BIOGEOGRAPHY

Out of China: Distribution history of *Ginkgo biloba* L.

Yunpeng Zhao,[1] Juraj Paule,[2] Chengxin Fu[1] & Marcus A. Koch[2]

1 *Laboratory of Systematics, Evolutionary Botany and Biodiversity, College of Life Sciences, Zhejiang University, Hangzhou 310058, China*

2 *University of Heidelberg, Institute of Plant Science, Department of Biodiversity and Plant Systematics, Im Neuenheimer Feld 345, 69120 Heidelberg, Germany*

Author for correspondence: *Marcus A. Koch, mkoch@hip.uni-hd.de*

Abstract *Gingko biloba* L. is one of the most mysterious plant species, which continues to attract the interest of scientists and the public since many centuries. However, our knowledge of its evolutionary history and worldwide distribution is limited. Herein we are combining evidence from a previous phylogeographic analysis of *Ginkgo biloba*'s range in China using cpDNA and AFLPs with new data from old, early introduced in Korea, Japan, Europe and Northern America with the aim to unravel the early human-mediated introduction history of this species. We provide evidence that *Ginkgo biloba* reached Japan via different routes from China during the last two millennia most likely by human-mediated dispersal. Based on AFLP data, all individuals originally introduced to Europe and North America (e.g., by Kaempfer in Europe in the 1720s) are genetically similar to one of the Korean accessions raising, the question of the validity of the origin of Kaempfer's original material, which was said to be brought from Japan to Europe in the early 18th century. Multiple introduction into Korea and Japan in concert with an out-crossing mating system has maintained high levels of gene diversity; this is also true for the European and North American trees. In addition, the trees outside the original Chinese Pleistocene refugia, due to their relatively small gene-pool, have a reduced number of AFLP fragments and no exclusive alleles.

Keywords AFLP data; distribution history; *Gingko biloba*; human propagation

■ INTRODUCTION

The introductory history of plant species is of interest to plant scientists for many reasons. Since Columbus re-discovered the American continent and global trading routes became established, a large number of organisms have been dispersed worldwide along various transcontinental trading routes. An example is *Ginkgo biloba* L., the Maidenhair Tree, first introduced into Europe in the early 18th century. Engelbert Kaempfer (1651–1716), a German physician and botanist, visited Japan from 1690 to 1692 during a mission financed by the Dutch East-India Company, where he saw the *Ginkgo* tree (1691) and described it in his work *Amoenitatum exoticarum* (1712). It is said that he brought some seeds to Utrecht, and seedlings were cultivated and the first European cultivated tree was planted in 1730 in Utrecht, Netherlands.

Ginkgo biloba is regarded as a "living fossil" and is the only extant representative of the isolated gymnosperm family Ginkgoaceae (Harris, 1874; Traula 1967, 1968; Uemura 1997; Zhou, 2003). *Ginkgo* is a long-lived dioecious tree. The oldest individuals known are estimated to be approximately 1000 to 3000 years old (He & al., 1997). At its native sites the seeds are eventually dispersed by carnivores such as the leopard-cat (*Felis bengalensis*) or badgers which may be attracted by the smell of rotting flesh emited from the ripening sarcotesta (Jiang & al., 1990; Del Tredici & al., 1992). Seedlings are rarely observed in natural stands, however, where the species occurs and their occurrence seems to depend on open and sunny places in the woodlands (Del Tredici & al., 1992; Y. Xiang & al., 2000; Z. Xiang & al., 2001, 2003; B. Xiang & al., 2006, 2007).

From 1730 onwards, *Ginkgo* trees were increasingly planted all over Europe: Geetbets (Belgium) 1730 (independently brought by missionaries from China), Anduze (France) 1750, Padova (Italy) 1750, Slavkov (Czech Republic) 1758, Kew (United Kingdom) 1762, Vienna (Austria) 1770, Daruvar (Croatia) 1777, Harbke (Germany) 1781, Montpellier (France) 1788, etc. In 1784 the first *Ginkgo* tree was planted in North America, in Philadelphia (http://www.xs4all.nl/~kwanten/more.htm). All of these trees are male individuals. The first recorded female tree was from near Geneva, Switzerland; in 1814 its scions were grafted onto a male tree in the botanical garden of Montpellier, where the first seed had grown. Similar records of grafted female scions are known from other botanical gardens, e.g., the Botanical Garden of Vienna. During the following two centuries, *Ginkgo* was continuously introduced from Japan and maybe also China, to Europe and North America.

It was long thought among Western botanists that the genus *Ginkgo* was probably extinct in the wild and preserved only via human propagation (Sargent, 1897; Wilson 1914, 1919; and discussed in Del Tredici & al., 1992). When the occurrence of relic populations in China became obvious, questions about true wild populations, remained open (Del Tredici & al., 1992). Recently, some data on the phylogeographic history of *G. biloba* in China were presented by Gong & al. (2008). Chloroplast DNA sequence variation indicated that Pleistocene glaciations forced *Gingko* into two refugia in southwestern China, and in eastern China in the West Tianmu Mountains. Particularly, the status

of the population from Eastern China was controversial (Del Tredici & al., 1992; Lin & Zhang 2004). Our recent cpDNA and AFLP study confirmed, that this population represents offspring from a second refugium and represents a highly diverse gene pool. However, AFLP data indicated that Central China was colonised multiple times, mostly from these Eastern areas. It also appeared that cpDNA variation outside China is largely reduced: in Korea and Japan we only found the most prominent Chinese cpDNA type and only one singleton and derived haplotype was found in Tsukuba, Japan. We took this as first evidence that *G. biloba* was introduced from China to Korea and Japan in different historical periods (Gong & al. 2008). It is said that *G. biloba* was introduced into Japan from China between the late 13th and the early 14th century (Satoh & al., 2002, 2003). A larger time interval was proposed by Tsumara & Ohba (1997), showing an introduction during the Kamakura and Muromachi eras (A.D. 1192–1573). This fits well with that Eastern Chinese populations at Tianmu Mountain and its surrounding areas have been the site of human cultural and agricultural activities, and that *Ginkgo* propagation and conservation was practiced by Buddhist monks for approximately 1500 years in the gardens and forests surrounding their temples (Del Tredici & al., 1992).

In this paper we aim to provide genetic data on the European introductory history of *G. biloba*. We used AFLP and cpDNA sequence variation in order to (1) test for multiple and early introduction from China into Korea and Japan; (2) explore the genetic source of the oldest European and Northern American trees; and (3) provide data on the genetic variation in areas of introduction compared to its natural stands in China.

Although our data are far from being comprehensive, we hope this work will stimulate further research to unravel the biological and evolutionary questions concerning this fascinating tree.

■ MATERIALS AND METHODS

Population sampling. — In total we analysed 145 individuals, originating from 13 natural populations: China (92 individuals), Korea (11), Japan (18), Europe (14) and North America (10) (Fig. 1). Our aim was to sample the oldest trees outside China known (Table 1). In a previous study, we analysed the phylogeography of *G. biloba* within China (Gong & al., 2008) and worked with incomplete datasets for AFLPs and cpDNA sequence variation outside China. Herein we aim to add the missing information (AFLP data for Korean, Japanese and American samples; cpDNA data for various European samples) (Table 1) and to address open questions concerning *G. biloba*'s early introduction history.

