POPULATION STRUCTURE AND GENETIC RELATIONSHIPS OF TAXA IN THE *LARIX GMELINII* COMPLEX IN CHINA

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ABSTRACT

Allozyme variation was conducted to elucidate the population structure and genetic relationships of three closely related larch taxa *Larix gmelinii*, *L. olgensis* and *L. principis-rupprechtii* in China. Most populations were randomly outcrossing, with only a few populations showing significant heterozygote deficit at individual loci. Deviations from linkage disequilibria within populations were not significant among the 8 polymorphic loci. Nei's genetic distance among populations within each taxa was 0.002, whereas the genetic distance among the taxa was 0.01, with *L. gmelinii* and *L. olgensis* being most closely related. Allozyme diversity was very similar within the three taxa (mean $H_e = 0.101$), and taxa showed very limited genetic divergence among populations ($F_{ST} = 0.009 \sim 0.02$). The extent of genetic differences suggests that these three taxa have differentiated very recently and are best regarded as subspecies of *L. gmelinii*. The pattern of genetic divergence suggests that *L. principis-rupprechtii* diverged first. This may have occurred after populations of *L. gmelinii* became isolated at high elevation in the south, as the main body of the population moved north at lower elevation at a time of climate warming.

Key words: *Larix gmelinii*, *L. olgensis* , *L. principis-rupprechtii*, genetic relationship, population structure

INTRODUCTION

Larix is a genus of deciduous conifers with an extensive and circumpolar distribution in the northern hemisphere (OSTENFELD & LARSEN 1930). Within north-eastern China three taxa are currently recognised. *L. gmelinii* Rupr occurs in the far north of China in the Xingan mountains at elevations below 1200 m. (Fig. 1). It represents an extension of a northern and very widely distributed species that penetrates south into China. The second taxon, *L. olgensis* Henry, has a range that extends north from Korea, through the Changbei mountains as far as latitude 45° 20' N within China at elevations between 500 and 1800 m. In the northern part of its range, *L. olgensis* meets *L. gmelinii*. The third taxon, *L. principis-rupprechtii* Mayr occupies a disjunct geographic distribution located to the southwest in the high mountain regions of Hebei and Shanxi provinces at elevations between 1400 and 2500 m.

All three of these taxa are important timber species in north-east China, and have been the subject of extensive provenance trials aimed at delineating seed zones and developing seed transfer rules (MA & TAO 1992). However, there remains uncertainty about the population genetic structure and genetic relationships of these taxa. There is also debate about the taxonomic status of the three units. *L. olgensis* and *L. principis rupprechtii* have been regarded by some as subspecies of *L. gmelinii* (OSTENFELD & LARSEN 1930) whereas they are treated as separate species by Chinese taxonomists (WANG & ZHANG 1992).

A previous study of restriction fragment length polymorphism using southern blotting and a library of cpDNA probes failed to detect any variation for cpDNA among these three *Larix* taxa (TANG *et al.* 1995). This technique has limited sensitivity due to the low rate of base substitution in the cpDNA genome (WOLFE *et al.* 1987, CLEGG *et al.* 1994). In recently differentiated taxa, analysis of allele frequency differences at polymorphic allozyme loci are expected to be much more informative about evolutionary relationships than analyses of cpDNA variation (CRAWFORD 1983). To clarify uncertainties in classification within *Larix* we report here an analysis of divergence within and among taxa at polymorphic isozyme nuclear markers. Analysis of population structure for allozyme

