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Genetic relationship among Eurasian and American *Larix* species based on allozymes

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Genetic variation at 16 allozyme loci was studied in both American (*Larix occidentalis* Nutt., *L. laricina* (Du Roi) C. Koch, *L. lyallii* Parl.) and Eurasian (*L. sibirica* Ledeb., *L. gmelinii* Rupr., *L. olgensis* A. Henry, *L. kaempferi* (Lamb.) Carr. (= *L. leptolepis* (Sieb. et Zucc.) Endl.), *L. kamtschatica* (Rupr.) Carr. and *L. decidua* (Mill.)) larch species. Species with a limited range, such as *L. olgensis* and *L. lyallii*, had lower genetic variation than species with a wider range. Population differentiation within species was of the same order of magnitude among species. The resulting phylogeny indicates a clear separation between American and Eurasian species. This result is in agreement with recent palaeontological findings that suggest that gene flow between American and Eurasian species has been unlikely since the last glaciation.

Keywords: allozyme polymorphism, *Larix*, phylogeny.

Introduction

The genus *Larix* (Pinaceae) comprises some 10 species of deciduous trees with varying geographical importance (Bobrov, 1972). For instance, *L. sibirica* and *L. gmelinii* are a main component of the Siberian forest, whereas the natural range of *L. decidua* is mostly limited to the mountainous areas of western and central Europe. Similarly, on the American continent *L. laricina* is found over most of the Canadian boreal forests, whereas *L. lyallii* and *L. occidentalis* are confined to the Rocky Mountains and the northern part of the Cascade Range. The phylogeny and phylogeography of *Larix* is still poorly known, and recent studies based on chloroplast DNA RFLPs (Kisanuki *et al*., 1995; Qian *et al*., 1995) did not yield an easily interpretable pattern. Not too surprisingly, given the weak morphological differentiation between species and the ease of artificial hybridization, the resulting phylogenies were not congruent with classical taxonomy based on cone bract morphology. Perhaps more surprisingly, nor was the species clustering always consistent with what could be expected based on current knowledge of glacial history. Some 60 years ago Hultén showed that the forests of Siberia and Alaska were so different that they could not have merged in the Pleistocene (Hultén, 1937). This was recently confirmed by Elias *et al*. (1996), who used under-sea cores of the Bering Strait to demonstrate that Beringia, the Bering land bridge that was submerged some 11 000 years ago, was a treeless Arctic tundra during the last glaciation, particularly during its maximum 22 000–14 000 years ago. This does not preclude gene flow between the two continents, through pollen or seeds, but it certainly reduces its likelihood. Furthermore, it seems consistent with divergence estimates obtained from molecular phylogenies of broad-leaved species, that indicate a divergence during the Miocene (Qiu *et al*., 1995; Hasebe *et al*., 1998; Wen *et al*., 1998). Hence, most probably, although *Larix* might have been continuously distributed over high latitudes some million years ago (LePage & Basinger, 1995), extant American larch populations originate from glacial refugia located south of the ice sheet (Whitlock, 1995) and, consequently, differentiation between them and Siberian species would be expected. Contrary to that expectation, *L. laricina* and *L. occidentalis* clustered with *L. sibirica* in Qian *et al*.'s (1995) phylogenetic analysis (the variation was too limited to draw any conclusion from Kisanuki *et al*.'s (1995) study).

The genetic variation at 16 allozyme loci in three North American and seven Eurasian *Larix* species was analysed. The genetic variation within species was assessed, the genetic distance between species estimated and a phylogenetic tree constructed. The use of allozyme data for phylogenetic reconstruction has a weak resolution compared with RFLP or sequence data because

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of the limited number of loci available. However, the possibility of analysing a large number of individuals makes this approach quite reasonable for a first, large scale, investigation (Qiu *et al.*, 1995).

