populations is correlated with geographical gradients, Pearson correlations between statistics of variation (A_S and H_e) and geographic co-ordinates (latitude and longitude) for each population were analysed. Stepwise regression analysis of A_S and H_e in relation to the two independent variables (latitude and longitude) were further assessed separately. Both analyses were conducted using SPSS 13·0 for Windows (SPSS Inc., 2004).

In addition, in order to evaluate whether the sampled populations have experienced recent bottlenecks, Wilcoxon's sign-rank test (Piry *et al.*, 1999) under the infinite allele model (IAM) and the stepwise mutation model (SMM) was each performed using BOTTLENECK 1·2·02 software (Cornuet and Luikart, 1996).

Genetic differentiation between populations. Genetic differentiation between populations was determined using Weir and Cockerham's F_{ST} (1984). The significance of F_{ST} was tested for the 95 % and 99 % confidence intervals based on 1000 permutations. The significance of F_{ST} at each locus was tested using the log-likelihood (G) -based exact test (Goudet *et al.*, 1996). Pairwise-F_{ST} was also evaluated and its significance was tested by applying the adjusted sequential Bonferroni correction based on 8700 permutations. All estimates of F_{ST} and their tests of significance were performed using the FSTAT software.

Patterns of population genetic structure. The geographical structure of the genetic variation in nuclear DNA of *F. mandshurica* was investigated extensively using various approaches. First, the Bayesian approach that clusters 'unclassified' individuals into inferred clusters (Pritchard *et al.*, 2000) was implemented using STRUCTURE 2·2 software (Pritchard *et al.*, 2007). A total of 10 000 Markov Chain Monte Carlo iterations, after a burn-in period of 10 000 iterations, using all the individuals, were run ten times for each number of genetic clusters (K, ranging from 1 to 13) from the admixture model. Both

TABLE 1. *List of the sampling sites of 30 natural populations of* Fraxinus mandshurica *in North-east China*

