Molecular markers used for genetic studies in Japanese Larch (Larix kaempferi (Lamb.) Carr.)

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NNEXT Non-native Tree Species for European Forests - Experiences, Risks and Opportunities (FP1403)

Molecular markers used for genetic studies in Japanese Larch (*Larix kaempferi* (Lamb.) Carr.)

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1. General remarks

Larch is one of the most abundant conifers in the northern hemisphere where it grows at both high latitudes and high elevations. It comprises around ten species distributed over North America, Europe and Asia.

Japanese larch is native on Honshu Island, where it grows at 1300 to 2900 m elevation. Faster growing than European larch at early stage, it has usually been preferred in Western Europe's oceanic areas. Presently, hybrid larch (*Larix* x *eurolepis*, hybrid between European and Japanese larches), which is more resistant to cold and drought, tends to replace Japanese larch in the reforestation of northern Europe.

Since 2009, infection of Japanese larch by the *Phytophtora ramorum* has led to a severe decline of the species in the British Isles. For this reason, the species is no more planted there.

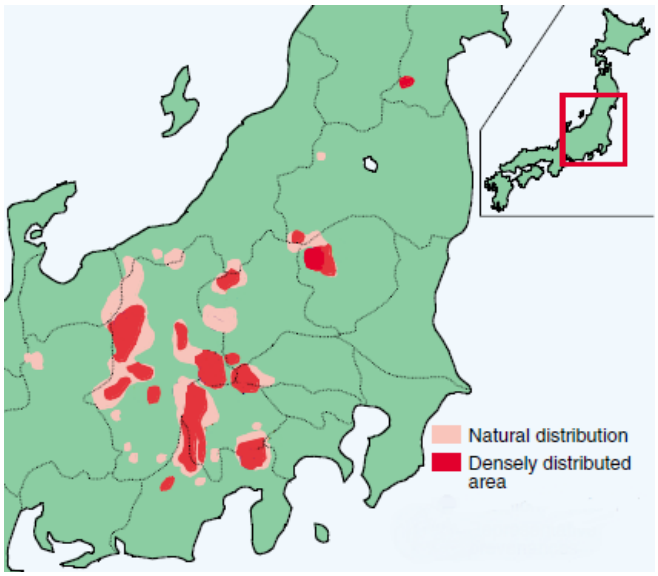

Figure 1. Natural distribution of Japanese Larch in Japan (in Hoshi 2004)

Due to literature scarcity on the use of molecular markers to implement genetic studies on *Larix kaempferi,* references in the present document are often given for related species (*Larix sibirica, Larix gmelinii, Larix decidua, Larix eurolepis*) when available.

2. Isozymes

Genetic studies on *Larix kaempferi* based on isozyme markers have been generally used to identify interspecific hybrid seeds (embryos) between *Larix kaempferi* and *Larix decidua* (e.g. Bergmann and Ruetz 1987, Häcker and Bergmann 1991, Tröber and Hasemann 2000). Isozyme patterns are very similar for the two species in the number of scored loci, but for some loci the relative position of bands differs. For both species the same analysis method can be applied. As detailed studies on genetic variation for *Larix kaempferi* are missing, references in Table 1 refer not only to *Larix kaempferi*, but also to *Larix decidua.*

Material for protein extraction (only Larix kaempferi)

Proteins were extracted from dormant buds and seeds (both endosperm and embryos) (Bergmann and Ruetz 1987, Häcker and Bergmann 1991, Tröber and Hasemann 2000).

Protein extraction and separation protocols

Isozyme extraction, separation by starch gel electrophoresis and staining of gels were carried out based on standard procedures decribed by Siciliano and Shaw (1976), Cheliak and Pitel (1985), Häcker and Bergmann (1991), Müller-Starck and Starke (1993) and Konnert and Maurer (1995).

Important results (only Larix kaempferi and hybrids)

Estimation of the proportion of hybrid seed from seed orchards consisting of *Larix kaempferi* and *Larix decidua*

clones was possible (Bergmann and Ruetz 1987, Häcker and Bergmann 1991, Tröber and Hasemann 2000) based on the loci SKDH-A and NADH-A. At these two loci the two larch species could be unambiguously distinguished by the position of bands in the zymogram and, therefore, the proportion of hybrids and selfings (individual and clonal) could be exactly determined.