Materials and methods

Sampling

The species investigated, the number of populations per species and their locations are given in Table 1. Figure 1 gives the distribution range of all *Larix* species. Seed samples were obtained from four populations of *L. occidentalis*, three populations of *L. laricina and* one population of *L. lyallii*. Each of the eight populations was characterized by seeds from at least nine trees. These seeds were planted and grown into seedlings, which were then used for protein electrophoresis. Samples from 15 populations of *L. sibirica*, six of *L. gmelinii*, three of *L. olgensis*, two of *L. decidua* and one each of *L. kaempferi* (= *L. leptolepis*) and *L. kamtschatica* were analysed. In Eurasian *Larix* species adult needles and seedlings were analysed. A full description of the Eurasian samples is given in Semerikov *et al.* (1998).

Allozymes

Twelve enzyme systems representing 16 protein loci were examined: glutamic-oxaloacetic transaminase (GOT, EC 2.6.1.1, two loci), glutamate dehydrogenase (GDH EC 1.4.1.2), isocitrate dehydrogenase (IDH, EC 1.1.1.42), diaphorase (DIA, EC 1.8.1.4), phosphoglucoisomerase (PGI, EC 5.3.1.9, two loci), phosphogluconate dehydrogenase (6-PGD, EC 1.1.1.44), shikimate dehydrogenase (SKDH, EC 1.1.1.25), glucose-6-phosphate dehydrogenase (G6PDH, EC 1.1.1.49), phosphoglucomutase (PGM, EC 5.4.2.2, two loci), superoxide dismutase (SOD, EC 1.15.1.1, two loci), fluorescent esterase (EST-F, EC 3.1.1.1) and formate dehydrogenase (FDH, EC 1.2.1.2). Mendelian segregation for these allozymes was shown in *Larix* species by Cheliak & Pitel (1985), Fins & Seeb (1986) and Lewandowski & Mejnartowicz (1990). In GOT and PGD, third and second zones of electrophoretic mobility, respectively, were observed, but they were not considered here as their reproducibility was weak. Finally, homology of the loci among species was established on the basis of the common alleles.

Protein extraction and polyacrylamide gel electrophoresis were carried out according to Shurkhal *et al.* (1992) and Semerikov & Matveev (1995). Needles (150 mg) and insoluble PVP (150 mg) were ground with liquid nitrogen in a mortar, and mixed with extraction buffer. The extraction buffer was composed of 1 M sucrose, 5.7 mM L-ascorbic acid, 8.3 mM DL-cysteine, 0.02 M dithiothreitol, and 1.5 mM aminocapronic acid dissolved in electrode buffer diluted 1 : 1.7. One mL Tween-80 was added to 100 mL of this solution and after 2–14 h at 4°C the mixture of extraction buffer, ground needles and PVP was filtered through nylon filters. The supernatant was centrifuged after adding a small amount of CCl_4 in order to improve the centrifugation of the supernatant from small tissue particles. Extracts from seedlings were prepared by grinding needles in 0.15 mL extraction buffer and centrifuging with CCl_4. Electrophoresis in 7% polyacrylamide gel in the tris-EDTA-borate system was conducted. The electrode buffer (pH 8.0) was: 116 mM tris, 3.5 mM EDTA, 161 mM boric acid and the gel buffer (pH 8.6) was 118 mM tris, 3.5 mM EDTA, 118 mM boric acid. Histochemical staining was carried out using standard methods (Harris & Hopkinson, 1976).

Data analysis

The within-species genetic variation was analysed using GENEPOP (version 2; Raymond & Rousset, 1995) and FSTAT (version 1.2; Goudet, 1995).

Hardy–Weinberg expectations The fit of genotypic distributions to Hardy–Weinberg expectations was tested by the exact test proposed by Guo & Thompson (1992). The overall significance for each locus was estimated by Fisher's combined probability test (Fisher, 1954). According to this test, if P-values are obtained for each locus separately under the null hypothesis, then $2\sum_{i=1}^{n}\log(P_i)$ is distributed according to a χ^2 distribution with n degrees of freedom, where n is the number of loci and P_i is the P-value for the ith locus. Heterozygote deficiencies or excesses were tested using an exact test (Rousset & Raymond, 1995).