Code	Name	Longitude (E)	Latitude (N)	Altitude (m)	DBH (cm)	Height (m)	Forest type (dominant species)
X1	Jianxin	129 °28′	48 °42′	200–310	21·90 ± 7·61	19·0 ± 7·1	MPC (*Picea koraiensis*)
X2	Shuiyuan	130 °00′	48 °12′	990–1100	24·63 ± 7·75	23·0 ± 5·6	DBF (*Betula platyphyla*, *Populus davidiana*)
X3	Pingshan	129 °15′	48 °01′	900–910	36·71 ± 5·52	23·9 ± 3·1	MPC (*Picea koraiensis*)
X4	Chaoyang	128 °50′	47 °52′	350–400	30·80 ± 5·68	22·3 ± 3·3	MPC (*Pinus koraiensis*)
X5	Cuiluanhe	128 °33′	47 °44′	160–240	31·33 ± 6·85	23·3 ± 3·2	MPC (*Pinus koraiensis*)
X6	Meixi	129 °05′	47 °35′	840–870	25·86 ± 8·41	25·4 ± 6·6	MPC (*Pinus koraiensis*)
X7	Jinshantun	129 °41′	47 °26′	480–520	32·42 ± 5·50	23·5 ± 4·1	MPC (*Pinus koraiensis*), DBF (*B. platyphyla*)
X8	Dailing	129 °04′	47 °00′	850–930	31·16 ± 16·45	20·2 ± 3·0	MPC (*Pinus koraiensis*), DBF (*B. platyphyla*)
W1	Dongfanghong	133 °07′	46 °13′	800–820	33·77 ± 3·87	23·3 ± 4·1	MPC (*Larix gmelinii*), DBF (*Populus* spp.)
W2	Yanggang	132 °14′	45 °49′	680–820	24·94 ± 5·71	20·4 ± 3·1	MPC (*L. gmelinii*), DBF (*Populus* spp)
L1	Shuangyashan	131 °02′	46 °33′	100–110	28·95 ± 5·44	20·1 ± 2·5	DBF (*Phellodendron amurense*)
L2	Boli	130 °24′	45 °42′	300–420	37·18 ± 4·66	23·8 ± 0·8	DBF (*P.davidiana*, *Juglans mandshurica*)
L3	Bamiantong	130 °49′	44 °44′	500–560	32·59 ± 6·88	20·8 ± 2·1	DBF (*J. mandshurica*, *P. davidiana*)
L4	Muling	128 °58′	44 °31′	500–580	33·05 ± 5·96	21·8 ± 1·0	DBF (*B. platyphyla*, *Tilia amurensis*)
Z1	Laoyeling	127 °34′	45 °25′	350–450	30·75 ± 12·17	18·0 ± 4·1	MFS (*Pinus koraiensis*, *J. mandshurica*)
Z2	Laoshan	127 °32′	45 °21′	320–480	29·72 ± 9·81	24·9 ± 4·4	DBF (*Fraxinus mandshurica*)
Z3	Hufeng	128 °57′	44 °55′	400–480	36·44 ± 7·59	28·3 ± 3·3	DBF (*F. mandshurica*)
Z4	Daguokuigang	127 °12′	44 °55′	520–600	16·82 ± 7·03	16·1 ± 5·2	DBF (*Populus* spp., *J. mandshurica*)
Z5	Datudingzi	127 °09′	44 °54′	500–600	18·32 ± 7·20	17·1 ± 6·0	DBF (*F. mandshurica*, *J. mandshurica*)
C1	Hongshi	127 °02′	43 °50′	320–400	26·78 ± 10·94	21·0 ± 4·5	DBF (*Quercus mongolica*, *J. mandshurica*)
C2	Jiaohe	127 °08′	43 °48′	500–600	33·92 ± 5·19	22·0 ± 1·6	DBF (*J. mandshurica*, *Ulmus* spp.)
C3	Wangqing	129 °32′	43 °47′	250–320	30·97 ± 3·82	22·8 ± 2·3	DBF (*J. mandshurica*, *Ulmus* spp.)
C4	Dunhua	127 °59′	43 °21′	440–460	24·01 ± 6·35	20·6 ± 2·9	DBF (*B. platyphyla*, *Q. mongolica*)
C5	Fengman	127 °20′	42 °56′	320–380	24·45 ± 5·28	16·1 ± 3·0	DBF (*J. mandshurica*, *B. platyphyla*)
C6	Fusong	127 °33′	42 °50′	420–480	20·94 ± 6·65	19·1 ± 3·0	DBF (*J. mandshurica*, *B. platyphyla*)
C7	Lushuihe	127 °37′	42 °43′	700–800	19·41 ± 4·43	12·9 ± 2·1	MFS (*Larix olgensis*, *Picea koraiensis*)
C8	Jiguanshan	126 °13′	42 °16′	300–500	34·70 ± 7·11	23·9 ± 2·6	MFS (*L. olgensis*, *Picea koraiensis*)
C9	Qingyuan	124 °55′	42 °06′	560–620	24·34 ± 6·58	20·7 ± 2·7	DBF (*Betula* spp., *Ulmus* spp)
C10	Laotudingzi	125 °01′	41 °18′	100–150	23·29 ± 4·22	21·3 ± 4·5	MFS (*L. olgensis*, *B. platyphyla*)
C11	Baishilazi	124 °47′	40 °43′	300–500	39·23 ± 7·72	23·4 ± 5·0	MFS (*L. olgensis*, *B. platyphyla*)

Altitude is the range occupied by individuals within each population; DBH (diameter at breast height) and height are the mean ± s.d. Forest types for the stands sampled were classified as deciduous broadleaved forests (DBF), mixed forest stands with deciduous broadleaved and conifer species (MFS), or mixed stands with planted conifer species (MPC).

the correlated allele frequencies model and the independent allele frequency model were tested in this study. Second, the spatial analysis of molecular variance (SAMOVA) algorithm, based on a simulated annealing procedure, was used to define clusters (groups) of populations that are geographically homogeneous and maximally differentiated from each other (Dupanloup *et al.*, 2002). The program (SAMOVA 1·0) was run for 1000 iterations for each number of clusters (K, ranging from 2 to 13). For each K, the configuration producing the maximum values of F_{CT}, the proportion of total genetic variance due to differentiation between clusters of populations, was retained as the best grouping of populations based on IAM and SMM. Third, barriers analysis (Manni *et al.*, 2004) based on Monmonier's (1973) algorithm, was used to directly identify genetic barriers between populations. All the barriers were calculated using the Barriers 2·2 software (Manni and Guérard, 2004) with significance tested by means of 1000 bootstrap matrices of D_A (Nei *et al.*, 1983) that were computed using Microsatellite Analyzer (MSA) 4·05 software (Dieringer and Schlötterer, 2003). Fourth, neighbor-joining (NJ) tree analysis and principal co-ordinates analysis (PCoA), based on D_A with 1000 bootstraps, were performed using POPULATION 1·2·28 software (Langella, 2002) and GENALEX 6 software (Peakall and Smouse, 2006), respectively. Fifth, to test for isolation by distance (IBD; Wright, 1943), we examined the association between the matrix of the natural logarithm of geographic distance and pairwise population differentiation $[F_{ST}/(1 - F_{ST})]$ (Rousset, 1997) using the Mantel test (Mantel, 1967) with 9999 random permutations among all the sampled populations; the values were estimated using GENALEX 6 software.