DNA extraction and AFLP analysis. — Total genomic DNA was extracted from 50–75 mg dried leaf tissue following the procedure of Doyle & Doyle (1987) and Doyle (1991) with minor modifications (Gong & al., 2008). Leaf tissue was

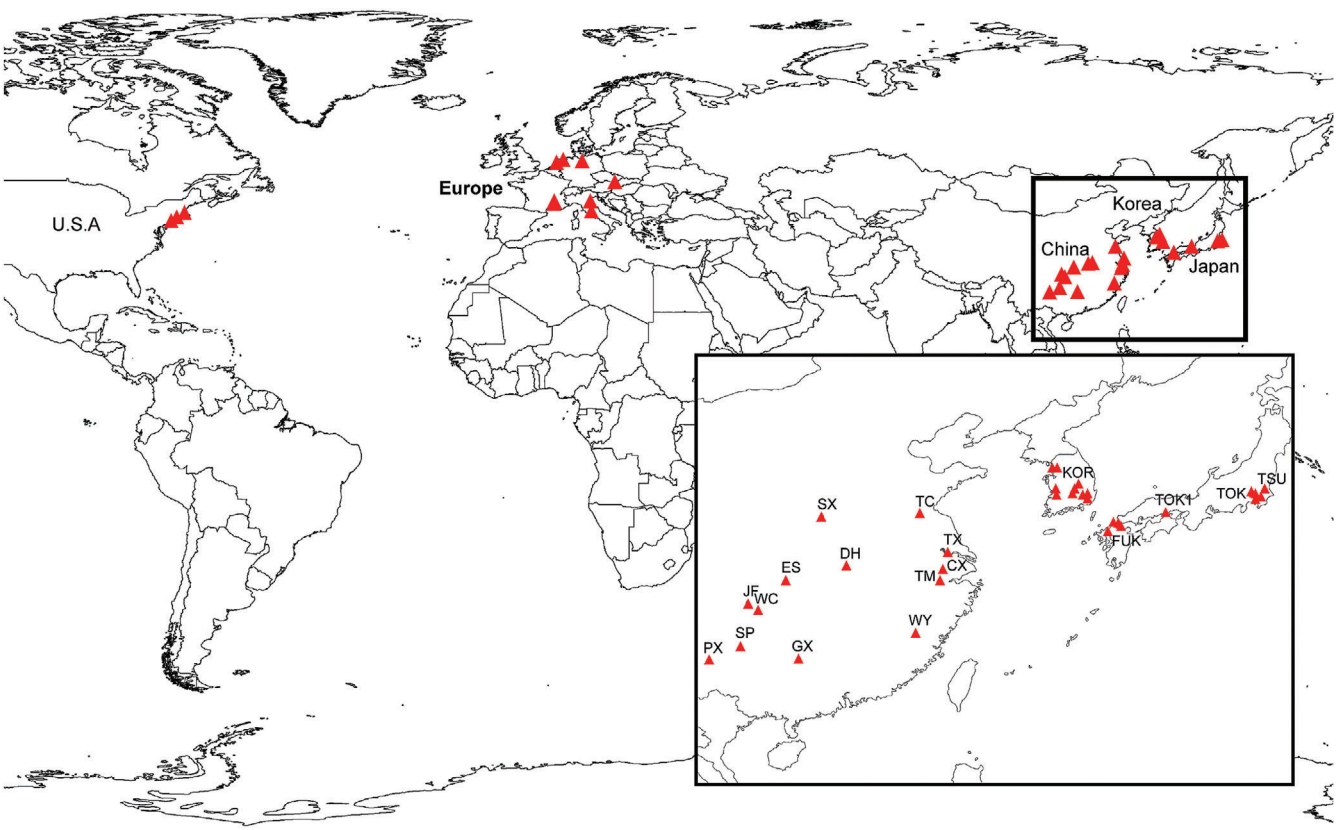

Fig. 1. Distribution of *Ginkgo* accessions and populations analysed herein. For details refer to Table 1.

Table 1. Accession data of *Ginkgo biloba* from Eastern Asia, Europe and North America analysed for AFLP and cpDNA variation. Remarks provide data on voucher information (Chinese populations), assumed age, tree dimensions and historical aspects. DBH, trunk diameter at breast height.

No(s).	Code	Origin	Sex	cpDNA-type	Remarks
	SW China				
1–7	ES1–7	Enshi, Hubei		D, E	Voucher No. GW2004ES173 (HZU)
8–15	JF1–8	Mt. Jinfo, Chongqing		A, B, E	Voucher No. FG2003JF135 (HZU)
16–24	PX1–9	Panxian, Guizhou		D, E	Voucher No. QL2002PX104 (HZU)
25–33	SP1–9	Shanping, Guizhou		E	Voucher No. GW2004SP183 (HZU)
34–41	WC1–8	Wuchuan, Guizhou		C, D, E	Voucher No. GD2001WC011 (HZU)
	Central China				
42–46	DH1–5	Mt. Dahong, Hubei		E	Voucher No. GY2002DH021 (HZU)
47–52	GX1–6	Lingchuan, Guangxi		D, E	Voucher No. GD2001GX001 (HZU)
53–59	SX1–7	Songxian, Henan		E	Voucher No. GY2002SX067 (HZU)
	East China				
60–66	CX1–7	Changxing, Zhejiang		E, G	Voucher No. YG2005CX037 (HZU)
67–72	TC1–6	Tancheng, Shandong		E	Voucher No. GY2001TC080 (HZU)
73–80	TM1–8	Mt. Tianmu, Zhejiang		E, F, I	Voucher No. CC2006TM301 (HZU)
81–88	TX1–8	Taixing, Jiangsu		E	Voucher No. GY2001TX057 (HZU)
89–92	WY1–4	Mt. Wuyi, Fujian		E	Voucher No. GY2001WY094 (HZU)
	Japan				
93–94	TSU1,2	Tsukuba University, Ibaraki	?,?	E, H	~34 years
95–96	TSU3,4	Tsukuba, Ibaraki	♀, ♀	E, H	~34 years
97	TOK1	Korakuen Park, Okayama	?	E	girth = 4.5 m, height = 20 m
98	TOK2	Ishigamimae, Ome City, Tokyo	♀	E	girth = 7.1 m, height = 25 m
99	TOK3	Nakagamityo, Akishima City, Tokyo	♀	E	~400 years, girth = 6.5 m, height = 22 m
100	TOK4	Iyamachi, Fuchu City, Tokyo	?	E	girth = 6 m, height = 18 m
101	TOK5	Hiramachi, Hachioji City, Tokyo	♂	E	~800 years, girth = 7 m, height = 30 m
102	TOK6	Ooi, Shinagawa-ku, Tokyo	♂	E	~800 years, girth = 6.5 m, height = 25 m
103	TOK7	Motoazabu, Minato-ku, Tokyo	♂	E	~800 years, girth = 10 m, height = 25 m, the largest one in Tokyo
104	FUK1	Ikazuchi Shrine, Maebaru City, Fukuoka	♀	E	~800 years, girth = 6.5 m, height = 25 m
105	FUK2	Jinguin, Kawara Town, Tagawa-gun, Fukuoka	♀	E	~1000 years, height = 40 m, girth = 6.2 m
106	FUK3	Eigen-ji Temple, Nougata City, Fukuoka	♀	E	~450 years, with chichi, girth = 4.5 m, height = 18 m
107	FUK4	Okitama Shrine, Nougata City, Fukuoka	♂	E	~600 years, with chichi, girth = 6 m, height = 20 m
108	FUK5	Bank of Onga River, Kiyase, Nougata City, Fukuoka	♂	E	~600 years, with chichi, girth = 6 m, height = 20 m
109	FUK6	Ohara Shrine, Onga-gun, Fukuoka	♂	E	~600 years, girth = 6.9 m, height = 15 m, 2 trees
110	FUK7	Hananoki-zeki, Nougata City, Fukuoka	♂	E	~1000 years, with chichi, girth = 17.6 m, height = 28.4 m