Population differentiation Wright's F-statistics, F_{IS}, F_{IT} and F_{ST}, were estimated according to Weir & Cockerham (1984) and a 95% confidence interval was estimated by bootstrapping over loci. F_{IS} and F_{IT} are the correlations between two uniting gametes relative to the subpopulation and relative to the total population, respectively, and F_{ST} is the correlation between two gametes drawn at random from each subpopulation and measures the degree of genetic differentiation of subpopulations (Nei, 1987).

Phylogeny The software PHYLIP 3.5 (Felsenstein, 1993) was used for phylogenetic analysis. Following Hillis *et al.*'s (1996) recommendations we used Cavalli-Sforza & Edwards's (1967) genetic distance (other distances provided by the program GENDIST give roughly the same result). The Fitch–Margoliash method (Fitch & Margoliash, 1967) was used for tree building

Table 1 Location and description of the *Larix* populations used in the present study

Pop.	Collection site	Lat., long.	*N*	Material analysed
L. sibirica Ledeb.				
1	Vizhaj River, near Ivdel	61°10′N, 60°20′E	47	Needles of mature trees
2	Severka, near Ekaterinburg	56°50′N, 60°15′E	50	Needles of different-aged trees
3	Sob River	67°00′N, 65°40′E	96	35 open-pollinated trees
4	Poluy River	66°10′N, 68°30′E	50	19 open-pollinated trees
5	Iremel Mt.	54°30′N, 56°40′E	48	Needles of different-aged trees
6	Ajat railway station	57°05′N, 60°15′E	33	Needles of mature trees
7	Ongudai (Altai Mts)	51°00′N, 89°00′E	48	Seedling needles (1)
8	Numgi-Yaha River	66°43′N, 73°15′E	48	Needles of mature trees
9	Hadutte-Yaha River	67°27′N, 77°30′E	45	Needles of mature trees
10	Hadita-Yaha River	67°00′N, 69°50′E	44	11 open-pollinated trees
11	Yada-Yahoda-Yaha River	67°05′N, 71°40′E	52	24 open-pollinated trees
12	Ust-Kut (Upper Lena River)	56°50′N, 105°40′E	50	Needles of mature trees
13	Mergen river	51°40′N, 95°40′E	60	Seedling needles†
14	Chadan settlement	51°10′N, 92°00′E	31	Seedling needles†
25	Pangodi settlement	66°10′N, 74°00′E	32	Seedling needles†
L. gmelinii Rupr.				
16	Severobaikalsk settlement	55°40′N, 109°24′E	50	Needles of mature trees
17	Severomuysk settlement	56°10′N, 113°40′E	50	Needles of mature trees
18	Chara settlement	56°50′N, 118°20′E	48	Needles of different-aged trees
19	Nerungri settlement	56°44′N, 124°42′E	48	Needles of different-aged trees
20	Skovorodino railway station	54°00′N, 124°00′E	48	Needles of different-aged trees
21	Urgal settlement	51°00′N, 132°50′E	48	Seedling needles†
L. decidua Mill.				
22	Bluhnbachtal (Austria)	47°28′N, 13°05′E	50	Seedling needles†
23	Muran (Slovakia)	49°07′N, 20°04′E	50	Seedling needles
L. olgensis A. Henry				
24	Olga Bay	43°40′N, 135°15′E	50	Needles of mature trees
25	Kavalerovo settlement	44°05′N, 135°20′E	51	Needles of different-aged trees
26	Shufan basaltic plateau	43°25′N, 131°20′E	50	Seedling needles†
L. kaempferi (Lamb.) Carr‡				
27			51	Needles of mature trees
L. kamtchatica (Rupr.) Carr.				
28		47°30′N, 142°40′E	50	Needles of mature trees
L. laricina (Du Roi) C. Koch				
29	Five Fingers, New Brunswick	47°52′N, 67°36′W	50	Seeds from at least nine trees
30	Kingsclear, New Brunswick	45°97′N, 66°80′W	54	Seeds from at least nine trees
31	Burke Lake Bog, MI, USA	42°48′N, 84°22′W	18	Seeds from at least nine trees
L. occidentalis Nutt.				
32	Christina Lake (950 m) BC	49°05′N, 118°18′W	52	Seeds from at least nine trees
33	Nakusp (850 m) BC	50°14′N, 117°42′W	48	Seeds from at least nine trees
34	Seedlot 05080 (1219 m)	50°31′N, 115°53′W	47	Seeds from at least nine trees
35	Seedlot 27260 (1379 m)	49°27′N, 119°18′W	37	Seeds from at least nine trees
L. lyallii Parl.				
36	Monica Meadows (2195 m)	50°25′N, 116°42′W	29	Seeds from at least nine trees

†Grown from seeds of bulked collection. ‡60-year-old artificially generated stand in south of Sakhalin island.

