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Recolonization and radiation in *Larix* (Pinaceae): evidence from nuclear ribosomal DNA paralogues

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Abstract

Gene paralogy frequently causes the conflict between gene tree and species tree, but sometimes the coexistence of a few paralogous copies could provide more markers for tracing the phylogeographical process of some organisms. In the present study, nrDNA ITS paralogues were cloned from all but one species of *Larix*, an Eocene genus having two sections, *Larix* and *Multiserialis*, with a huge circumboreal distribution and an Eastern Asia–Western North America disjunction, respectively. A total of 96 distinct clones, excluding five putative pseudogenes or recombinants, were obtained and used in the gene genealogy analysis. The clones from all Eurasian species of section *Larix* are mixed together, suggesting that recolonization and recent morphological differentiation could have played important roles in the evolution of this section. In contrast, the species diversification of the Eurasian section *Multiserialis* may result from radiation in the east Himalayas and its vicinity, considering extensive nrDNA founder effects in this group. Our study also suggests that the distribution pattern analysis of members of multiple gene family would be very useful in tracking the evolutionary history of some taxa with recent origin or rapid radiation that cannot be resolved by other molecular markers.

Keywords: biogeography, founder effect, *Larix*, radiation, recolonization, nrDNA ITS paralogues

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Introduction

The climatic cooling over the past 65 million years, especially climate oscillations in the Quaternary, has had a significant influence on the geographical distribution and diversification of plant species (Comes & Kadereit 1998; Hewitt 2000). Many arctic or boreal plants were assumed to be derived from ancestral stocks that occurred on high mountains to the south in both Asia and North America (Hultén 1937; Weber 1965; Hedberg 1992; Murray 1995). This hypothesis is strongly supported by recent phylogeographical studies with molecular markers, which have provided new insights into locations of glacial refugia and routes of postglacial expansion of the boreal species (Abbott *et al*. 1995, 2000; Tremblay & Schoen 1999; Gugerli *et al*. 2001; Abbott & Brochmann 2003). However, most of these studies focused on the population process of a single species, such as silver fir (Konnert & Bergmann 1995), common beech (Demesure *et al*. 1996), Scots pine (Sinclair *et al*. 1999) and ponderosa pine (Johansen & Latta 2003). Similar studies at higher taxonomic levels and in a broad range are very essential to reveal the history and evolution of the arctic or boreal flora. Also, a good understanding of diversification process of a flora component, such as a genus, will provide the baseline for drawing generalisations about the diversification as a whole (Anthony Verboom *et al*. 2003).

Larix, a main component of the boreal forest, is one of the four Pinaceae genera with a very large range (Farjon 1990). However, it is a young genus with an Eocene origin (LePage & Basinger 1991, 1995; Wang *et al*. 2000). This genus contains about 15 species (Fu *et al*. 1999), 10 of which are widely accepted and classified into two morphologically distinct groups, i.e. the sections *Larix* and *Multiserialis* (Farjon 1990). Section *Larix*, characterized by short bract scales and nonpendulous branchlets, has a circumboreal distribution, including occurrence at or near the limit of trees in North America and Siberia. In contrast, section *Multiserialis*, with long bract scales and pendulous branchlets, is restricted to montane and subalpine habitats, showing a typical Eastern Asia–Western North America disjunction. It is interesting that all Asian species of section *Multiserialis*

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are centralized in the Himalayan–Hengduan Mountains, a diversity hot spot that has many groups with adaptive radiation, such as *Pedicularis* and *Primula*. Among them, *Larix potaninii* has differentiated into four varieties. Several recent molecular studies showed that genetic differentiation is low among these long-bracted larch species (Semerikov *et al*. 2003; Wei & Wang 2003, 2004) but, surprisingly, founder effects have been observed in the nrDNA evolution of the four varieties of *L. potaninii* (Wei *et al*. 2003). The short evolutionary history, wide distribution, rapid morphological diversification and molecular evidence of *Larix* mentioned above lead us to deduce that recolonization and radiation might have occurred in the evolution of the genus. Further molecular studies are obviously needed to explore this deduction.