Table 1. Continued.

No(s).	Code	Origin	Sex	cpDNA-type	Remarks
	Korea				
111	KOR1	Won ri, Cheongdo-eup, Cheongdo-gun, Gyeongsangbuk-do	♀	E	~800 years, DBH ~1.8 m, height ~28 m
112	KOR2	Churyang-ri, Daedeok-myeon, Gimcheon-si, Gyeongsangbuk-do	♀	E	~400 years, DBH ~1.1 m, height ~37 m
113	KOR3	Seongdang-ri, seongdang-myeon, Iksan-si, Jeollabuk-do	♀	E	~500 years, DBH ~1.0 m, height ~15 m
114	KOR4	Nongso-ri, Okseong-myeon, Gumi-si, Gyeongsangbuk-do	♀	E	~400 years, DBH ~1.9 m, height ~21.6 m
115	KOR5	Joryong-ri, Daedeok-myeon, Gimchon-si, Gyeongsangbuk-do	♀	E	~500 years, DBH ~1.8 m, height ~28 m
116	KOR6	Hapyeong-ri, Maejeon-myeon, Cheongdo-gun, Gyeongsangbuk-do	♀	E	~450 years, DBH ~1.2 m, height ~27 m
117	KOR7	Daejeon-ri, Iseo-myeon, Cheongdo-gun, Gyeongsangbuk-do	♂	E	~400 years, DBH ~1.4 m, height ~29 m
118	KOR8	Jangsu-dong, Namdong-gu, Incheon	♂	E	~800 years, DBH ~1.4 m, height ~30 m
119	KOR9	Sungkyunkwan Univ, Myeongnyun-dong, Jongno-gu, Seoul	♂	E	~400 years, DBH ~1.2 m, height ~21 m
120	KOR10	162 Gyesan-dong, Gyeyang-gu, Incheon	♀	E	~500 years, DBH ~1.6 m, height ~25 m
121	KOR11	Mahyeon-ri, Gongdeok-myeon, Gimje-si, Jeollabuk-do	♀	E	~650 years, DBH ~1.5 m, height ~15 m
	Europe				
122	EUF2	Montpellier, Jardin des Plantes, France	♂	E	ca. 1788, girth = 2.8 m
123	EUF3–1	Anduze, La Bambouseraie Prafrance, France	♂	E	ca. 1750, girth <4.88 m, height ~30 m
124	EUF3–2	Anduze, La Bambouseraie Prafrance, France	♂	E	height >30 m, ~255 years
125	EUG1–1	Botanical Garden Jena, Germany	♂	E	1792–1794, Goethe-Gingko, height = 21 m
126	EUG3–2	Hannover, Royal Park Herrenhausen, Germany	♂	E	1826
127	EUG3–3	Hannover, Royal Park Herrenhausen, Germany	♂	E	1843
128	EUG3–4	Hannover, Royal Park Herrenhausen, Germany	♂	E	1826
129	EUI1–1	Padova, Hortus Simplicium, Padova University, Italy	♂	E	1750
130	EUN1	Harderwijk, Stadspark, Academiestraat, The Netherlands	♂	E	said to be planted in 1735 by Linnaeus
131	EUN3–2	Utrecht, De Oude Hortus, Lange Nieuwstraat, The Netherlands	♂	E	ca. 1730, maybe the oldest Gingko outside Asia
132	HBV1	Vienna University, Austria	♂	E	ca. 1770, basal branches, not grafted
133	HBV2	Vienna University, Austria	♂	E	ca. 1770, basal branches, grafted on HBV1 by Joseph von Jacquin
134	HBV4	Vienna University, Austria	♂	E	ca. 1886
135	HBV5	Vienna University, Austria	♂	E	ca. 1886, grafted
	North America				
136–141	NY1–6	New York Botanical Gardens	♂	E	60 years
142	PHI1	The Woodlands Cemetery, Philadelphia, Pennsylvania	♂	E	ca. 1784, the oldest one in U.S.
143	PHI2	Original Harvard University Botanical Garden, Boston, Massachusetts	♂	E	ca. 1850
144	PHI3	Original Harvard University Botanical Garden, Boston, Massachusetts	♀	E	ca. 1850
145	PHI4	Mount Auburn Cemetery, Boston, Massachusetts	♂	E	1901

grinded in a Precellys 24 (Bertin technologies) homogenizator. Two units of ribonuclease A per extraction were added to the isolation buffer. The DNA pellet was washed twice with 70% ethanol and dissolved in 50 µl TE-buffer. The concentration of the sample was measured using the NanoDrop ND-1000 spectrophotometer (Peqlab) and prior to analysis each sample was diluted with ddH_2O to a final DNA concentration of 100 ng/µl.

AFLP analysis was performed according to Vos & al. (1995) and modified by Gong & al. (2008). The analysis was carried out using *Eco*RI-AC and *Mse*I-CC as pre-selective primers and six selective primer combinations: *Eco*RI-ACG (TET)/*Mse*I-CCTG, *Eco*RI-ACG (FAM)/*Mse*I-CCAG, *Eco*RI-ACG (HEX)/*Mse*I-CCAA, *Eco*RI-ACG (TET)/*Mse*I-CCAC, *Eco*RI-ACG (FAM)/*Mse*I-CCTA and *Eco*RI-ACC (HEX)/*Mse*I-CCTG. Three differentially fluorescence-labelled primer pairs were multiplexed (2 µl TET, 2 µl FAM, 5 µl HEX) and diluted 30 times with ddH_2O, of which 6 µl were taken and mixed with 0.2 µl ET-ROX 550 size standard. After 2 min denaturation at 95°C, samples were run on a MegaBase 500 automated sequencer (Amersham Biosiences). Raw data were scored and exported as a presence/absence matrix using Genemarker v.1.6 (SoftGenetics LLC).