(procedure KITCH). Bootstrap values were calculated by generating 500 samples with the program SEQBOOT, which were used as input for the program KITCH. A consensus tree was obtained from these 500 bootstrap replicates with the program CONSENSE. The tree was drawn using TREEVIEW (Page, 1996).

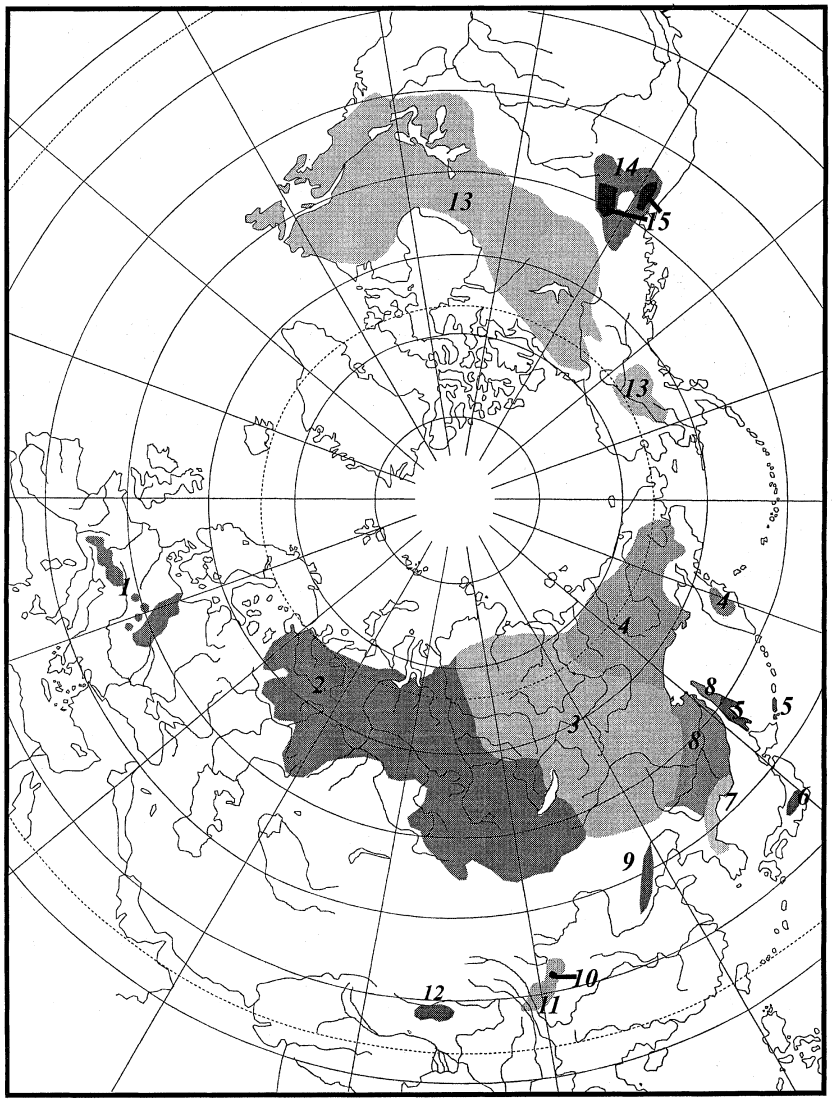

Fig. 1 Distribution of *Larix* taxa. (1) *L. decidua*; (2) *L. sibirica*; (3) *L. gmelinii*; (4) *L. cajanderi*; (5) *L. kamtschatica*; (6) *L. kaempferi* (= *L. leptolepis*); (7) *L. olgensis*; (8) putative hybrid *L. gmelinii* × *L. olgensis*; (9) *L. principis rupprechtii*; (10) *L. mastersiana*; (11) *L. potaninii*; (12) *L. griffithiana*; (13) *L. laricina*; (14) *L. occidentalis*; (15) *L. lyallii*. Adapted from Bobrov (1972).