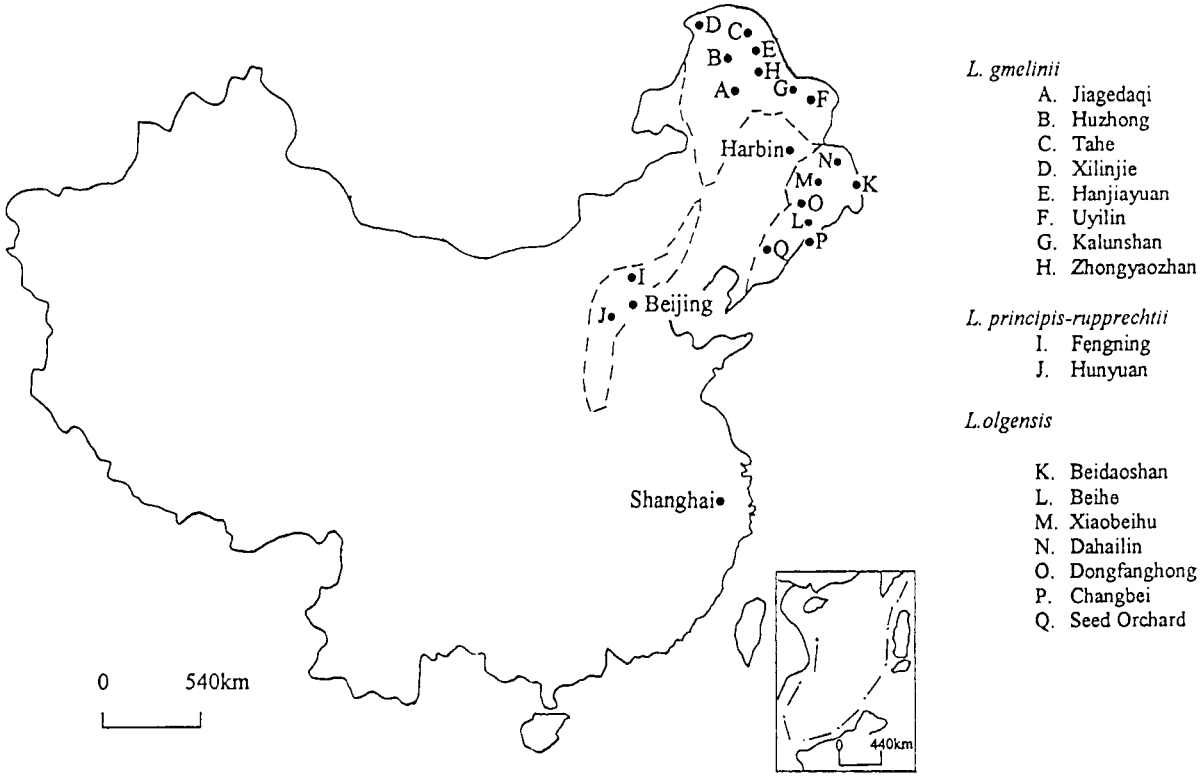

Figure 1. Natural distribution of the three *Larix* taxa in China and the locations of those populations investigated in this study.

loci was also used to determine whether the taxa differ significantly in population genetic diversity or show different degrees of population genetic divergence.

MATERIALS AND METHODS

Seed preparation

Open pollinated seeds were collected from at least 20 maternal parents within eight natural populations of *L. gmelinii*, six natural populations of *L. olgensis* and two natural populations of *L. principis-rupprechtii*. In addition, one population of *L. olgensis* was sampled from a seed orchard in Liaoning Province. Details of the population locations are shown in Table 1 and Fig. 1. Samples of *L. gmelinii* and *L. olgensis* cover most of their distributions in China, but only two populations of *L. principis-rupprechtii*, located in the Northern seed zone (MA 1990), were available (Fig. 1).

Electrophoresis

Mature seeds were surface-sterilized using H_2O_2 for 20 minutes, and germinated for four days prior to analysis. Macrogametophyte tissue and embryo were isolated and analysed in adjacent lanes to facilitate recognition of heterozygotes. Electrophoresis was conducted according to CHELIAK & PITEL (1984a) on 11 % starch gels. Gels were stained for six enzyme systems: aspar-tate aminotransferase (AAT; E.C.2.6.1.1); malate dehydrogenase (MDH; E. C. 1.1.1.37); 6-phosphogluconate dehydrogenase (6PGD; E.C. 1.1.1.44); phosphoglucomutase (PGM; E.C. 2.7.5.1), phosphoglucose isomerase (PGI; E.C.5.3.1.9); and shikimic acid dehydrogenase (SDH; E.C.1.1.1.25).