RESULTS

Genetic diversity within populations

All the allelic diversity statistics within populations were very variable for each locus (Table 2): N_A from 4 (fm06) to 49 (FEMSATL4 and FEMSATL19); A_S from 2·945 (fm06) to 23·968 (fm14); H_e from 0·064 (fm13) to 0·939 (fm14); and H_T from 0·065 (fm13) to 0·947 (fm14).

At the mutilocus estimates (Table 3), total N_A was 270, and A_S and H_e were 11·063 ± 7·053 and 0·564 ± 0·284, respectively. In contrast to the other populations, the population located in the most northerly part of the Xiaoxing'anling Mountains (Jianxin) displayed distinct characteristics with respect to its intra-population genetic diversity; it had the lowest values of N_A, A_S and H_e.

Wright's inbreeding coefficient within populations (F_{IS}) showed no significant deviation from zero at any of

TABLE 2. *Genetic characteristics of nine SSR loci for all sampled individuals from the 30 natural populations of* Fraxinus mandshurica

Locus	RAS (bp)	N_A	A_S	H_e	H_T	F_{IS}	F^*_{ST}
fm04	74–152	36	10·125	0·695	0·699	−0·017	0·005
fm06	107–115	4	2·945	0·145	0·146	0·043	0·008
fm13	77–111	13	3·329	0·064	0·065	0·024	0·005
fm14	152–225	37	23·968	0·939	0·947	0·065	0·009
M2-30	200–286	34	16·074	0·707	0·712	0·033	0·007
FEMSATL4	164–269	49	22·668	0·865	0·874	0·005	0·011
FEMSATL16	189–226	18	6·523	0·502	0·506	0·069	0·008
FEMSATL19	174–296	49	14·854	0·572	0·579	0·148	0·012
FR16	192–261	30	7·432	0·585	0·604	−0·005	0·032

RAS, range of allele sizes; N_A, total number of detected alleles; A_S, allele richness based on the minimum population size of 43 diploid individuals (86 gene copies); H_e, average expected heterozygosity; H_T, total gene diversity; F_{IS}, Wright's inbreeding coefficient, where 5400 randomizations were used to test for departure from Hardy–Weinberg genotypic proportions – values were not significant at any locus at the nominal level (5 %); F_{ST}, genetic differentiation based on allele identity, where * indicates significant differentiation at each locus ($P < 0·05$) according to the log-likelihood (G) -based exact test (Goudet *et al.*, 1996).

TABLE 3. *Statistics of genetic diversity for the 30 natural populations of* Fraxnius mandshurica *at the multilocus estimates*