In each experiment running 48 samples on the DNA sequencer, six standards (previously analysed samples; Gong & al. 2008) and one repeat of the sample were always applied in order to score constancy and repeatability of banding patterns.

cpDNA sequence analysis. — For DNA amplification of the plastids, *trnK* IGS and *trnS-trnG* IGS, we used the same DNA as used for AFLP analysis. Amplification and sequencing strategy followed Gong & al. (2008). PCR was performed using a GeneAmp 9700 PCR DNA Thermal Cycler (Perkin Elmer). Thermal cycling started with a denaturation step lasting 5 min at 94°C, followed by 35 cycles each comprising 1 min denaturation at 94°C, 30 s annealing at 52°C (for the *trnK* IGS) or 58°C (for the *trnS-trnG* IGS), and 2 min elongation at 72°C. Amplification ended with an elongation phase lasting 10 min at 72°C, and a final hold at 10°C. Amplicons were purified using a PCR purification kit (Quiagen). Cycle sequencing was done with the DYEnamic ET Terminator Cycle Sequencing Kit (Amersham Biosciences) using the PCR primers for the cycle sequencing reaction. Samples were resolved in 10 µl Loading Solution and then run on a MegaBace 500 Sequencer (GE Healthcare Life Sciences).

Data analysis. — As a measure of within-population diversity, we assessed the total number of fragments (F_T), and the percentage of polymorphic fragments (Poly%) for all individual populations from China and the samples from Japan, Korea, Europe and North America (Table 2). The number of private fragments was counted for each population (F_{PP}) and each geographical region (F_{PR}) of *G. biloba* in China using AFLPDAT (Ehrich, 2006). Accordingly, gene diversity was measured as Nei's genetic diversity.

Hierarchical structuring of genetic variation among populations was determined by an analysis of molecular variance (AMOVA) with WINAMOVA v.1.55 (Excoffier & al., 1992) (Table 3). Significance levels of the variance components were based on 1000 permutations.

Phylogenetic relationships were inferred using the distance-based neighbour-net method (implemented in SplitsTree v.4, Huson & Bryant, 2006) based on uncorrected p-distances (Fig. 2). In comparison to generally used tree building methods, a network method allows to visualise potentially conflicting signals, which may be caused by homoplasy, reticulation or hybridisation.

Principal coordinate analysis (PCoA) was performed to calculate principal coordinates from pairwise Euclidian distances between individual genotypes (MVSP v.3.1, Kovach Computing Services, Anglesey, Wales) (Fig. 3).

As an alternative approach, the population structure was examined by genetic admixture analysis using the programs STRUCTURE v.2.2 (Pritchard & al., 2000) and BAPS v.3.2 (Corander & al., 2003, 2004, 2006) (Fig. 4). The data were

Table 2. Genetic diversity based on AFLP data among the various *Ginkgo* populations.

Populations	F_T	Poly%	F_{PP}	F_{PR}	Gene diversity
SW China	99			2	
1. ES	90	23.85	2		0.104
2. JF	87	21.10			0.076
3. PX	85	22.93			0.078
4. SP	88	22.02			0.088
5. WC	89	21.10			0.085
Central China	94			1	
6. DH	85	20.18			0.101
7. GX	87	20.18			0.098
8. SX	85	17.43	1		0.078
Eastern China	99			0	
9. CX	89	22.94			0.093
10. TC	80	6.42			0.027
11. TM	90	24.77			0.103
12. TX	86	13.76			0.044
13. WY	84	17.43			0.096
Japan	89			0	
14. TSU	78	33.94			0.182
15. TOK	83	14.68			0.060
16. FUK	87	28.44			0.118
Korea	89			0	
17. KOR	89	41.28			0.123
Europe	84			0	
18. EUR	84	19.27			0.068
North America	84			0	
19. NY	82	13.76			0.059
20. PHI	82	30.27			0.171

F_T, total number of fragments; Poly%, percentage of polymorphic fragments; F_{PP}, number of private fragments per population; F_{PR}, number of private fragments per geographic region. Genetic diversity calculated as Nei's measure of heterozygosity.

Table 3. Analysis of molecular variance (AMOVA) of all analyzed samples of *G. biloba* based on the AFLP data.

Source of variation	df	Sum of squares	Variance of components	Percentage of variation	F_{ST}
Among regions	6	163.446	1.09429	16.30**	
Within regions	138	775.478	5.61940	83.70**	
Total	144	938.924	6.71369		0.16299**
Among populations	19	339.393	1.81224	27.42**	
Within populations	125	599.531	4.79625	72.58**	
Total	144	938.924	6.60849		0.27423**

**, $P < 0.001$

analysed with STRUCTURE with K (number of groups) ranging from 2 to 5, with 10 replicate runs for each K, and a burn-in period of 2×10^4 and 1×10^5 iterations. The "no admixture model" and uncorrelated allele frequencies were chosen for the analysis. The likelihood of Ks ranging from 1 to 10 was calculated using the R-script Structure-sum (Ehrich, 2006). For a detailed description of the above used parameters, refer to Ehrich & al. (2007) and Evanno & al. (2005). BAPS was run with the most likely number of groups (K) set to 2–5 as revealed by the R-script analysis. Each run was replicated three times.

Chloroplast DNA sequences were analysed for those individuals not included in our previous phylogeographic survey (Gong & al., 2008). The haplotypes were identified accordingly and are shown in Table 1.

■ RESULTS

AFLP data and high reproducibility. — A total of 145 individuals of *G. biloba* (92 individuals from 13 Chinese populations, 14 European individuals, 18 Japanese individuals, 11 Korean individuals, 10 North American individuals) were successfully scored for the six selective primer combinations, producing 109 repeatable fragments with sizes ranging from 75 to 500 bp (Table S1 in the Electronic Supplement to this article). Out of the 109 fragments, 85 fragments (77.98%) were polymorphic. The mean repeat accuracy of all experiments amounts to 98.93%; the mean scoring error was therefore below 1.1%. Furthermore, we observed a 99% accuracy and repeatability when comparing the standard samples with former results (Gong & al., 2008). The experiments showed the general quality and reliability of the AFLP marker system and the possibility to add more individuals in future studies.

cpDNA data indicate limited gene flow out of China. — With the exception of two Japanese individuals carrying cpDNA haplotype H (Table 1), all remaining individuals from outside China are characterised by cpDNA haplotype E. It was shown earlier (Gong & al., 2008) that haplotype H directly derived from haplotype E and was not found elsewhere.

Haplotype E is widespread in China and is the most common haplotype. We can hypothesize for the maternal lineage, and thus for seed dispersal, that *Ginkgo* migrated out of China, but most likely not from its two primary centres of cpDNA genetic diversity as we found none of the rare and refugia specific cpDNA haplotypes outside China. This might be an indicator for human propagation not from the primary centres of genetic diversity in the refugia but from neighbouring or other regions in China possibly surrounding Buddhists' temples.