Data Analysis

Data on allele frequencies at each locus in each population were used to calculate mean number of alleles per locus (A), percent polymorphic loci (P (99 %) criterion), expected (H_e) and observed (H_o) heterozygosity. Deviations from Hardy-Weinberg equilibrium at each locus, and tests of linkage disequilibrium were conducted using GENEPOP package (RAYMOND & ROUSSET 1995).

Population genetic structure was analysed using the FSTAT package (version 1.2; GOUDET 1995). The spatial pattern of genetic variation within taxa was tested for evidence of isolation by distance using the method introduced by SLATKIN (1993). The spatial heterogeneity in terms of F_{ST} was also tested using Mantel's test (MANTEL 1967), and the exact probability (P-value) under the null hypothesis was calculated for

Table 1. Location and sample size of the 17 *Larix* populations investigated using allozyme analysis.

Species	Population	Latitude (N)	Longitude (E)	Seeds analyzed
Larix gmelinii	Huzhong	51° 56′	123° 42′	90
	Tahe	52° 30′	124° 45′	90
	Xilinjie	53° 20′	122° 10′	90
	Hanjiayuan	52° 15′	125° 45′	90
	Uyilin	48° 30′	129° 26′	90
	Kalunshan	49° 58′	127°30′	54
	Zhongyaouzhan	50° 45′	125° 07′	56
	Jigedaqui	50° 24′	124° 07′	126
Larix principis-rupprechtii	Fengning	41° 12′	116° 32′	121
	Hunyuan	39° 32′	112° 41′	75
Larix olgensis	Beidaoshan	44° 00′	131° 07′	120
	Beihe	42° 25′	128° 08′	174
	Xiaobeihu	44° 01′	128° 50′	198
	Dahailin	44° 28′	129° 48′	288
	Dongfanghong	42° 39′	128° 06′	139
	Changbei	41° 26′	128° 11′	209
	Seed orchard	41° 54′	124° 06′	188

Table 2. Measures of allozyme variability in 17 populations of three *Larix* taxa.

Species	Population	A	$P_{(99\%)}$	H_o	H_e
Larix gmelinii	Jiagedaqi	2.12	50	0.106	0.102
	Huzhong	2.25	87	0.100	0.102
	Tahe	2.25	50	0.081	0.094
	Xilinjie	2.37	87	0.096	0.108
	Hanjiayuan	2.00	62	0.075	0.078
	Uyilin	2.12	75	0.085	0.089
	Kalunshan	2.37	62	0.126	0.115
	Zhongyaouzhan	2.12	75	0.104	0.107
	Mean	**2.20**	**68**	**0.097**	**0.100**
Larix principis-rupprechtii	Fengning	1.87	37	0.103	0.117
	Hunyuan	2.00	62	0.104	0.134
	Mean	**1.93**	**49**	**0.103**	**0.126**
Larix olgensis	Beidaoshan	2.12	75	0.072	0.071
	Beihe	2.12	62	0.076	0.076
	Xiaobeihu	2.12	50	0.067	0.077
	Dahailin	1.87	62	0.101	0.125
	Dongfanghong	2.28	71	0.091	0.090
	Changbei	2.12	75	0.083	0.105
	Seed orchard	2.14	71	0.141	0.135
	Mean	**2.11**	**66**	**0.090**	**0.097**

*: A - average number of alleles per locus; $P_{(99\%)}$ - percentage of polymorphic loci where the frequency of the most common allele is < 0.99; H_o and H_e - observed and expected hetorozygosities, respectively.

the observed sample (GOUDET 1995). Neighbour-Joining method (see the PHYLIP package; FELSENSTEIN 1989) was applied to draw the tree of genetic relationships among the three larch taxa on the basis of the matrix of Nei's genetic distances (D) among populations (NEI 1972). One thousand individual trees were

constructed from 1000 distance matrices generated by bootstrap resampling (SEQBOOT), and a final unrooted consensus tree was constructed using the computer program CONSENSE (FELSENSTEIN 1989).