Population	N_O	N_A	A_S (mean ± s.d.)	H_e (mean ± s.d.)	F_{IS} (mean ± s.d.)
X1, Jianxin	48	64	6·817 ± 4·918	0·477 ± 0·290	0·049 ± 0·320
X2, Shuiyuan	48	93	9·949 ± 6·825	0·549 ± 0·302	0·056 ± 0·082
X3, Pingshan	48	106	11·274 ± 6·903	0·550 ± 0·284	0·058 ± 0·113
X4, Chaoyang	48	116	12·304 ± 7·330	0·561 ± 0·288	0·035 ± 0·122
X5, Cuiluanhe	48	97	10·290 ± 6·491	0·504 ± 0·296	0·081 ± 0·307
X6, Meixi	48	97	10·290 ± 7·013	0·553 ± 0·276	0·108 ± 0·095
X7, Jinshantun	48	97	10·419 ± 6·831	0·560 ± 0·278	0·026 ± 0·086
X8, Dailing	48	93	9·884 ± 6·223	0·539 ± 0·277	−0·005 ± 0·043
W1, Dongfanghong	48	102	10·828 ± 7·265	0·542 ± 0·29	0·039 ± 0·083
W2, Yanggang	48	99	10·580 ± 7·347	0·555 ± 0·284	−0·015 ± 0·046
L1, Shuangyashan	48	101	10·790 ± 6·189	0·559 ± 0·276	0·048 ± 0·108
L2, Boli	48	87	9·299 ± 6·276	0·573 ± 0·267	0·046 ± 0·104
L3, Bamiantong	48	96	10·327 ± 6·386	0·574 ± 0·295	0·070 ± 0·144
L4, Muling	48	104	11·161 ± 6·941	0·587 ± 0·281	0·022 ± 0·079
Z1, Laoyeling	48	113	12·017 ± 7·475	0·580 ± 0·295	0·075 ± 0·073
Z2, Laoshan	48	108	11·549 ± 7·171	0·569 ± 0·313	0·020 ± 0·054
Z3, Hufeng	48	111	11·838 ± 7·071	0·572 ± 0·275	0·013 ± 0·050
Z4, Daguokuigang	48	100	10·647 ± 6·407	0·560 ± 0·296	0·040 ± 0·101
Z5, Datudingzi	48	115	12·221 ± 7·379	0·576 ± 0·266	0·035 ± 0·081
C1, Hongshi	48	116	12·464 ± 7·130	0·600 ± 0·276	0·050 ± 0·084
C2, Jiaohe	43	107	11·889 ± 6·367	0·590 ± 0·270	0·027 ± 0·103
C3, Wangqing	48	103	10·964 ± 7·384	0·554 ± 0·299	0·056 ± 0·085
C4, Dunhua	48	110	11·778 ± 7·484	0·582 ± 0·294	0·002 ± 0·059
C5, Fengman	48	113	12·037 ± 6·825	0·566 ± 0·282	0·023 ± 0·083
C6, Fusong	48	120	12·636 ± 7·730	0·588 ± 0·295	0·064 ± 0·101
C7, Lushuihe	48	103	10·999 ± 7·414	0·550 ± 0·307	0·049 ± 0·081
C8, Jiguanshan	48	117	12·436 ± 8·399	0·581 ± 0·289	0·048 ± 0·118
C9, Qingyuan	48	116	12·416 ± 6·819	0·602 ± 0·269	0·004 ± 0·052
C10, Laotudingzi	48	99	10·631 ± 6·646	0·557 ± 0·289	0·057 ± 0·149
C11, Baishilazi	48	104	11·168 ± 7·159	0·605 ± 0·262	-0·010 ± 0·121
Overall	1435	270	11·063 ± 7·053	0·564 ± 0·284	0·041 ± 0·128

N_O, number of individuals of the genotype per population; N_A, total number of detected alleles; A_S, allelic richness based on the minimum population size of 43 diploid individuals (86 gene copies); H_e, expected heterozygosity; F_{IS}, Wright's inbreeding coefficient, showing no significant deviations from zero at the multilocus estimates based on 5400 randomizations ($P > 0·05$).

the loci ($P > 0·05$; Table 2) or multilocus estimates ($F_{IS} = 0·041 \pm 0·128$, $P > 0·05$; Table 3), suggesting that the HWE was adhered to in each population. Tests of genotypic LD between pariwise-loci also showed no significant deviation from zero ($P > 0·05$). In addition, based on Wilcoxon's sign-rank test, there was no significant excess of heterozygosity in any of the populations studied under the IAM ($P > 0·05$) and SMM ($P > 0·05$).

Pearson correlation analysis showed that intra-population genetic diversity statistics (A_S and H_e) were significantly negatively correlated to latitude ($R = -0·642$, $P < 0·001$ and $R = -0·618$, $P < 0·001$; Fig. 2A, B). In contrast, although A_S displayed a significant correlation with longitude ($R = -0·471$, $P < 0·05$), H_e did not ($P > 0·05$). Furthermore, stepwise regression analysis of A_S dependent on latitude and longitude showed that only latitude