Nuclear ribosomal DNA (nrDNA) occurs as tandemly repeated units at one or several loci with copy numbers varying from several hundreds to thousands per haploid genome. In contrast to the evolution of single genes, members of the rDNA gene family do not evolve independently, but in a concerted manner with the underlying molecular process of gene conversion and unequal crossing over (Muir *et al*. 2001). As a part of the rDNA unit, the internal transcribed spacer (ITS) region has developed into a ubiquitous tool for phylogenetic reconstruction at lower taxonomic levels in angiosperms as a result of the rapid concerted evolution within and among component subunits, fast evolution rate, and short length and availability of universal primers (Baldwin *et al*. 1995). This region, however, is much longer in gymnosperms (Liston *et al*. 1996; Maggini *et al*. 1998), especially Pinaceae (≈ 1550–3660 bp), and generally has extensive intra- and inter-genomic variation in both length and sequence (Karvonen & Savolainen 1993; Quijada *et al*. 1998; Gernandt *et al*. 2001). The high heterogeneity of gymnospermous ITS region significantly influences its phylogenetic use. Fortunately, the region has faster concerted evolution in *Larix* than in some old genera of Pinaceae such as *Pinus* and *Picea* (Wei *et al*. 2003), making it possible and relatively easy to obtain a good sampling of different paralogues for a robust phylogenetic analysis. In this study, we cloned and sequenced the nrDNA ITS region from all but one species of *Larix* to investigate its intra- and inter-genomic variation in the young conifer genus. The gene genealogy obtained from phylogenetic analysis of ITS paralogues was further used to trace the evolutionary history and phylogeography of larch species.

Materials and methods

Plant materials

All recognized species and most varieties of *Larix* (Farjon 1990; Fu *et al*. 1999) were sampled except *Larix lyallii*, a long bract species endemic to subalpine regions of the Cascade and Rocky Mountains of North America. *Larix lyallii* is a close relative of *Larix occidentalis*, considering the morphological similarities, geographical proximity, interspecific gene flow (Carlson & Theroux 1993) and the identical ITS sequence (AF041346–AF041347) shared by the two species (Gernandt & Liston 1999); it has been considered as the sister species or even a subspecies of *L. occidentalis* (Nadeem *et al*. 2003). Following *Flora of China* (Fu *et al*. 1999), here we keep *Larix olgensis* and *Larix speciosa* as two independent species. The ITS sequences of *Pseudotsuga* species were retrieved from GenBank (AF041350, AF041353). The origins of materials are shown in Table 1. Voucher specimens have been deposited in the herbarium of Institute of Botany, Chinese Academy of Sciences (Beijing, China).

DNA extraction, gene amplification, cloning and sequencing

Total DNA was extracted from silica gel-dried needles using the cetyltrimethylammonium bromide (CTAB) method, following the protocol of Rogers & Bendich (1988), and used as a template in a polymerase chain reaction (PCR) amplification. The nrDNA ITS region was amplified using primers ITS1N (5′-GTCGTAACAAGGTTTCCGTAGG-3′) on the 18S gene and ITS4 of White *et al*. (1990). The PCR amplification was conducted in a Peltier Thermal Cycler (PTC-200; MJ Research, MA), with a reaction volume of 25 μL containing 5–50 ng of DNA template, 6.25 pmol of each primer, 0.2 mM of each dNTP, 2 mM $MgCl_2$ and 0.75 Units of *Taq* DNA polymerase (TakaRa Biotech Co., Dalian, China). The PCR cycles were as follows: 1 cycle of 4 min at 70 °C, 4 cycles of 40 s at 94 °C, 20 s at 52 °C, and 150 s at 72 °C, followed by 36 cycles of 20 s at 94 °C, 20 s at 52 °C, and 150 s at 72 °C, with a final extension step for 10 min at 72 °C. The PCR products were separated by 1.5% agarose gel electrophoresis. The only strong band was excised in order to separate the target product from nonspecific PCR products with very low molecular weight and purified using the GFX PCR DNA and Gel Band Purification Kit (Amersham Biosciences, Buckinghamshire). The purified PCR products were then cloned with the pGEM-T Easy Vector System II (Promega, WI).