RESULTS

The six enzyme stains used resolved eight polymorphic loci (HU 1998): *Aat-1* (2 alleles), *Aat-2* (2 alleles), *Aat-3* (expressed only in macrogametophyte tissue, 4 alleles), *Mdh-1* (2 alleles), *6Pgd-2* (2 alleles), *Pgm* (1 locus 4 alleles), *Pgi* (1 locus 3 alleles), *Sdh* (1 locus 4 alleles). Banding patterns are the same as *L. laricina* for enzymes PGI, 6PGD, MDH, AAT, and PGM (CHELIAK & PITEL 1984b), and the same as *L. decidua* for enzymes MDH and SDH (LEWANDOWSKI & MEJNARTOWICZ 1990). Genetic diversity statistics summarised in Table 2 reveal a mean of 2.13 alleles per locus, 65 % polymorphic loci and a gene diversity of 0.101 over all sampled populations within the Chinese larch complex.

Deviations from linkage equilibrium within populations were not significant. No consistent, significant deviations from Hardy-Weinberg equilibrium were found for most loci over all populations. However for a few populations at some loci there was a significant heterozygote deficit (Table 3). The seeds analysed were mostly derived from essentially randomly outcrossing populations.

Genetic differentiation among populations within taxa, as measured by F_{ST}, was very small but significant (Table 4). However only 1.2 %, 0.9 % and 1.9 % of allozyme variation was accounted for by differences among populations in *L. gmelinii*, *L. principis-rupprechtii* and *L. olgensis* respectively. When estimated numbers of migrants (Nm) between populations (inferred from F_{ST}) was regressed on geographic distance no relationship was found for *L. gmelinii* ($b > 0$ for multiple loci), but a significant negative relationship was found for *L. olgensis* ($b < 0$ and $r = -0.637$, $P < 0.01$ for multiple loci), indicating significant isolation by distance (Table 5). The above analysis was also consistent with MANTEL's test results (MANTEL 1967). The probability (P-value) under the null hypothesis was $P = 0.792$ for *L. gmelinii* and $P = 0.033$ (<5%) for *L. olgensis*.

Nei's genetic distances calculated from frequencies

Table 3. Tests of Hardy-Weinberg equilibrium for each locus in each population. Type-I error probabilities were listed for rejecting null hypothesis for only heterozygote deficit (Deficit) or excess (Excess). Bold characters indicate significant values ($P < 0.05$). Symbol '–' stands for monomorphic loci in this sample of embryos; Δ: lack of data.

Locus		*L. gmelinii*							
		Jiagedaqi	Hushong	Tahe	Xilinjie	Hanjiayuan	Uyilin	Kalunshan	Zhongyaozhan
Pgi	F_{IS}	–	-0.011	–	– 0.053	-0.017	-0.043	-0.093	-0.028
	Deficit		ns		ns	ns	ns	ns	ns
	Excess		ns		ns	ns	ns	ns	ns
Mdh-1	F_{IS}	–	-0.035	–	-0.011	–	-0.006	–	–
	Deficit		ns		ns		ns		
	Excess		ns		ns		ns		
6-pgd-2	F_{IS}	–	–	–	–	–	–	–	–
Aat-1	F_{IS}	–	-0.011	-0.023	-0.017	–	–	–	-0.019
	Deficit		ns	ns	ns				ns
	Excess		ns	ns	ns				ns
Aat-2	F_{IS}	0.129	0.796	–	0.388	-0.023	1	-0.010	0.488
	Deficit	ns	**0.000**		ns	ns	**0.000**	ns	ns
	Excess	ns	ns		ns	ns	ns	ns	ns
Pgm	F_{IS}	-0.063	-0.000	0.075	0.229	0.005	0.036	-0.141	-0.131
	Deficit	ns	ns	ns	**0.000**	ns	ns	ns	ns
	Excess	ns	ns	ns	ns	ns	ns	ns	ns
Sdh	F_{IS}	0.010	-0.102	0.203	0.029	0.094	-0.079	-0.054	0.268
	Deficit	ns	ns	ns	ns	ns	ns	ns	ns
	Excess	ns	ns	ns	ns	ns	ns	ns	ns

Table 3. (continued).