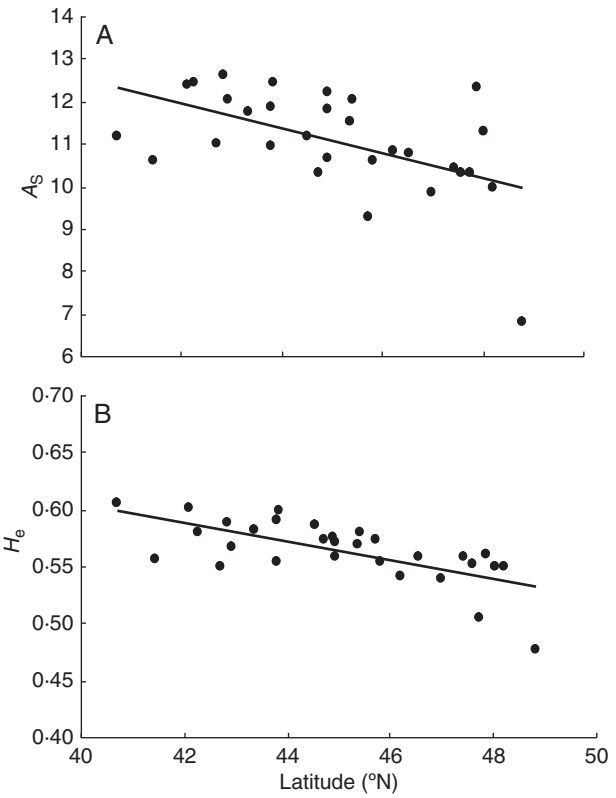

FIG. 2. Pearson correlation analysis showing that (A) the allele richness (A_S) was significantly correlated with latitude ($R = -0{\cdot}642$, $P < 0{\cdot}001$), and (B) that the expected heterozygosity (H_e) was also significantly correlated with latitude ($R = -0{\cdot}618$, $P < 0{\cdot}001$).

significantly contributed to the stepwise regression equation ($R^2 = 0{\cdot}292$, $F = 11{\cdot}567$, $P < 0{\cdot}001$), whereas the longitude was excluded. Stepwise regression analysis using H_e as the dependent variable on latitude and longitude produced a similar result, with only latitude contributing significantly to the stepwise regression equation ($R^2 = 0{\cdot}382$, $F = 17{\cdot}326$, $P < 0{\cdot}001$).

Genetic differentiation between populations

Although population differentiation was significant at each locus ($P < 0{\cdot}05$; Table 2), the average F_{ST} value at multilocus estimates was 0·010, ranging from 0·007 to 0·018 and from 0·007 to 0·021 for confidence intervals of 95 % and 99 %, respectively. This indicates that the population genetic differentiation was extremely low. Furthermore, about 40 % of the pairwise-F_{ST} values were significant ($P < 0{\cdot}05$), and the greatest values were between Jianxin and all the other populations.

Patterns of population genetic structure

The Bayesian clustering approach did not allow us to clearly identify genetic structure. Log-likelihood of the multilocus genotypic data [ln(X/K)] progressively declined as the number of assumed genetic clusters (K) increased from 1 to 13. This suggests that the optimal value of K is 1 (data not shown). The SAMOVA found no difference between IAM and SMM. As revealed by SAMOVA based on SMM, the optimal number of groups of populations (K) was two, because F_{CT} values decreased progressively as K was increased from 2 to 13 and reached a maximum at $K = 2$ (Fig. 3A). In this case, only the Jinxin population maximally differentiated from the others, while the rest clustered together and there was no clear genetic differentiation. Similarly, the first significant barrier, based on Monmonier's algorithm, maximally differentiated Jianxin from all the others (Fig. 3B). When the potential second, third, etc. barriers were attempted in Monmonier's algorithm, they showed no significance between mountain ridges. The NJ tree and PCoA also illustrated unclear clusters among all the sampled populations, with the exception of the Jinxin population, which was maximally differentiated from the rest (data not shown).

At the level of NEC, no significant pattern of IBD based on the Mantel permutation test was apparent among the 30 populations ($R^2 = 0{\cdot}017$, $P > 0{\cdot}05$; Fig. 4A). However, a weak but significant trend for IBD was observed among the 29 populations when Jianxin was excluded ($R^2 = 0{\cdot}048$, $P < 0{\cdot}001$; Fig. 4B).

DISCUSSION

Intra-population genetic diversity and associated geographical patterns

The genetic diversity within populations of *F. mandshurica* in North-east China (NEC) is relatively high, although it is slightly lower than the previous findings for *F. mandshurica* var. *japonica* in northern Japan (Goto *et al.*, 2006) and findings from the closely related species *F. excelsior* in Europe (e.g. Heuertz *et al.*, 2001, 2003, 2004*b*; Morand *et al.*, 2002; FRAXIGEN, 2005) determined on the basis of a number of shared microsatellite loci. There was no evidence to indicate that the expected heterozygosity (H_e) was significantly greater than the expected equilibrium gene diversity (H_{EQ}) based on Wilcoxon's sign-rank test under two models (IAM and SMM). Thus, there was no evidence of recent bottlenecks associated with any of the natural populations. This is probably because all the sampled populations were established too many generations ago to have experienced recent bottlenecks, as suggested by Amos and Balmford (2001).