AFLP data show reduced genetic diversity outside China. — Based on AFLP data and similar to the cpDNA data, genetic diversity is decreasing outside China. This is most obvious when considering the overall number of fragments detected (F_T, Table 2). Chinese populations and regions revealed up to 99 fragments. In contrast, we scored only 90% of the original diversity (89 fragments) in Japan and Korea and 85% (84 fragments) in Europe and North America. This genetic depletion is also evident in terms of private fragments, which exclusively exist in single populations or areas. This level of genetic variation was only observed in *Ginkgo* plants from China.

AFLP data show relative high gene diversity outside China. — Unexpectedly, gene diversity at the larger scale of whole regions (Europe, America, Japan, Korea, China) exhibited no dramatic loss of genetic variation (Table 2). This can be best explained by the dioecious breeding biology of the species, which maintains high levels of heterozygosity and gene diversity, or by the extreme longevity of this species. Our AFLP data does not allow assumptions on overall heterozygosity, but high levels of heterozygosity had been demonstrated in earlier isozyme analysis (Tsumara & Ohba, 1997). Consequently, AMOVA showed that most genetic variation is distributed within regions and populations with 83.7% and 72.6% ($P < 0.001$), respectively (Table 3).

Multiple immigration events to Japan but limited introductions to Europe and North America. — Results from Network analysis are congruent with genetic diversity analysis (Fig. 2). As molecular variance analysis (Table 3) showed high levels of diversity within population/region, the network analysis showed an unresolved star-like structure with few internal nodes, indicating a low amount of diversity between the populations or regions. The most important findings concern individuals from *Ginkgo* populations outside China: (1) Korean samples group at three different positions within all Chinese individuals; (2) Japanese samples are found throughout the network at various positions; (3) European samples are

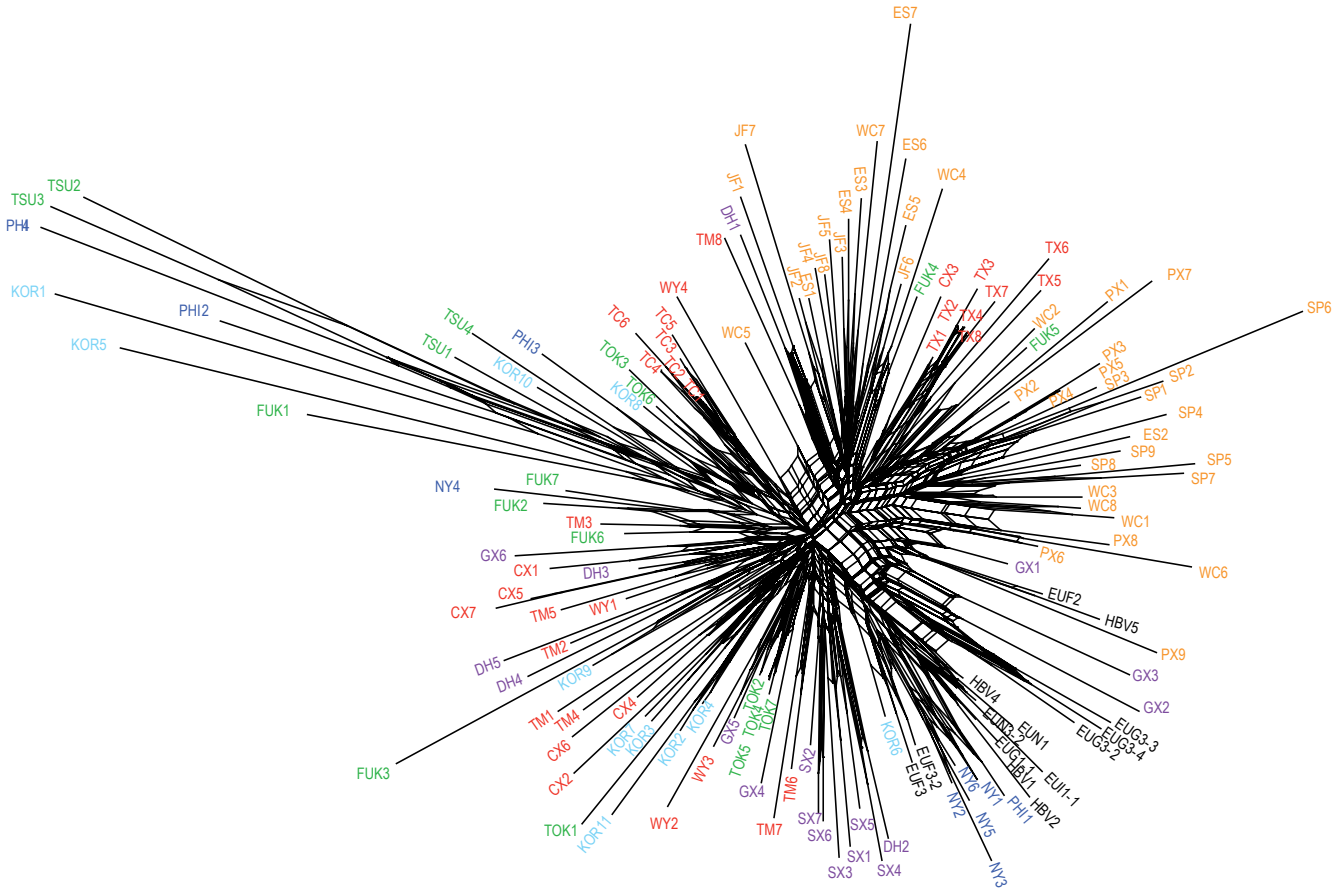

Fig. 2. Phylogenetic relationships inferred using the distance-based neighbour-net method as implemented in SplitsTree 4. Accession codes follow Table 1 (red, eastern China; violet, Central China; orange, southwestern China; green, Japan; blue, U.S.; light blue, Korea; black, Europe).

all clustering closely together with one Korean sample and few individuals from China and, in general, are more close to individuals from Central China then from the putative Chinese refugia; (4) North American individuals are close either to the European samples, or Philadelphian to Japanese and Korean individuals.

The PCoA analysis (Fig. 3) was conducted to highlight potential artefacts that could be caused by the phylogenetic approach. The first three axes explain 24% of the observed variation (axis 1: 10.3%; axis 2: 7.1%; axis 3: 6.4%, not shown). Most results are congruent to those of network analysis. However, American individuals are extremely close to the European material; and, furthermore, materials from Philadelphia group either with the European samples as well or cluster with Japanese/Korean samples.

STRUCTURE analysis revealed the most clear assignment pattern of genetic diversity, and the results are consistent with BAPS (Fig. 4). We followed the recommendations from the program, STRUCTURE, to build our interpretations on the *K*- value with the highest likelihood (in our case: $K = 4$, K equals the number of recognised genetic clusters), for which the ten runs provide a consistent result and individuals were clearly assigned to non-empty groups (K values ranging from 2–6, data shown in Fig. 4).