Results

The locus *G6pdh* of *L. laricina* gave an unusual zymogram pattern: in addition to the bands common for other *Larix* taxa some additional indistinct bands were also produced. Similar problems were encountered by Cheliak & Pitel (1985) who observed "non-genetic modification" of one of the alleles after storage of the seeds. Diffuse bands were found not only on zymograms of megagametophytes, but also when analysing seedlings, embryos and needles of young plants. Because the proteins in the leaves of the young plants could not have been produced before the seed germination, we concluded that the modification of the *G6pdh* enzyme was not produced by seed storage. *G6pdh* was not used in the phylogenetic analysis. A high frequency of 'null-alleles' was discovered at the *Skdh* locus of *L. occidentalis*, and their frequency was estimated from the homozygote frequency assuming Hardy–Weinberg equilibrium and using the EM algorithm (Lange, 1997).

Genetic variability, Hardy–Weinberg and within-species differentiation

The mean number of alleles, the percentage of polymorphic loci and the observed and expected heterozygosities are given in Table 2 (the allozyme frequency table is available from the first author). All three quantities were lowest in species with more restricted distribution areas, such as *L. lyallii* and *L. olgensis*. A significant heterozygote deficit was found at loci *G6pdh* ($P = 0.035$), *Skdh* ($P < 0.0001$) and *Idh* ($P < 0.0001$) in *L. occidentalis*, *Pgm-B* ($P = 0.0091$) in *L. laricina* and *Dia* ($P = 0.0025$) in *L. lyallii*. The deficit of heterozygotes at *Skdh* can be explained by the incorrect scoring of null-allele heterozygotes as homozygotes of

Table 2 Genetic variability at 16 loci in American and Eurasian populations of larches (standard errors in parentheses)†

Population	Mean sample size per locus	Mean no. of alleles per locus	Percentage of polymorphic loci‡	Mean heterozygosity	
				Observed	Expected§
L. laricina					
Five Fingers	50.0 (0.0)	1.7 (0.2)	53.3	0.073 (0.042)	0.079 (0.042)
Kingsclear Nursery	54.0 (0.0)	1.7 (0.3)	40.0	0.102 (0.047)	0.100 (0.047)
Burke Lake Bog	17.9 (0.1)	1.3 (0.2)	26.7	0.052 (0.040)	0.048 (0.036)
L. occidentalis					
Christina Lake	51.4 (0.3)	1.5 (0.2)	43.8	0.115 (0.044)	0.161 (0.055)
Nakusp	47.9 (0.1)	1.6 (0.2)	43.8	0.146 (0.052)	0.170 (0.055)
05080	47.6 (0.2)	1.6 (0.2)	43.8	0.112 (0.043)	0.130 (0.050)
27260	36.9 (0.1)	1.5 (0.2)	43.8	0.121 (0.047)	0.140 (0.050)
L. lyallii					
Monica Meadows	28.8 (0.1)	1.4 (0.1)	37.5	0.082 (0.040)	0.094 (0.042)
L. sibirica					
Ongudai	48.0 (0.0)	1.8 (0.2)	56.3	0.117 (0.043)	0.130 (0.049)
L. gmelinii					
Urgal	47.5 (0.2)	2.5 (0.3)	68.8	0.154 (0.049)	0.155 (0.049)
L. decidua					
Bluhnbachtal	63.8 (4.0)	2.0 (0.3)	50.0	0.125 (0.051)	0.126 (0.052)
L. olgensis					
Olga Bay	47.6 (2.3)	1.4 (0.2)	37.5	0.080 (0.036)	0.082 (0.037)
L. kaempferi					
Sakhalin	48.3 (2.6)	1.8 (0.2)	56.3	0.122 (0.041)	0.136 (0.047)