For each taxon, at least 30 clones with potential correct insertion (determined by digestion with *Eco*RI) were screened by comparing restriction fragments of *Msp*I, *Hae*III and *Hin*fI in order to find different kinds of clones. All distinct clones, designated as the abbreviation of taxon name plus clone number, were sequenced with the two PCR primers and several internal primers (Fig. 1), i.e. LITS3 (5′-CTTCTTGCCTCGAGATTTCC-3′), ITS1R1 (5′-CATAACAAGCACACCCATCAC-3′), ITS1R2 (5′-CCTCGTGCAAGACAAAGCAC-3′), and P2N (5′-GAGAGCCGAGATATCCGTTG-3′), using the ABI Prism Bigdye Terminator Cycle Sequencing Ready Reaction Kit

Table 1 Sources of materials used in the present study

Taxa (abbreviation)	Sources (individuals)	Clone no.*	GenBank accession no.
Eurasian *Larix*			
Sect. *Multiserialis*			
L. griffithii (*Gri*)	Linzhi/Gongbu/Linzhi, Xizang, China (3)	1, 4, 5/2, 3, 9/13, 34, 35	AY523413–AY523418; AY603159–AY603161
L. mastersiana (*Mas*)	Wolong Natural Reserve, Sichuan, China (1)	1, 2, 5, 10, 25	AY523419–AY523423
L. potaninii var. *australis* (*Aus*)	Lijiang, Yunnan, China (1)	1, 2, 18, 20, 7, 17	AY188526–AY188529; AY188550–AY188551
L. potaninii var. *chinensis* (*Chi*)	Qinling, Shaanxi, China (1)	1, 5, 9, 12, 26, 27	AY188530–AY188534; AY188552
L. potaninii var. *himalaica* (*Him*)	Jilong, Xizang, China (1)	5, 7, 9, 13, 18, 22, 14	AY188535–AY188540; AY188553
L. potaninii var. *potaninii* (*Pot*)	Kangding, Sichuan, China (1)	1, 2, 3, 5, 8, 10, 16	AY188541–AY188547
L. speciosa (*Spe*)	Jianchuan, Yunnan, China (3)	3, 4, 6, 9, 18, 20, 25/7, 8, 10, 23/9, 13, 16, 17, 18, 19, 23	AY523424–AY523430; AY603162–AY603172
Sect. *Larix*			
L. decidua (*Dec*)	Campus of Michigan State University, USA (1)	2, 8, 12, 14, 18	AY523431–AY523435
L. gmelinii var. *gmelinii* (*Gme*)	Botanic Garden, Institute of Botany, Beijing (1)	5, 12	AY523436–AY523437
L. gmelinii var. *principis-rupprechtii* (*Pri*)	Wolong Natural Reserve, Sichuan, China (1)	4, 8, 12, 20	AY523438–AY523441
L. kaempferi (*Kae*)	Cultivated in Linzhi, Xizang, China (1)	1, 2, 6, 15, 24, 31	AY523442–AY523447
L. olgensis (*Olg*)	Botanic Garden, Institute of Botany, Beijing (1)	19, 26	AY523448–AY523449
L. sibirica (*Sib*)	Altai Mountains, Xinjiang, China (3)	1, 4, 9, 15/2, 3, 4, 24/2, 4, 6, 18, 23	AY523450–AY523453; AY603173–AY603181
North American *Larix*			
Sect. *Multiserialis*			
L. occidentalis (*Occ*)	Washington State, USA (1)	1, 2, 4, 5, 35	AY523454–AY523458
Sect. *Larix*			
L. laricina (*Lar*)	Aboretum of Shenyang, Liaoning, China (1)	1, 2, 6, 18, 19, 30	AY188548–AY188549; AY523459–AY523462
Pseudotsuga species			
P. menziesii	Gernandt & Liston (1999)	Direct sequencing	AF041353
P. sinensis	Gernandt & Liston (1999)	Direct sequencing	AF041350

*Clones from different individuals of the same taxon are separated by slants.