The populations from southwestern China are clearly separated from the remaining populations and individuals. Eastern and Central China are differentiated similarly, and postglacial and even human-mediated colonisation from East to Central China seems obvious. The Japanese individuals are either indistinguishable from these Central/Eastern Chinese populations or close to some individuals from Korea indicating multiple immigrations from the original source areas in China. Interestingly, one individual from Korea (KOR6) resembles a genotype from Eastern China (CX) not found in Japan, but there are also genotypes in Korea (KOR1, KOR5) close to materials from Japan (TSU, FUK1) and not found in China. These results confirm putative colonisation of Japan via Korea. All European materials and most North American trees are grouped with the Korean sample with no corresponding representatives in Japan ($K = 4$; Fig. 4). Finally, individuals from Philadelphia are closest to Korean/Japanese material and cannot be assigned more precisely (Fig. 4). Only one individual from Philadelphia (PHI1), representing the oldest tree found in the United States (Table 1), groups with the oldest *Ginkgo*s from Europe.

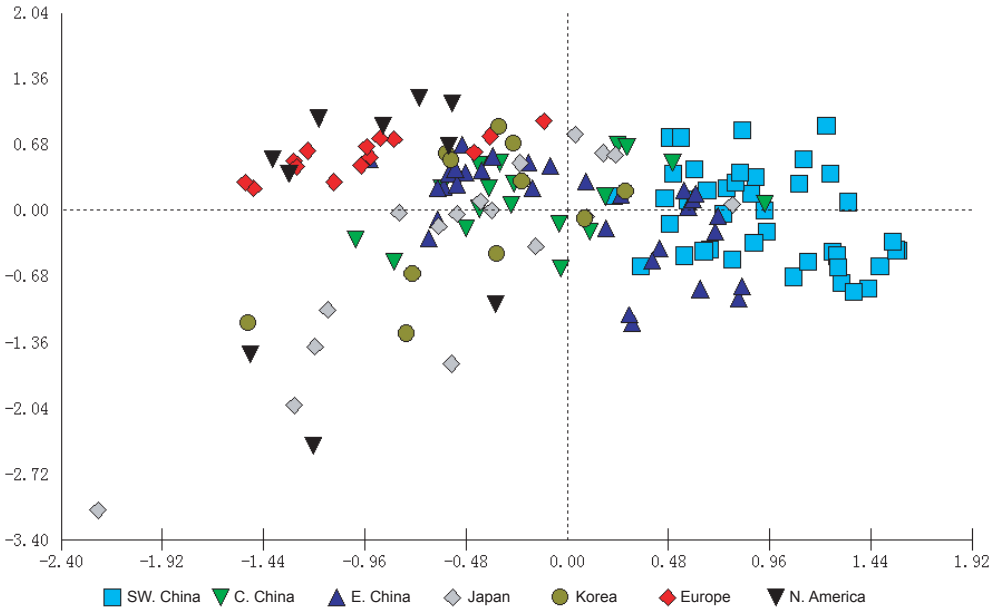

Fig. 3. Principal coordinate analysis (PCoA) using pairwise Euclidian distances between individual genotypes (a version of this figure with accession codes included is provided as Fig. S1 in the Electronic Supplement).

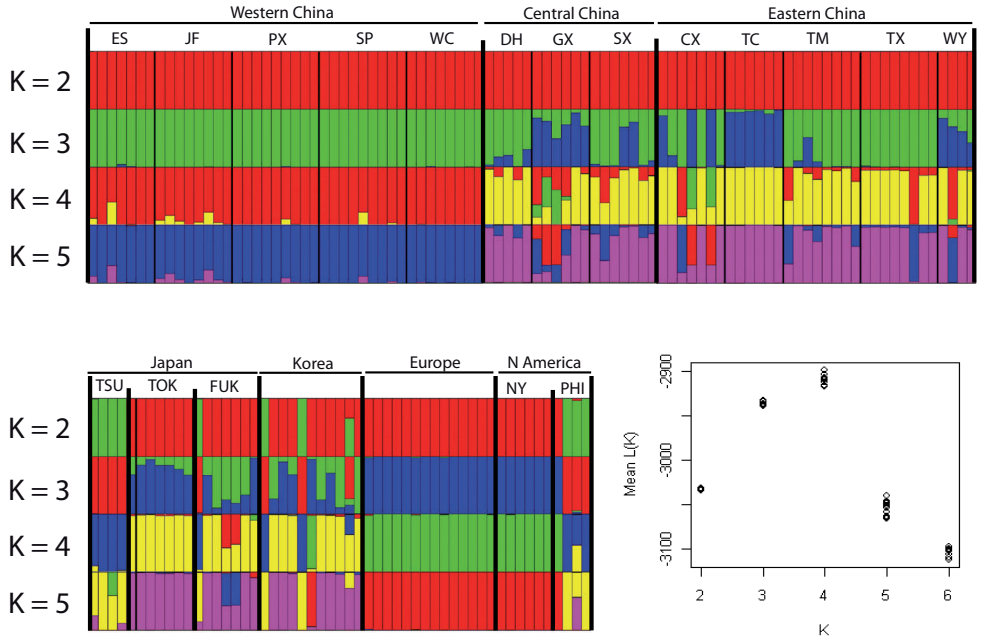

Fig. 4. Population structure examined by genetic admixture analysis using the programs STRUCTURE with *K* ranging from 2 to 5 (*K* equals the number of recognized genetic clusters). The likelihoods of *K*s ranging from 2 to 6 using the R-script Structure-sum (Ehrich, 2006) are shown demonstrating *K* = 4 with the highest likelihood.

■ DISCUSSION

There are few studies providing evidence on the evolutionary history of *G. biloba* outside China. The most comprehensive, but not fully documented and published results are based on mitochondrial *nad2* DNA sequence variation (Satoh & al., 2002, 2003; Satoh & Hori, 2004). This work analysed old *Ginkgo*s with a trunk diameter at breast height greater than 2 m from China, Korea and Japan. A total of 18 mtDNA types were characterised (ca. 300 trees from Japan with 14 types including 7 unique ones; 58 trees from Korea with 4 types all of them also distributed in China and Japan; 20 trees from China with 11 types including 4 unique ones). The latter suffered from under-sampling of Chinese materials and, consequently, we have to assume that (1) the number of *nad2* DNA sequence types unique to China will rise drastically when sample size is increased, and (2) more types found only in Japan are most likely to occur in China as well (Satoh, pers. comm. from 2009). However, Satoh & Hori's data can be standardised (the numbers of types divided by the numbers of individuals), and then become fully consistent with the cpDNA data (Gong & al., 2008). The preliminary results suggest multiple introductions from China to Japan along various routes (directly or via Korea).

An AFLP analysis of two different cultivars of *G. biloba* from Europe, America and China (Wang & al., 2006), did not reveal a consistent biogeographic pattern. This can be easily explained by extensive and multiple introductions from Japan and China during the last 200 years leading to complex and reticulate distribution patterns of genetic diversity. Moreover, it was shown that genetic diversity among cultivars from Europe and America (14) exceeds that among Chinese cultivars (7) by approximately 10% (measured as percentage of polymorphic bands). This is comparable with our results on old and early introduced trees in the 18th century showing also very high genetic diversity compared to its genetic source area in China (Tables 2–3).