†*G6pdh* was scored in all populations, except *L. laricina*. Data of Eurasian populations take into account the *Fdh* locus.
‡A locus is considered polymorphic if more than one allele is detected.
§Unbiased estimate (see Nei, 1987).

active alleles. However, heterozygote deficit at other loci cannot be attributed to the presence of null-alleles with certainty, because these were not observed among the genotyped individuals. Even if this explanation seems to be the most plausible, other explanations such as the Wahlund effect cannot *a priori* be ruled out. Wright's *F*-statistics for the different species are given in Table 3. With F_{ST} values lower than 0.1, population differentiation was generally not very pronounced. Population differentiation was, however, more conspicuous in *L. occidentalis* and *L. sibirica* (0.100 and 0.079) than in *L. laricina* and *L. gmelinii* (0.026 and 0.021).

Phylogeny

A consensus tree was constructed from 500 Fitch–Margoliash dendrograms (Fig. 2) and the bootstrap method was used to evaluate the support for the different groupings. Because a rather limited number of loci was analysed (only 15), results should be interpreted with care even if some of the groupings are strongly supported. The American larches are clearly separated from the Eurasian ones, the Japanese larch being more closely related to the latter than to the former. *Larix occidentalis* and *L. lyallii* always grouped together, regardless of the distance or tree-building method used and the consensus tree constructed on the data, after excluding the *Skdh* and *Idh* loci, gave a similar topology to that obtained when these loci were included (results not shown). In both phylogenetic trees American larches are grouped together, separately from other species.

Discussion

Genetic variability and population differentiation

The genetic variation found in the *L. laricina* populations in the present study (H_{exp} = 0.048–0.100) was lower than those reported previously (H_{exp} = 0.220, Cheliak *et al.*, 1988; H_{exp} = 0.151, Ying & Morgenstern, 1991; H_{exp} = 0.104, Liu & Knowles, 1991). This difference may be a consequence of the location of the populations at the eastern limit of the distribution

Table 3 Wright's *F*-statistics at all loci in *Larix laricina* (three populations), *L. occidentalis* (four populations), *L. sibirica* (15 populations) and *L. gmelinii* (six populations)