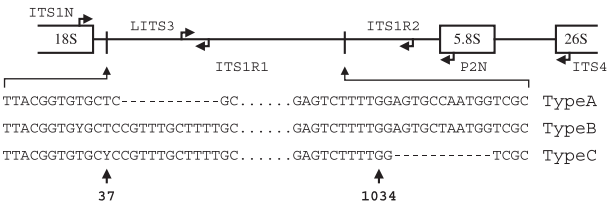

Fig. 1 The internal transcribed spacer (ITS) region of *Larix* showing primer positions and directions and the three different sequence types. Numbers below the vertical arrows indicate base positions in the aligned ITS dataset of the ingroup (*Larix*).

(Applied Biosystems, Foster City, CA). After purification through precipitation with 95% EtOH and 3 M NaAc (pH 5.2), the sequencing reaction products were applied to an ABI 377 automatic sequencer (PE Applied Biosystems, Inc., CA).

Data analysis

Sequence alignments were made with CLUSTAL X (Thompson *et al*. 1997) and BIOEDIT version 5.09 (Hall 1999) and refined manually. The alignments are available from the authors upon request. MEGA version 2.1 (Kumar *et al*. 2001) was applied to calculate GC content and genetic distance according to Kimura's two-parameter model (Kimura 1980). The aligned data set of the ITS region was analysed with the maximum parsimony method using PAUP version 4.0 (Swofford 1998). Two *Pseudotsuga* species, *P. menziesii* and *P. sinensis*, were used as outgroups because the sister relationship between *Pseudotsuga* and *Larix* was supported by most previous studies (Price *et al*. 1987; Wang *et al*. 2000). All character states were specified as unordered and equally weighted with indels as missing data. Heuristic search was implemented with 100 random addition sequence replicates, tree-bisection-reconnection (TBR) branch swapping, the MULTREES option and a maximum of 1000 trees saved per round. To evaluate relative robustness of the clades found in the most parsimonious trees, the bootstrap analysis (Felsenstein 1985) employed 100 replicates using the heuristic search with the MULTREES option, TBR branch swapping and a maximum of 100 trees saved per round. Based on the same data set, a maximum likelihood analysis was also performed by quartet puzzling (Strimmer & Haeseler 1996) using TREEPUZZLE 5.0 (available at http://www.tree-puzzle.de), with 1000 puzzling steps, Hasegawa–Kishono–Yano (HKY) substitution model and transition/transversion parameter estimated from data.

Results

At least four distinct ITS clones were detected from each sample except *Larix griffithii*, *L. olgensis* and one individual of *Larix gmelinii* (Table 1). Based on the restriction profiles, the frequency of the clones ranged from 10% to 50% (not including some unique ones). A total of 101 distinct clones were obtained and completely sequenced. The full-length ITS region of *Larix* ranged from 1653 to 1773 bp, and the alignment of the ingroup sequences was 1808 bp long. Most length variations came from the ITS1 region. For example, *Aus*-17 and *Chi*-26 had a major deletion (> 100 bp) between nucleotide (nt) positions 756 and 871, and *Gme*-5 had a long gap of 29 bp at nts 1294–1322. Other two 11-bp deletions were located at nts 39–49 and 1036–1046, respectively. The rest gaps in the alignment were not longer than 6-bp. In contrast, the 5.8S coding region and ITS2 of *Larix* had a conserved length of 161–162 bp and 231–233 bp, respectively.