In contrast, intra-population genetic diversity significantly decreased as latitude increased (Fig. 2); this was robustly supported by stepwise regression analysis. The tendency for genetic variation to decline along latitudinal gradients is probably the result of biome change during the mid-Holocene (Yu *et al.*, 1998, 2000; Member of China Quaternary Pollen Data Base, 2000). Based on a set of 113 sites of fossil pollen taxa dated to 6000 BP (^{14}C) including *Fraxinus* species, Yu *et al.* (1998) hypothesized that the forest biomes of eastern China had systemically expanded northwards during the mid-Holocene. Cool temperate forests in NEC were rapidly shifted about 4° northward as a result of both higher temperate and humidity

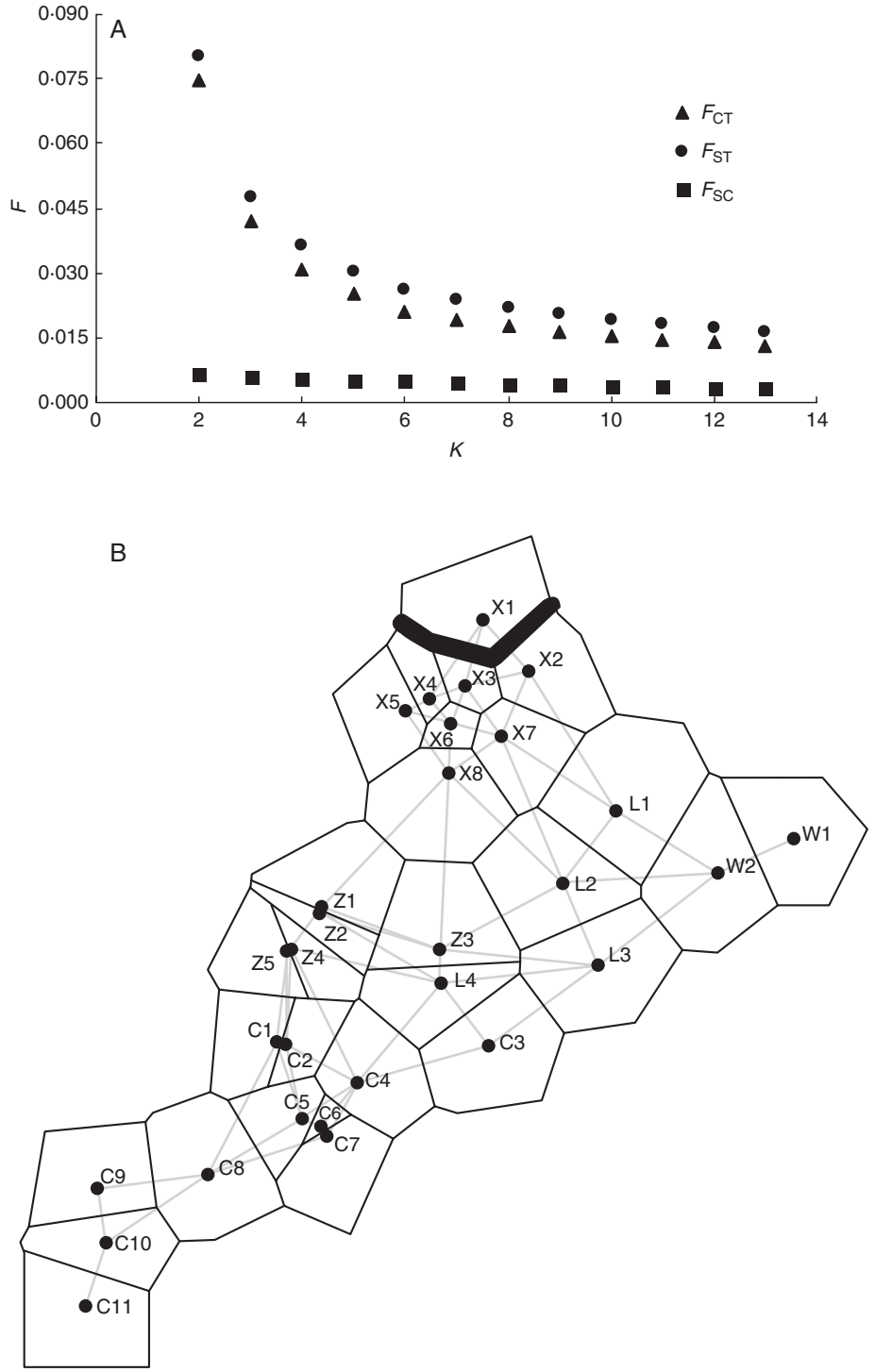

FIG. 3. (A) Fixation indexes (F) as a function of the user-defined number of groups of populations (K) for 30 natural populations of *Fraxinus mandshurica* using SAMOVA based on the stepwise mutation model (SMM) in terms of 1000 iterations. F_{CT}, F_{ST} and F_{SC} represent genetic differentiation between groups of populations, between populations overall, and between populations within groups, respectively. (B), Barrier analysis based on Monmonier's algorithm and significance tested by means of 1000 bootstrap matrices of D_A (Nei *et al.*, 1983). The first significant barriers (95 %) confirmed that there was only one distinct population: Jianxin (X1) was maximally differentiated from all the others.

during this historical episode (Yu *et al.*, 2000). As a result, genetic diversity may have gradually decreased during the one-dimensional (northward) colonization process (e.g. Austerlitz *et al.*, 2000).