Locus	F_{IS}	F_{IT}	F_{ST}
L. laricina			
Got-B	0.004 (0.003)†	0.000 (0.005)	−0.004 (0.009)
Dia	−0.179 (0.182)	−0.164 (0.150)	0.015 (0.068)
Pgi-B	0.018 (0.036)	0.021 (0.001)	0.005 (0.034)
6-Pgd	−0.020 (NA)	0.012 (NA)	0.031 (NA)
Skdh	0.100 (0.103)	0.110 (0.111)	0.011 (0.010)
Pgm-A	0.002 (0.003)	−0.009 (0.001)	−0.010 (0.002)
Pgm-B	0.070 (0.241)	0.096 (0.217)	0.038 (0.031)
Est-F	−0.008 (0.008)	−0.015 (0.021)	−0.007 (0.027)
Fdh	−0.083 (0.037)	−0.027 (0.012)	0.054 (0.025)
Mean	0.003	0.029	0.026
	(0.023–0.107)‡	(−0.042–0.124)	(0.012–0.043)
L. occidentalis			
G6pdh	0.092 (0.144)	0.205 (0.114)	0.132 (0.094)
6-Pgd	−0.137 (0.078)	−0.096 (0.073)	0.037 (0.041)
Skdh	0.784 (0.127)	0.844 (0.146)	0.204 (0.122)
Pgm-A	0.140 (0.070)	0.258 (0.126)	0.142 (0.157)
Pgm-B	0.022 (0.075)	0.019 (0.076)	−0.003 (0.008)
Est-F	0.133 (0.078)	0.225 (0.133)	0.101 (0.094)
Idh	0.450 (0.046)	0.493 (0.035)	0.081 (0.073)
Mean	0.178	0.260	0.100
	(−0.005–0.405)	(0.072–0.473)	(0.063–0.136)
L. sibirica			
Got-A	0.048 (0.046)	0.148 (0.060)	0.104 (0.031)
Got-B	0.050 (0.043)	0.103 (0.051)	0.056 (0.022)
Dia	0.035 (0.073)	0.118 (0.058)	0.087 (0.031)
Pgi-B	−0.004 (0.037)	0.039 (0.041)	0.043 (0.018)
G6pdh	0.037 (0.061)	0.119 (0.057)	0.085 (0.018)
6-Pgd	0.002 (0.051)	0.059 (0.051)	0.057 (0.021)
Skdh	0.042 (0.093)	0.096 (0.078)	0.057 (0.018)
Pgm-A	−0.046 (0.032)	−0.011 (0.029)	0.033 (0.010)
Sod-A	0.100 (0.050)	0.148 (0.049)	0.054 (0.014)
Est-F	0.131 (0.045)	0.216 (0.041)	0.098 (0.019)
Mean	0.059	0.134	0.079
	(0.020–0.093)	(0.081–0.171)	(0.058–0.091)
L. gmelinii			
Got-A	−0.028 (0.009)	−0.025 (0.008)	0.003 (0.004)
Got-B	0.344 (0.195)	0.391 (0.222)	0.057 (0.035)
Dia	0.003 (0.058)	0.045 (0.053)	0.043 (0.023)
Pgi-B	0.062 (0.081)	0.063 (0.081)	0.002 (0.007)
G6pdh	0.012 (0.063)	0.009 (0.061)	−0.003 (0.003)
6-Pgd	0.247 (0.177)	0.259 (0.188)	0.012 (0.013)
Skdh	−0.001 (0.073)	0.014 (0.067)	0.014 (0.015)
Pgm-A	0.046 (0.055)	0.055 (0.056)	0.009 (0.009)
Pgm-B	0.147 (0.169)	0.150 (0.162)	0.005 (0.008)
Sod-A	0.036 (0.014)	0.054 (0.044)	0.019 (0.008)
Est-F	−0.026 (0.038)	0.007 (0.042)	0.033 (0.028)
Mean	0.024	0.044	0.021
	(0.001–0.052)	(0.026–0.064)	(0.009–0.031)

†Standard deviation in parentheses.
‡95% bootstrap confidence interval.

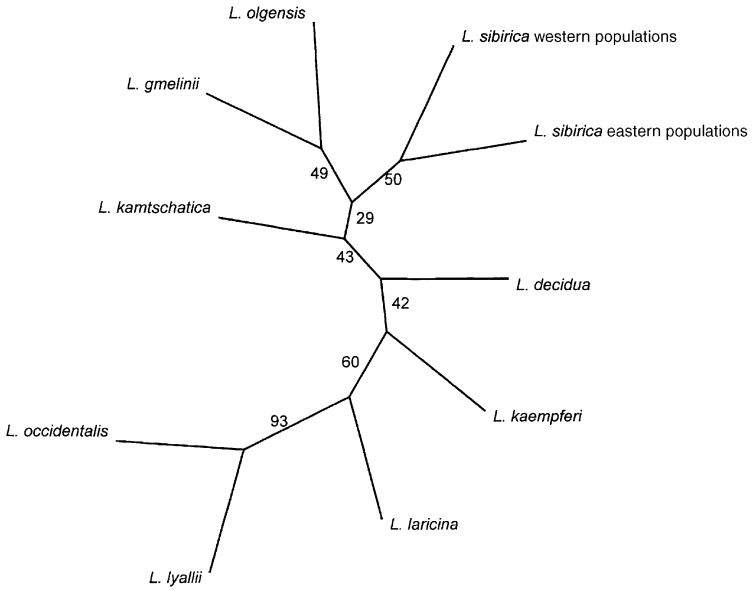

Fig. 2 Unrooted consensus Fitch–Margoliash dendrogram, based on allele frequencies at 15 allozyme loci, illustrating the genetic distances between *Larix* species. Figures give percentage bootstrap support for 500 replicate runs. The Cavalli-Sforza and Edwards genetic distance was used.