Based on the two 11-bp indels in the ITS1 region, all sequences could be divided into three types, i.e. A, B and C (Fig. 1). All clones from the two North American larches, *Larix laricina* and *L. occidentalis*, belonged to type A, with a deletion between nts 39 and 49, while those from the Eurasian section *Larix* had type B sequences without deletions. Interestingly, 47 out of 58 clones from the Eurasian section *Multiserialis* possessed type C sequences with a deletion at nts 1036–1046, but the other 11 — *Aus*-17, *Chi*-26, *Him*-5, *Him*-22, *Spe*-3(1), *Spe*-10(2), *Spe*-16(3), *Spe*-18(1), *Spe*-19, *Spe*-23(3) and *Spe*-25(1) — belonged to type B. According to the investigation on ITS sequences from *L. potaninii* and its varieties, the three clones, *Him*-22, *Aus*-17 and *Chi*-26, would be pseudogenes because of their low GC content, high substitution rates, unique positions in the phylogenetic trees or significant length variations, and *Aus*-7 is a putative recent recombinant (Wei *et al*. 2003). Following this analysis, we found that *Spe*-3(1) obtained in the present study might also represent a pseudogene. Although its GC content (55.7%) was not much lower than the mean value (56.7%), the average pairwise distance (0.035 ± 0.004) between *Spe*-3(1) and other conspecific clones was remarkably higher than that (0.009 ± 0.001) among the latter ones. Also, in the molecular phylogeny including all sequences, *Spe*-3(1) was always located at a basal position with a marked long branch length. The average genetic distance between *Larix* and *Pseudotsuga* was 0.201 ± 0.012. When all clones from the same taxon (section, species or variety) were treated as a group, the intragenomic sequence divergence of the Eurasian section *Multiserialis* ranged from 0.003 ± 0.001 to 0.025 ± 0.002. Excluding the five putative pseudogenes or recombinants (*Him*-22, *Aus*-17, *Spe*-3(1), *Chi*-26 and *Aus*-7), the value decreased to 0.002 ± 0.001 to 0.009 ± 0.001, which was slightly lower than that (0.008 ± 0.001 to 0.014 ± 0.001) of the Eurasian section *Larix*. The intragenomic sequence divergence of the two North American species, *L. laricina* and *L. occidentalis*, was 0.005 ± 0.001 and 0.016 ± 0.002, respectively.

The alignment of the 101 clones from *Larix* and two sequences of *Pseudotsuga* had 1932 characters. A total of 362 sites were phylogenetically informative, of which 166 were from the ingroups. In order to avoid long branch attraction,

the five putative pseudogenes or recombinants, i.e. *Aus*-17, *Chi*-26, *Him*-22, *Spe*-3(1) and *Aus*-7, were deleted from the data set. The heuristic search generated 1000 most parsimonious trees with tree length = 914 steps, consistency index = 0.7867 and retention index = 0.9050. The strict consensus tree is shown in Fig. 2, in which all *Larix* clones were divided into two lineages, one leading to the North American species (clade A) and the other to the Eurasian ones. The Eurasian lineage was further separated into two strongly supported monophyletic groups, corresponding to section *Larix* (clade B) and section *Multiserialis* (clade C), respectively (Fig. 2). In clade A, all clones from the short-bracted species *L. laricina* were nested within those from *L. occidentalis*, a species with long bracts. Although clade B had a high bootstrap value (86%), no species relationships were resolved. Clones from all *Larix* species (*L. decidua*, *L. gmelinii*, *L. kaempferi*, *L. olgensis* and *L. sibirica*) were mixed together. Within this clade, *Gme*-12 separated out first, another six clones, namely *Kae*-15, *Sib*-2(2), *Sib*-4(3), *Sib*-15(1), *Sib*-24(2) and *Dec*-14, separated out next as a cluster, and the remaining clones, except four from *L. sibirica*, formed a strongly supported monophyletic group (bootstrap value = 98%). The topology in clade C is interesting. All clones from *L. potaninii* var. *chinensis*, var. *himalaica* and *L. griffithii* formed a robustly supported monophyletic group, respectively, but those from *Larix mastersiana*, *L. potaninii* var. *potaninii* and var. *australis* mixed together. The clones from *L. speciosa* did not cluster together, but most of them had basal positions together with *L. griffithii* clones in Clade C. Excluding outgroups, the strict consensus of unrooted most parsimonious trees generated from a further analysis was nearly identical to Fig. 2 in topology. Also, the phylogenetic tree constructed from the quartet puzzling analysis was topologically very similar to the parsimonious trees obtained (tree not shown).