Locus		*L. principis-ruprechtii*			*L. olgensis*					
		Fengning	Hunyuan	Beidaoshan	Beihe	Xiaobeihu	Dahailin	Dongfang-hong	Changbei	Seed orchard
Pgi	F_{IS}	0.124	0.373	-0.017	0.129	0.025	-0.096	0.009	-0.039	-0.039
	Deficit	ns	**0.010**	ns	ns	ns	ns	ns	ns	ns
	Excess	ns	ns	ns	ns	ns	ns	ns	ns	ns
Mdh-1	F_{IS}	-0.030	-	-0.030	-0.018	-	-0.021	-0.026	0.746	-0.086
	Deficit	ns		ns	ns		ns	ns	**0.000**	ns
	Excess	ns		ns	ns		ns	ns	ns	ns
6-pgd-2	F_{IS}	-	-	-0.021	-0.021	-	-	-0.026	-0.039	-0.068
	Deficit			ns	ns			ns	ns	ns
	Excess			ns	ns			ns	ns	ns
Aat-1	F_{IS}	-	0.282	-0.021	-0.006	-	-	-	-0.027	-
	Deficit		ns	ns	ns				ns	
	Excess		ns	ns	ns				ns	
Aat-2	F_{IS}	-	-0.021	0.426	-0.039	0.508	0.433	-0.013	0.215	0.663
	Deficit		ns	**0.006**	ns	**0.000**	**0.000**	ns	**0.022**	**0.023**
	Excess		ns	ns	ns	ns	ns	ns	**ns**	ns
Pgm	F_{IS}	0.098	-0.056	-0.097	-0.038	0.057	0.273	-0.029	0.102	-0.105
	Deficit	ns	ns	ns	ns	ns	**0.000**	ns	ns	ns
	Excess	ns	ns	ns	ns	ns	ns	ns	ns	ns
Sdh	F_{IS}	-	-	-	-0.003	-0.018	0.161		-	
	Deficit				ns	ns	**0.007**			
	Excess				ns	ns	ns			

Table 4. Estimates of F_{ST} for 7 polymorphic loci over all populations within each of the three larch taxa. - : monomorphic locus ;* - $P < 0.05$; ** - $P < 0.01$.

Locus	*L. gmelinii*	*L. principis-rupprechtii*	*L. olgensis*
Pgi	0.022±0.013**	0.001	0.005±0.005**
Mdh-1	0.015±0.011**	0.009	0.019±0.011**
6-pgd-2	0.000±0.003	-	0.015±0.011**
Aat-1	0.005±0.006**	0.079**	0.012±0.005**
Aat-2	0.001±0.007	0.015**	0.006±0.005**
Pgm	0.016±0.016**	0.005	0.008±0.008**
Sdh	-0.002±0.002	-	0.164±0.076**
Over all loci	**0.012±0.007****	**0.009****	**0.019±0.013****

of allozyme alleles showed a very close genetic relationship between populations both within taxa (mean D = 0.0022) and to a lesser extent among taxa (mean D = 0.0124). Distances among populations within each taxa were: 0.0025 ± 0.0018 in *L. gmelinii*, 0.0020 in *L. principis-rupprechtii*, and 0.0022 ± 0.0016 in *L. olgensis*. Distances between taxa were slightly larger than those within taxa: 0.0143 ± 0.0045 between *L. gmelinii* and *L. principis-rupprechtii*, 0.0075 ± 0.0041 between *L. gmelinii* and *L. olgensis*, 0.0089 ± 0.0013 between *L. olgensis* and *L. principis-rupprechtii*. *L. gmelinii* and *L. olgensis* are more closely genetically related to each other than to *L. principis-rupprechtii*.