A few other studies analysed genetic variation within *G. biloba* focusing on refugia in China (Shen & al., 2006), and using multiple molecular markers (Gong & al., 2008), or analysing RAPD polymorphisms in individuals from the eastern United States (Kuddus & al., 2002). Various analyses (e.g., Kuddus & al., 2002; Wang & al., 2006) are highly rudimental and do not allow major and far-reaching conclusions. However, in these studies, it is obvious that the *Ginkgo* trees were cultivated outside China and are therefore considered to be introduced by man. These population outside of China exhibit high levels of genetic diversity as measured with nuclear encoded markers (RAPD, AFLP). This is not true for the cpDNA (Gong & al., 2008), which indicates that *Ginkgo* was introduced from a limited gene pool resource into the various countries, not considering individuals from the putative relic populations in western and eastern China (Gong & al., 2008). This finding fits well with our observation that most genetic variation is distributed within populations but not between them (Tables 1–2; Figs. 2–3). The genetic differentiation of *Ginkgo* populations detected by AFLP analysis (F_{ST} = 0.27, Table 3) is comparable to other conifers or tree species estimated by biparental inherited markers, e.g., F_{ST} = 0.054–0.300 (compilation of various wind-pollinated tree species; Newton & al., 1999), 0.116 (compilation of various *Picea* and *Pinus* species; Petit & al., 2005) or F_{ST} = 0.22 (*Cathaya argyrophylla*, Pinaceae; Wang & Ge, 2006). Compared to the nuclear data genetic differentiation analysed by cpDNA sequences (F_{ST} = 0.35; Gong & al., 2008) is lower based on maternally inherited markers. Moreover, cpDNA-based estimates are usually higher with F_{ST} > 0.6 (Newton & al., 1999; Petit & al., 2005). However, this discrepancy can be best explained by the combination of two factors: their evolutionary history and breeding system. The distribution of common haplotype E and the restricted distribution of unique haplotypes indicate less genetic differentiation among the populations.

The AFLP data do not allow major conclusions on the relatedness of single trees. However, they provide some relevant information. The Botanical Garden of Vienna, has a large male tree from around 1781 (HBV1). A female shoot was grafted on this plant by Joseph von Jacquin (Jacquin, 1819), and with this experiment he was able to demonstrate sex constancy of these particular branches. These kinds of experiments were repeated at various places in a similar way. However, it was unclear wheather this female branch still exists in Vienna. We therefore analysed putative material from the original branch (HBV2) and found that it is virtually identical with HBV1; it is not possible to determine that it is grafted material. Leaf material of this female branch kept in Herbarium W might be analysed in future.

The high levels of genetic variation combined with strict outbreeding will complicate any detailed reconstruction of *Ginkgo*'s introduction history. This is obvious when analysing American trees sampled at New York Botanical Garden with an age of approximately 60 years. These trees cluster more closely with the old European trees (Figs. 3–4). It is likely, that in principle, the European or Japanese (see Fig. 2) material served as a genetic source, but the variation among these few American trees from New York is comparable to that found in all other old European trees.

■ CONCLUSIONS

We were able to show that *Ginkgo biloba* was introduced to Japan along various routes (via Korea or directly from China). It was also shown that the oldest trees in Europe and North America represent genotypes from a gene pool which migrated most likely from China via Korea. Furthermore, it is unlikely that major immigration took place from the two main refugia in China as indicated by cpDNA and AFLP data. Finally, in all regions and continents, we found similar and high levels of genetic variation reflecting the outcrossing breeding system, but also the high levels of human impact on the distribution of genetic variation.

■ ACKNOWLEDGEMENTS

We are grateful to all institutions and individuals who sent us leaf materials of European *Gingko* specimens and provided us with valuable information about the history of the sampled individuals and their mode of propagation (Table 1). Our special thanks go to Ming-Shui Zhao (National Nature Reserve of Mt. Tianmu, China), Xie Lin (Zhejiang Forestry Institute, China), Yang-Mei Tan (Chongqing Herbal Medicine Institute, China), Tetsukazu Yahara (Kyushu University, Japan) and Joongku Lee (Korea Research Institute of Bioscience and Biotechnology, South Korea) for their kind help with the field work. We further thank Peter Del Tredici (Arnold Arboretum of Harvard University) and Zhi-Yan Zhou (Nanjing Institute of Geology and Palaeontology, Chinese Academy of Sciences) for providing material and advice. We thank Masaya Satoh (University of Tokushima, Japan) for sharing results and detailed information on mitochondrial *nad2* data. We are also grateful to Ingrid Jordon-Thaden for discussions on the final manuscript. This work was supported in part by the National Basic Research Program of China (973 Program 2007CB411600) and the National Science Foundation of China (Grant 30670139).

■ LITERATURE CITED

Corander, J., Marttinen, P. & Mätyniemi, S. 2006. Bayesian identification of stock mixtures from molecular marker data. *Fishery Bull.* 104: 550–558.

Corander, J., Waldmann, P., Marttinen, P. & Sillanpää, M.J. 2004.

BAPS 2: Enhanced possibilities for the analysis of genetic population structure. *Bioinformatics* 20: 2363–2369.

Corander, J., Waldmann, P. & Sillanpää, M.J. 2003. Bayesian analysis of genetic differentiation between populations. *Genetics* 163: 367–374.

Del Tredici, P., Ling, H. & Yang, G. 1992. The Ginkgos of Tian Mu Shan. *Conservation Biol.* 6: 202–209.

Doyle, J.J. 1991. DNA protocols for plants: CTAB total DNA isolation. Pp. 283–293 in: Hewitt G.M. & Johnston A. (eds.), *Molecular techniques in taxonomy*. Berlin: Springer.

Doyle, J.J. & Doyle J.L. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochem. Bull.* 19: 11–15.

Ehrich, D. 2006. AFLPdat: A collection of R functions for convenient handling of AFLP data. *Molec. Ecol. Notes* 6: 603–604.

Ehrich, D., Gaudeul, M., Assefa, A., Koch, M.A., Mummenhoff, K., Nemomissa, S., IntraBioDiv consortium & Brochmann, C. 2007. Genetic consequences of Pleistocene range shifts: Contrast between the Arctic, the Alps and the East African mountains. *Molec. Ecol.* 16: 2542–2559.

Evanno, G., Regnaut, S. & Goudet, J. 2005. Detecting the number of clusters of individuals using the software STRUCTURE: A simulation study. *Molec. Ecol.* 14: 2611–2620.

Excoffier, L., Smouse, P.E. & Quattro, J.M. 1992. Analysis of molecular variance inferred from metric distances among DNA haplotypes: Applications to human mitochondrial DNA restriction data. *Genetics* 131: 479–491.

Gong, W., Chen, C., Dobes C., Fu, C.X. & Koch, M.A. 2008. Phylogeography of a living fossil: Pleistocene glaciations forced *Ginkgo biloba* L. (Ginkgoaceae) into two refuge areas in China with limited subsequent postglacial expansion. *Molec. Phylog. Evol.* 48: 1094–1105.