Discussion

Although marked intragenomic heterogeneity of nrDNA ITS has been reported from *L. potaninii* (Wei *et al*. 2003), the present study shows that the intraspecific nrDNA diversity is significantly lower in *Larix* than in two Cretaceous genera *Pinus* and *Picea*. Except for some putative pseudogenes with great sequence divergence and length variation, such as *Aus*-17, *Chi*-26, *Him*-22 and *Spe*-3(1), the other clones of *Larix* are relatively conserved in length, and the average pairwise sequence divergence within a species or variety ranges only from 0.003 ± 0.001 to 0.016 ± 0.002. In contrast, intra- or inter-genomic nrDNA ITS variants with length variation of several hundreds base pairs are frequent in *Pinus* and *Picea* (Maggini *et al*. 1998; Quijada *et al*. 1998). The low complexity of nrDNA paralogues in *Larix* is also reflected by the clear grouping of different ITS sequence types in the strict consensus tree (Fig. 2). For example, all type A sequences form clade A, comprising the North American species, while clade B, the Eurasian section *Larix*, has pure type B sequence. In Clade C, the Eurasian section *Multiserialis*, most clones belong to type C, but there are still a few type B sequences. The coexistence of type B sequence in both sections of Eurasian larches, especially in the four putative pseudogenes, implies that this sequence type could represent an ancient nrDNA paralogue in the common ancestor of *Larix* or Eurasian *Larix*.

The split of the present ITS phylogeny of *Larix* into a North American and an Eurasian clade and subdivision of the latter into two sections are highly congruent with the results of most previous molecular and allozyme analyses (Gernandt & Liston 1999; Semerikov & Lascoux 1999; Semerikov *et al*. 2003; Wei & Wang 2003, 2004). In the Eurasian section *Larix*, clones from all species are mixed together. This clone distribution pattern represents the deep paralogy (Bailey *et al*. 2003), i.e. deep nrDNA ITS paralogues resulting from duplication and divergence prior to speciation events. In contrast, clones from each species or variety of the Eurasian section *Multiserialis*, except *L. mastersiana*, *L. speciosa* and two varieties of *L. potaninii*, form a monophyletic group, demonstrating the phenomenon of shallow paralogy (Bailey *et al*. 2003). The great difference between the two Eurasian sections in the clone distribution could be attributed to their different evolutionary histories. Actually, the two sections separated early and then evolved independently (LePage & Basinger 1995; Wei & Wang 2003, 2004).

The deep paralogy of nrDNA ITS copies in the Eurasian section *Larix* might suggest that recent recolonization and morphological differentiation have played important roles in the evolution of this section. According to the congruent result of all molecular analyses, including the present one, genetic differentiation is low among species of the Eurasian section *Larix* (Semerikov *et al*. 2003; Wei & Wang 2003, 2004). Besides, this section only has type B ITS sequence. Richardson *et al*. (2001) suggested that recent divergence among taxa would result in lower levels of genetic variation within a clade and a poorly resolved phylogeny. Also, recent divergence hypothesis of the Eurasian short-bracted larches is supported by their similar morphology and easier interspecific hybridization (Ostenfeld & Larsen 1930). Although extant species of this short-bracted section are distributed in north Asia and Europe, these regions were severely glaciated in the Quaternary. Postglacial recolonization and local divergence could be responsible for their present wide distribution and low genetic diversity. As both molecular and allozyme variability analyses of the Eurasian short-bracted larches indicated, some refugia were preserved in southern Siberia mountain ranges, south of the Urals and northern Kazakhstan, and at the end of glaciation, forest species extended into the north of the Urals and north Siberia (Semerikov *et al*. 1999;