An unrooted consensus tree showed the genetic relationships among fifteen populations (Fig. 2). Two populations, the seed orchard and Dongfanghong, were

Table 5. Estimates of the parameters *a* and *b* in the regression equation *Log* (effective number of migrants)=*a* + *b log* (geographic distance between populations), and *r*, the correlation coefficient between *Log* (effective number of migrants), and *Log* (geographic distance between populations) for two *Larix* taxa. **: $P < 0.01$.

Locus	*Larix gmelinii*			*Larix olgensis*		
	a	*b*	*r*	*a*	*b*	*r*
Pgi	1.496	-0.625	-0.249	1.796	-0.605	-0.511
Mdh-1	1.537	0.023	0.011	1.219	-0.585	-0.494
6-pgd-2	1.908	0.430	0.136	1.681	-1.347**	-0.605**
Aat-1	1.394	-0.033	-0.020	1.309	0.262	0.154
Aat-2	1.375	0.419	0.135	1.625	-0.276	-0.204
Pgm	1.026	0.404	0.243	2.169	-1.385**	-0.604**
Sdh	1.560	0.503	0.297	-	-	-
Over all loci	**1.443**	**0.027**	**0.013**	**1.843**	**-0.950****	**-0.637****

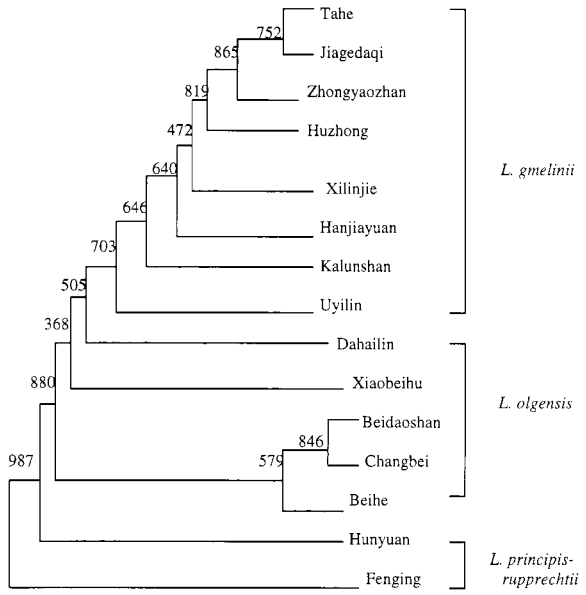

Figure 2. An unrooted consensus tree was constructed on the basis of 1000 Neighbour-Joining trees generated by bootstrap resampling (FELSENSTEIN 1989). The numbers at the forks indicated the number of times the group consisting of the populations which were to the right of that fork occurred among the trees, out of 1000 trees. Only those populations with data for all eight polymorphic loci were included.

not included in the analysis due to a lack of *Sdh* data. Populations Huanyuan and Fengning in *L. principis-rupprechtii* were separated from all investigated populations in both *L. olgensis* and *L. gmelinii* with a probability of 987/1000. Populations Beidaoshan, Changbei and Beihe in *L. olgensis* formed a subgroup that was separated from populations Xiaobeihu and Dahailin with a high probability (880/1000). Dahailin was separated from all investigated populations of *L. gmelinii* with an intermediate probability (505/1000) and from Xiaobeihu with a small probability (368 /1000), indicating a weak distinction between *L. olgensis* and *L. gmelinii*. populations Tahe, Jiagedaqi, Zhongyazhan, and Huzhong in *L. gmelinii* formed a subgroup with high probabilities (more than 819 /1000), and Xilinjie was separated from the subgroup with a small probability (472/1000). The rest populations in *L. gmelinii* were separated from the subgroup and Xilinjie with a probability of more than 640/1000. In conclusion, distinction of the three larch taxa can be generally viewed, but is very weak because of very small genetic distances among them.