range. A different picture might have been obtained had populations from the western part of the range been added to the present study. The low genetic variation observed in *L. lyallii* and *L. olgensis* could be explained by the narrowness of their ecological niche. *Larix lyallii* grows on poor and rocky sites, in cold, snowy and generally moist climates at high altitudes (upper subalpine to timberline ecotone) (Schmidt, 1995). Similarly, *L. olgensis* is found in small populations on rocky, steep, north-facing slopes. In either case, small population sizes and narrow ecological niches could easily be the cause of the low genetic diversity. Genetic differentiation was lower in *L. laricina* and *L. gmelinii* than in *L. occidentalis* and *L. sibirica*. The agreement with previous estimates is generally good (*L. occidentalis*, Fins & Seeb, 1986), except for *L. laricina* for which Cheliak *et al.* (1988), using populations sampled over a larger range, found a F_{ST} value of 5%. As for genetic diversity estimates, the discrepancy may simply be the consequence of the limited geographical range of our samples. Indeed Ying & Morgenstern (1991) also observed a low F_{ST} among populations restricted to New Brunswick.

Phylogeny

The main results of our study are: (i) the clear separation of American and Eurasian larches; (ii) the consistent grouping of *L. laricina* and *L. occidentalis*; and (iii) the lack of differentiation among Eurasian species. As was expected, based on recent palaeontological results (Elias *et al.*, 1996) showing that Beringia was a tundra, and thus *a priori* not conducive to the migration of trees such as larch, American and European larch species are clearly genetically differentiated, based on variation at isozyme loci. Furthermore, *L. lyallii* and *L. occidentalis* form a single and separated group, regardless of the genetic distance and tree building method used. The present range of these two species is not in contact with other species and they probably had refugia south of their present range during the last glaciation (Whitlock, 1995). In fact, the divergence between these two species and Eurasian ones is of the same order of magnitude as that observed between several eastern Asia-eastern North America disjunct broad-leaved species based on isozyme loci (table 6 in Qiu *et al.*, 1995). The corresponding divergence time, using Nei's method (Nei, 1987, p. 237), is ≈ 3–4 Myr. The differentiation between *L. laricina* and Eurasian species is somewhat less pronounced, which was expected, considering the present distribution range of the species. In that case the divergence could have occurred during the Pleistocene. An even less pronounced difference would probably have been observed had samples from Alaska been included in the study. These results might seem to conflict with the phylogeny based on bract morphology, for which *L. kaempferi* clusters with *L. lyallii* and *L. occidentalis*, whereas *L. laricina* clusters with other Eurasian species (LePage & Basinger, 1995). However, the two can easily be reconciled, if one assumes that allele frequencies at isozyme loci reflect recent demographic events, whereas bract morphology reflects more ancient history. Furthermore, LePage & Basinger (1995) observe that short-bract species commonly occupy low-altitude habitats, whereas long-bract species are found at high altitudes. Bract morphology would thus be related to fitness and evolve slowly. The fossil record from the high Arctic indicates that larches could have

dispersed between North America and northern Eurasia along the Beringia corridor some 40 Myr ago (LePage & Basinger, 1995). Our data do not allow us to draw any conclusions on the direction of dispersal, although migration from Eurasia into North America seems more likely, because *L. griffithiana* is apparently the oldest of the extant taxa of *Larix* (Sukaczew, 1924). No strong inference can be made within Eurasian species, the differentiation between them being too weak. We note, however, that *L. kaempferi* differs from others, notably *L. decidua*. Although they used 66 RFLP probe–enzyme combinations, Qian *et al*. (1995) observed no difference between *L. decidua*, *L. kaempferi* and *L. gmelinii*, but they detected differences between this group and *L. sibirica*. However, the overall differentiation was very small, the maximum number of nucleotide substitutions per site (*d*) being only 0.0096. In larch, mitochondrial DNA is maternally inherited, and thus dispersed only through seeds (Deverno *et al*., 1993; D. Prat, pers. comm.). A more pronounced genetic differentiation pattern is therefore expected. In a follow-up study the same populations, together with additional ones, will be analysed at mitochondrial DNA markers.

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