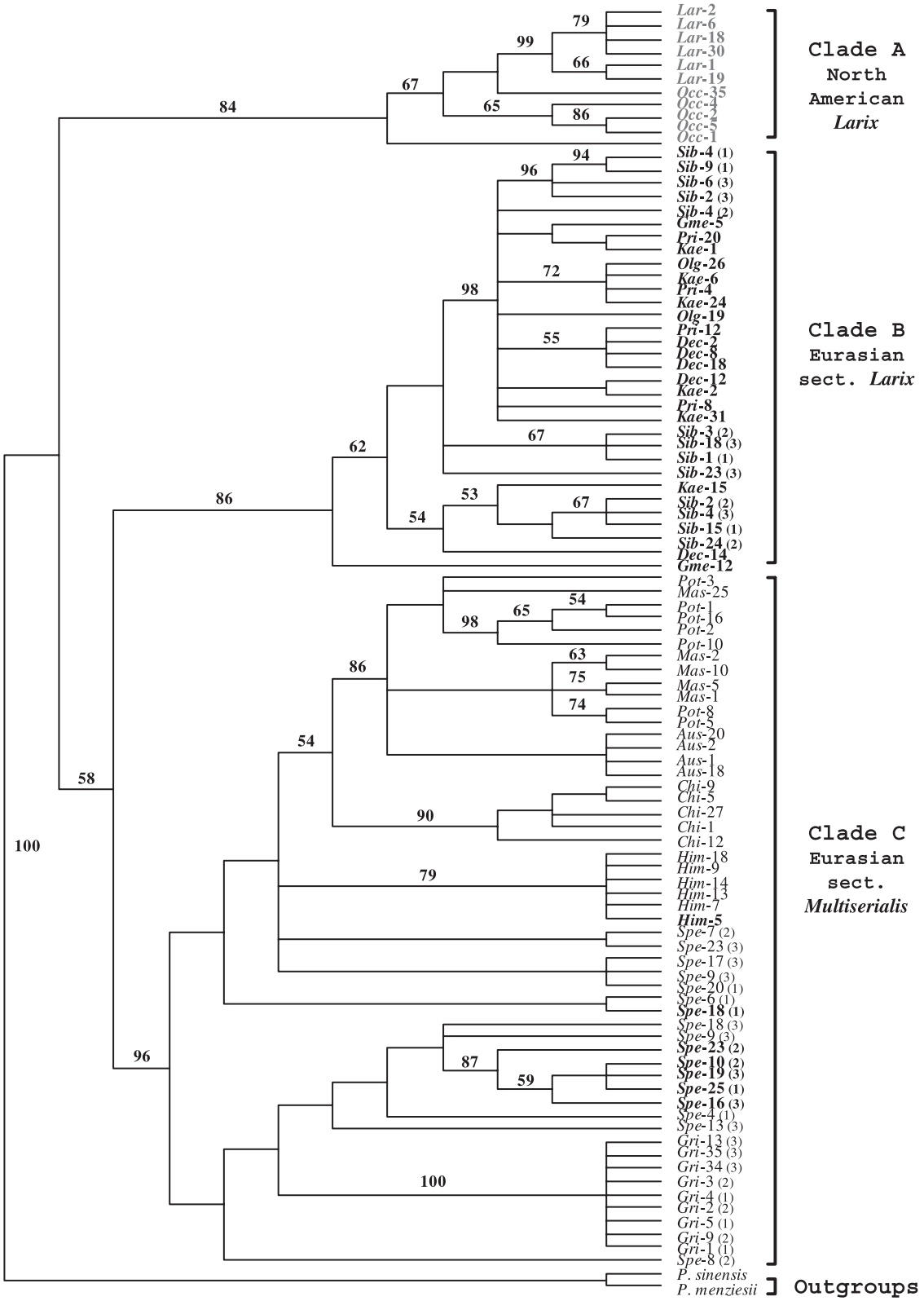

Fig. 2 Strict consensus tree of 1000 most parsimonious trees constructed from sequence analysis of *Larix* internal transcribed spacer (ITS) clones with *Pseudotsuga* species as outgroups. Bootstrap percentages greater than 50% are displayed on the branches. The numbers following taxa indicate different clones and those in brackets denote different individuals of the same taxon. The clones in grey letters represent sequence type A, those in bold type belong to type B, and the others are type C.

Semerikov & Lascoux 2003). In addition, Bennett (1997) suggested that at the time of the Last Glacial Maximum, northern Siberia and the Russian Far East were only locally glaciated. These conditions might explain the present huge distribution of *L. gmelinii* and *L. sibirica*, two important boreal forest species, as the result of rapid expansion from their refugia after the glaciation. As for *L. decidua*, the only European species with isolated populations in the Alps, Carpathians, Suderter and southern and central Poland (Scheepers *et al*. 2000), it would have been less successful than other species at migrating back into their previous distribution areas since the considerable glaciation in the Quaternary almost entirely covered northern Europe (Hewitt 2000).