Population structure

Extremely low levels of F_{ST} throughout NEC were identified, indicating that the effective migration rate per generation was high and that historical gene exchange

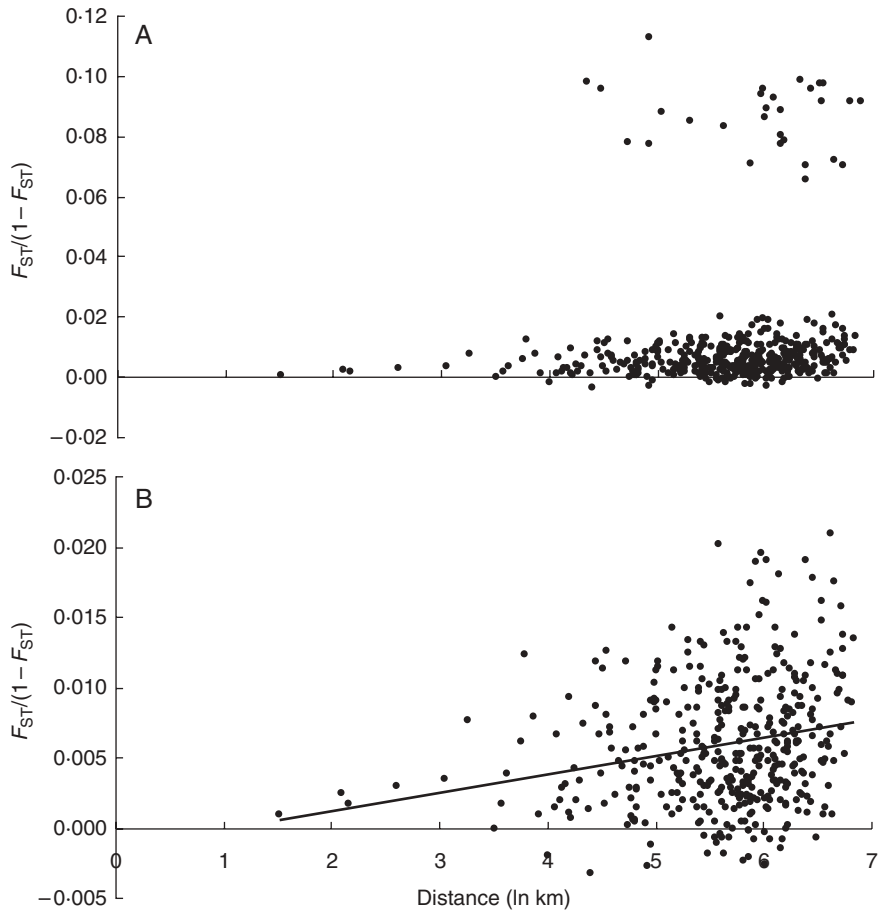

FIG. 4. (A) Isolation by distance (IBD) analysis based on the Mantel test (Mantel, 1967) with 9999 random permutations indicated no significant differences between the 30 *Fraxinus mandshurica* natural populations ($R^2 = 0{\cdot}017$, $P > 0{\cdot}05$); (B) The same data with X1 excluded: there is a weak but significant relationship between the remaining 29 populations ($R^2 = 0{\cdot}048$, $P < 0{\cdot}001$).

between populations occurred extensively. This is supported by previous studies using molecular-based pollen flow analysis (Heuertz *et al.*, 2003; Bacles *et al.*, 2005; Goto *et al.*, 2006), which detected extensive gene dispersal in ash species. Specifically, Bacles *et al.* (2005) demonstrated that the extensive pollen flow was sufficient to counteract the genetic drift that would be expected in severely reduced *F. excelsior* populations.