DISCUSSION

The results of this genetic analysis of both cpDNA and allozymes indicate very close genetic relationships among the three taxa *L. gmelinii*, *L. principis-rupprechtii* and *L. olgensis* within China. Values of Nei's genetic distance among taxa based on allozyme markers were very small (mean $D = 0.0124$). These results suggest a very recent and restricted divergence and are in line with previous work on cpDNA variation in the genus *Larix* (TANG *et al.* 1995, KISANUKI *et al.* 1995). On the evidence of these results it would be most reasonable to regard the three taxa as subspecies rather than accord them specific status.

Further support for the hypothesis of very recent divergence among *L. gmelinii*, *L. principis-rupprechtii* and *L. olgensis* comes from the results of artificial hybridisation experiments. Crosses among these taxa show little or no hybrid vigour indicating that they are genetically very similar. In contrast hybrids between *L. olgensis* and *L. leptolepis* Gord. show significantly enhanced F_1 performance that is currently being exploited in breeding programmes (WANG & DING 1989).

Analysis of genetic distances among taxa based on

isozyme variation indicate that *L. principis-rupprechtii* is more distantly related than are *L. olgensis* and *L. gmelinii*. One possible explanation for this pattern of divergence and the origin of *L. principis-rupprechtii* and *L. olgensis* in China is that these taxa were derived from populations of *L. gmelinii* that advanced much further south than the present range during a recent period of climatic cooling. When climatic warming occurred some of these populations may have migrated to higher elevations and become isolated on the mountain ranges in the southern latitudes. Here they diverged to become *L. principis-rupprechtii* and *L. olgensis*. Meanwhile the main body of the population migrated further to the north at lower elevation and gave rise to present day populations of *L. gmelinii*.

According to this hypothesis the isolation has been sufficiently long for limited divergence in the frequency of allozyme alleles to occur, but insufficient for the abnormally slow rate of base substitution or insertion/deletion in cpDNA differences to evolve (TANG *et al.* 1995, KISANUKI *et al.* 1995). This scenario would imply that the more southerly *L. principis-rupprechtii* may have been isolated for longer than the other two taxa and this would help to explain the greater genetic divergence of *L. principis-rupprechtii* and the closer genetic similarity of *L. olgensis* and *L. gmelinii* at isozyme loci.

The overall level of isozyme variation found in these taxa (mean gene diversity $H_e = 0.11$) is similar to that found in other widespread *Larix* species (FINS & SEEB 1986, CHELIAK *et al.* 1988, LIU & KNOWLES 1991, LEWANDOWSKI & MEJNARTOWICZ 1991). There are no major differences among the Chinese taxa in the level of isozyme variation seen. This implies that severe bottlenecking of the populations has not played a role in the origin of these taxa.

The analysis of genetic structure within taxa shows very small but significant genetic differences among populations accounting for between 1 % and 2 % of total genetic variation. These levels of differentiation are on the lower end of the range (2–8 %) found in studies of other widespread larch species (CHELIAK *et al.* 1988, LIU & KNOWLES 1991, YING & MORGENSTERN 1991, LEWANDOWSKI & MEJNARTOWICZ 1990, TIMERJANOV 1997, SEMERIKOV & LASCOUX 1999, SEMERIKOV *et al.* 1999). It is interesting that this differentiation shows no relationship with geographic distance in *L. gmelinii*, while in *L olgensis* there is clear evidence of an increase in genetic differentiation with geographic distance as expected if gene flow among populations is limited and there is isolation by distance. This difference in genetic structure between the taxa could be related to the more continuously distributed nature of the *L. gmelinii* populations compared with the *L. olgensis* populations that are isolated at higher elevation on a series of mountain ranges.

Comparison of the previous analysis of chloroplast variation (TANG *et al.* 1995, KISANUKI *et al.* 1995) with the present analysis of isozyme variation demonstrates the importance of choosing a molecular technique for studying the origins of taxa that is appropriate to the time scale involved. In Chinese *Larix* the divergence times of the taxa were too recent for analysis of sequence divergence in the cpDNA to be very informative. Over this time scale however divergence at polymorphic isozyme loci is sufficiently rapid that it can provide important clues for reconstructing evolutionary history.

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