In contrast, the species diversity of the Eurasian section *Multiserialis* might result from radiation in east Himalayas and its vicinity given the shallow paralogy of ITS clones from this section. As a diversity hotspot and one of the plant diversity centres of the world (Wilson 1992; Myers *et al*. 2001), the Himalayan regions have extremely complex climate, habit and topography, which could greatly hasten species diversification and radiation. Based on the analyses of the spatial pattern and origin of some characteristic taxa in this region, Sun (2002) suggested that quite a few groups in east Himalayas, especially in the Hengduan Mountains, migrated from boreal or arctic areas because of the world-wide temperature decrease from late Eocene and the dramatically climatic and topological changes in Asia in accompany with the uplift of the Tibetan Plateau (An *et al*. 2001), and then radiated in each genus, such as *Pedicularis*, *Aconitum*, *Saussurea* and *Gentiana*. The Eurasian section *Multiserialis* is also primarily distributed in the subalpine area of the Himalaya–Hengduan Mountains. Within the section, *L. speciosa* is endemic to north-west Yunnan and east Tibet, while *L. griffithii* is confined to the eastern Himalayas, especially south and east Tibet. In the overlap area, the two species can grow in the same stand. *Larix mastersiana* is limited to west Sichuan. Another species, *L. potaninii*, has been differentiated into four varieties. Var. *himalaica* and var. *chinensis* occur in the westernmost (south-west Tibet) and easternmost (Qinling Mountains near the margin of the Tibetan Plateau), respectively, whereas the other two, var. *potaninii* and var. *australis*, are distributed in the Hengduan Mountains and have a large sympatric region in south-west Sichuan and north-west Yunnan (Farjon 1990, 2001; Fu *et al*. 1999). According to the present and previous molecular studies (Wei & Wang 2003, 2004), the Eurasian section *Multiserialis* is a strongly supported monophyletic group, and has a low inter-specific (varietal) genetic differentiation. In several cases, however, all clones of a species (even a variety), such as *L. griffithii* and *L. potaninii* var. *himalaica*, form a clade. In addition, most clones of *L. speciosa* have basal positions in Clade C, except that *L. griffithii* is nested within them (Fig. 2). These phylogenetic relationships of ITS clones demonstrate strong founder effect in the evolution of nrDNA paralogues accompanying the species radiation. Although this section does not have many species like other large taxa with typical radiation, it could have experienced a radiation history as far as its complex morphological variations and variable habitats in a narrow range as well as the above molecular evidence are considered. In addition, its present distribution and early separation from the Eurasian short-bracted section as well as the Eocene origin of *Larix* indicate that southward migration of the Eurasian section *Multiserialis* occurred accompanying climatic deterioration from the later Eocene (Zachos *et al*. 2001). As for the mix-up of ITS clones from *L. mastersiana* and *L. potaninii* (var. *australis* and var. *potaninii*) (Fig. 2), two species with a sympatric distribution in west Sichuan, this may result from interspecific hybridization (Ostenfeld & Larsen 1930).

It is interesting that all clones from *L. laricina*, the only one short-bracted species in North America, form a robustly supported monophyletic group nested within the clones from *L. occidentalis* (Fig. 2), a long-bracted species confined to the Rocky Mountains and the northern part of the Cascade Range. Moreover, intragenomic sequence divergence is much lower in *L. laricina* (0.005 ± 0.001) than in *L. occidentalis* (0.016 ± 0.002). In order to eliminate the bias of sequence divergence estimation, we randomly sequenced 15 clones from *L. laricina* with primers ITS1, ITS3 and ITS1R2, and obtained the same result. The above molecular evidence suggests that *L. laricina* might have experienced a strong bottleneck effect, since this species occupies the plains territory of North America that was greatly affected by glaciation many times during the Pleistocene. However, further investigation of more individuals is needed to test this hypothesis.

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