Harris, T.M. 1935. The fossil flora of Scoresby Sound, East Greenland: 4, Ginkgoales, Coniferales, Lycopodiales, and isolated fructification. *Meddel. Gronland* 112: 1–176.

He, S.A., Yin, G. & Pang, Z.J. 1997. Resources and prospects of *Ginkgo biloba* in China. Pp. 37–383 in: Hori, T., Ridge, R.W., Tulecke, W., Del Tredici, P., Trémouillax-Guiller, J. & Tobe, H. (eds.), *Ginko biloba*: *A global treasure*. Tokyo, Heidelberg, New York: Springer.

Huson, D.H. & Bryant, D. 2006. Application of phylogenetic networks in evolutionary studies. *Molec. Phylog. Evol.* 23: 254–267.

Jacquin, J.F. 1819. *Ueber den Ginkgo*. Vienna: Gerold.

Jiang, M., Jin, Y. & Zhang, Q. 1990. [A preliminary study on *Ginkgo biloba* in Dahongshan region, Hubei]. *J. Wuhan Bot. Res.* 8: 191–193. [In Chinese.]

Kaempfer, E. 1712. *Amoenitatum exoticarum politico-physico-medicarum fasciculi V, quibus continentur variae relationes, observationes & descriptiones rerum Persicarum & ulterioris Asiae*. Lemgo: Meyer.

Kuddus, R.H., Kuddus, N.N. & Dvorchik, I. 2002. DNA polymorphism in the living fossil *Ginkgo biloba* from the eastern United States. *Genome* 45: 8–12.

Lin, X. & Zhang, D.H. 2004. Analysis of the origin of *Ginkgo* populations in Tianmu Mountains. *Sci. Silvae Sin.* 2: 28–31. [In Chinese with English abstract.]

Newton, A.C., Allnutt, T.R., Gillies, A.C.M., Lowe, A.J. & Ennos, R.A. 1999. Molecular phylogeography, intraspecific variation and the conservation of tree species. *Trends Ecol. Evol.* 14: 140–145.

Petit, R.J., Duminil, J., Fineschi, S., Hampe, A., Salvini, D. & Vendramin, G.G. 2005. Comparative organization of chloroplast, mitochondrial and nuclear diversity in plant populations. *Molec. Ecol.* 14: 689–701.

Pritchard, J.K., Stephens, M. & Donnelly, P. 2000. Inference of population structure using multilocus genotype data. *Genetics* 155: 945–959.

Sargent, C.S. 1897. Notes on cultivated conifers, I. *Gard. & Forest* 10: 390–391.

Satoh, M. & Hori, T. 2004. DNA polymorphism in old trees of *Ginkgo biloba* in eastern Asia. Poster abstract P22. International symposium on Asian plant diversity and systematics (July 29th–August 1st, 2004), National Museum of Japanese History, Sakura, Chiba Pref., Japan.

Satoh, M., Satoh, Y., Amou, H., Shibahara, R. & Hori, T. 2003. DNA polymorphism in old trees of *Ginkgo biloba* in Japan. *J. Pl. Res.* 116 (Suppl.): 46.

Satoh, M., Satoh, Y., Ogura, N. & Hori, T. 2002 Polymorphism of mitochondrial DNA of *Ginkgo biloba* in Japan. *J. Pl. Res.* 115 (Suppl.): 44.

Shen, L., Chen, X.Y., Zhang, X., Li, Y.Y., Fu C.X. & Qiu, Y.X. 2005. Genetic variation of *Ginkgo biloba* L. (Ginkgoaceae) based on cpDNA PCR RFLPs: Inference of glacial refugia. *Heredity* 94: 396–401.

Traula, H. 1967. Evolutionary trends in the genus *Ginkgo*. *Lethaia* 1: 63–101.

Traula, H. 1968. The phytogeographic evolution of the genus *Ginkgo* L. *Bot. Not.* 120: 409–422.

Tsumara, Y. & Ohba, K. 1997. The genetic diversity if isozymes and the possible dissemination of *Ginkgo biloba* in anicent times in Japan. Pp. 159–181 in: Hori., T., Ridge, R.W., Tulecke, W., Del Tredici, P., Trémouillax-Guiller, J. & Tobe, H. (eds.), *Ginko biloba: A global treasure*. Tokyo, Heidelberg, New York: Springer.

Uemura, K. 1997. Cenozoic history of *Ginkgo* in East Asia. Pp. 207–221 in: Hori, T., Ridge, R.W., Tulecke, W., Del Tredici, P., Trémouillax-Guiller, J. & Tobe, H. (eds.), *Ginko biloba: A global treasure*. Tokyo, Heidelberg, New York: Springer.

Vos, P., Hogers, R., Bleeker, M., Reijans, R., van de Lee, T., Hornes, M., Fritjers, A., Pot, J., Peleman, J., Kuiper, M. & Zabeau, M. 1995. AFLP: A new technique for DNA fingerprinting. *Nucleic Acids Res.* 23: 4407–4414.

Wang, H.W. & Ge, S. 2006. Phylogeography of the endangered *Cathaya argyrophylla* (Pinaceae) inferred from sequence variation of mitochondrial and nuclear DNA. *Molec. Ecol.* 15: 4109–4122.

Wang, L., Xing, S.Y., Yang, K.Q., Wang, Z.H., Guo, Y.Y. & Shu, H.R. 2006. Genetic relationships of ornamental cultivars of *Ginkgo biloba* analyzed by AFLP techniques. *Acta Genet. Sin.* 33: 1020–1026.

Wilson, E.H. 1914. Plantae wilsonianae. Cambridge: Harvard Univ. Press.

Wilson, E.H. 1919. The romance of our trees: II, The *Ginkgo*. *Gard. Mag.* 30: 144–148.

Xiang, B., Xiang, Z. & Xiang, Y. 2006. Investigation of wild *Ginkgo biloba* in Wuchuan County of Guizhou, China. *Guizhou Sci.* 24: 56–67.

Xiang, B., Xiang, Z. & Xiang, Y. 2007. Report on wild *Ginkgo biloba* in Qianzhong altiplano. *Guizhou Sci.* 25: 47–55.

Xiang, Y., Xiang, B., Zhao, M. & Wang, Z. 2000. A report on the natural forest with *Ginkgo biloba* population in West Tianmu Mountains, Zhejiang Province. *Guizhou Sci.* 18: 77–92.

Xiang, Z., Tu, C. & Xiang, Y. 2003. A report on *Ginkgo* resources in Panxian county, Guizhou province. *Guizhou Sci.* 21: 159–174.

Xiang, Z., Zhang, Z. & Xiang, Y. 2001. Investigation of natural *Ginkgo biloba* population on the Golden Buddha Mountains of Nanchuan, Chongqing. *Guizhou Sci.* 19: 37–52.

Zhou, Z.Y. 2003. Mesozoic Ginkgoaleans: Phylogeny, classification and evolutionary trends. *Acta Bot. Yunnan.* 25: 377–396. [In Chinese with English abstract.]