Although there is intra-population genetic diversity along latitudinal gradients, probably as a result of population expansion during the mid-Holocene, a report by China EPA (1998) suggested that wide refugia, rather than multiple small ones, existed across NEC during the last glacial maximum (LGM); these probably facilitated the retention of a high level of biodiversity for many native species. In this study, although the sampled populations were separated by up to 1000 km, only one admixed structure was detected using STRUCTURE analysis. The results suggest that the populations examined may be considered to be a single lineage; the extremely low F_{ST} value supports this suggestion. One possible explanation for the weak genetic structure exhibited by this species in NEC is that it occupied a wide range without separate refugia in the past. Similar results have been obtained in previous studies on widely distributed tree species (*F. excelsior*, Heuertz *et al.*, 2004*b*; *Betula maximowicsziana*, Tsuda and Ide, 2005). In both these studies, although several clusters were detected using STRUCTURE analysis over the whole species' range, further sub-structures could not be identified at the regional level, leading to the assumption that the species were dominated by a single lineage. The presence of a more homogeneous population and the survival of *F. mandshurica* in intermediate or higher latitudes in NEC are in accordance with recent findings relating to the phylogeography of other cold-tolerant tree species (e.g. Petit *et al.*, 2003; Palmé *et al.*, 2003; Lascoux *et al.*, 2004; Maliouchenko *et al.*, 2007). For example, Petit *et al.* (2003) investigated the variation of maternally inherited chloroplast DNA of 22 widespread European tree and shrub species and found that the species characterized by more boreal distributions exhibited low or medium levels of population differentiation, compared with other species. Palmé *et al.* (2003) found extremely low population differentiation and lack of phylogeographical structure in chloroplast DNA variation of the cold-tolerant tree species *Salix caprea*.

All approaches using SAMOVA, Monmonier's algorithm, PCoA, NJ trees and IBD showed that the Jianxin

population is divergent and isolated from the other populations. Considering the location of this population, this is not the result of geographical distance. Although the exact explanations are as yet unknown, a potential factor to account for the distinct genetic pattern in Jianxin might be the extremely low winter temperatures in this area; Zhao *et al.* (1991) analysed factors limiting the northern distribution of *F. mandshurica* associated with low winter temperatures, and demonstrated that the Jianxin area was a physiological stress zone for this species. Wang *et al.* (1994) further found that within the physiological stress zone in this area, normal metabolism of *F. mandshurica* was disrupted and numerous seedlings and young saplings could not survive because of damage caused by the extremely low temperatures. Therefore, genetic diversity within the Jianxin population might have been dramatically reduced when severe selection occurred because of the extreme temperature conditions. Even though some long-distance gene flow into this area may have been possible, establishment of immigrant gene resources might also have been restricted.

Although this study was conducted across five mountain ranges, no clear effect of the ridges of the mountains acting as significant barriers was detected; this was shown by the lack of genetic structure. Similar conclusions have been drawn for *Fagus sylvatica* across Europe on the basis of fossil and neutral molecular data (Magri *et al.*, 2006), as mentioned above. In contrast, previous studies have shown that mountain ridges have acted as significant barriers, causing the isolation of genetic lineages of plant species (e.g. *Pinus banksiana* in the USA, Godbout *et al.*, 2005; *Populus cathayana* in China, Lu *et al.*, 2006). Thus, whether mountain ridges act as effective barriers to gene flow and migration or not will vary according to the species involved and its mode of dispersal; the topography of the ridge will also influence the effect, as reviewed by Ohsawa and Ide (2008).

Conclusions and implications for conservation

This study has produced original data on the population genetics of *F. mandshurica* in its main and central range (NEC). These should be useful for various aspects of the conservation and management of sustainable populations of this endangered and national-priority protected species. Since it has a relatively high level of genetic variation within populations, but an extremely low level of differentiation among populations, it may not be necessary to take major steps to conserve genetic diversity in NEC. In contrast, the distinct population in Jianxin, exhibiting the lowest diversity and greatest differentiation from the other populations, requires priority conservation measures in order to avoid it becoming eradicated in its native habitat (e.g. management units; Moritz, 1994). The extremely low differentiation between populations across NEC can be attributed to extensive gene flow; this indicates that over this area the species constitutes a continuous genetic resource. In order to identify the most important genetic patterns and genetic resources for conservation, however, it is essential to examine both differentially inherited genetic markers exposed to selection pressures (e.g. chloroplast DNA; Heuertz *et al.*, 2004*a*) and to investigate different generations. The modern genetic structure of natural populations of *F. mandshurica* is unknown on the margins of its distribution (outside NEC). It also remains unclear whether this overall genetic pattern is unique to *F. mandshurica* or whether it occurs in other native tree species in NEC.

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