Table 1: List of enzymes, scored loci and number of alleles for *Larix decidua* and *Larix kaempferi*

Enzyme system	E.C. Number	Scored loci	No. of alleles*	References
Aspartate aminotransferase	2.6.1.1	AAT-A,-B -C	3, 4, 3	1,5,9
Diaphorase	1.8.1.4	Dia		9
Esterase	3.1.1.2	EST-A,-C	2, 4	1,3,4,9
Glutamate dehydrogenase	1.4.1.2	GDH-A	3	2,3,4,5,9
Glucose-6-phospate dehydrogenase	1.1.1.49	G6PDH-A	3	2,3,4,9
Glyceraldehyde-3-phosphate-dehydrogenase	1.2.1.9	G3PDH-A,-B,	4, 2	1
Isocitrate dehydrogenase	1.1.1.42	IDH-A,-B	1, 3	1,2,3,4,5,9
Leucine aminopeptidase	3.4.11.1	LAP-A,-B	4, 4	3,4,5
Malate dehydrogenase	1.1.1.37	MDH-A,-B,-C,-D	3, 3, 4, 3	1,2,3,4
Menadione reductase	1.6.99.2	MNR-B,-C,-D	1, 4, 2	1,3,4
Phosphoglucose isomerase	5.3.1.9	PGI-A,-B	2, 3	1,5, 6
Phosphoglucomutase	2.7.5.1	PGM-A	6	1,5,9
6-Phosphogluconate dehydrogenase	1.1.1.44	6PGDH-A,-B	3, 3,	1,2,5,9
Triose-phosphate isomerase	5.3.1.1	TPI-A,-B	1, 2	1
Shikimate dehydrogenase	1.1.1.25	SKDH-A	6	1, 2,3,4,5 6,7,8
Superoxide dismutase	1.15.1.1.	SOD-A,-B	1, 3	3,4,9
Sorbitol dehydrogenase	1.1.1.14	SrDH-A	2	3,4
NADH dehydrogenase	1.6.99.3	NDH-A	2	6

**- values for Larix decidua except SKDH-A and NADH-A which refer also to Larix kaempferi*

1-Beletti et al. 1996, 2-Maier 1992, 3-Lewandowski and Mejnartowicz 1988, 4- Lewandowski and Mejnartowicz 1992, 5-Müller-Starck and Felber 2010, 6-Häcker and Bergmann 1991, 7-Bergmann and Ruetz 1987, 8-Tröber and Haasemann 2000, 9-Semerikov and Lascoux 1999

3. Organelle DNA markers (chloroplast (cp) DNA, mitochondrial (mt)DNA)

Detailed information on markers used for DNA analyses from organelle and nuclear DNA from *Larix* species is given in Heinze et al. (2012). In the present guidelines, the focus is on *Larix kaempferi*. Thus, only primers working also for this species are included.

Loci and primers used

According to Heinze et al. (2012), chloroplast DNA (cpDNA) variation has been studied based on PCR-RFLPs (Semerikov and Lascoux 2003, Acheré et al. 2004), sequence variation (Wei and Wang 2003, Gros-Louis et al. 2005) and microsatellites (Semerikov and Lascoux 2003). Mitochondrial DNA (mtDNA) variation has been studied using direct PCR, PCR-RFLPs (Semerikov and Lascoux 2003, Acheré et al. 2004, Semerikov and Polezhaeva 2007) and sequencing (Gros-Louis et al. 2005).

Acheré et al. (2004) applied PCR-RFLP markers on cpDNA (paternally inherited) and mtDNA (maternally inherited) to identify European x Japanese larch hybrids. They used universal primers (Taberlet et al. 1991, Demesure et al. 1995, Dumolin-Lapégue et al. 1997, Petit et al. 1998). For cpDNA, ten out of 22 tested primer pairs gave clear amplification products in *Larix kaempferi* and *Larix decidua*. Only these primers are introduced in Table 2. Amplification products were digested with five restriction enzymes – *Taq*I, *Hap*II, *Hha*I, *Hae*III and *Bcl*I. For mtDNA eight of the eleven tested primer pairs amplify (see also table 2).

For PCR-RFLPs, Semerikov et al. (2003, 2006) and Semerikov and Lascoux (2003) used also published universal primers (Taberlet et al. 1991, Demesure et al. 1995, Dumolin-Lapégue et al. 1997, Parducci and Szmidt 1999) to amplify cpDNA and mtDNA fragments. cpDNA amplified fragments were cut with *Alu*I, *Hae*III, *Hin*fI, *Hpa*II, *Mbo*I, *Rsa*I, *Sf*13.

Material for DNA-extraction

DNA was extracted from buds, needles or germinated seed (Semerikov and Lascoux 2003, Semerikov et al. 2003, Acheré et al. 2004, Gros-Louis et al. 2005, Wei and Wang 2003, Polezhaeva et al. 2010, San Jose-Maldia et al. 2009).

DNA-extraction and amplification protocols

Total DNA was extracted from the mentioned tissue using:

- the CTAB protocol of Devey et al. (1996) cited in Ostrowska et al. (1998) (Semerikov and Lascoux 2003)
- the CTAB protocol of Rogers and Bendich (1988) (Wei and Wang 2003)
- the QIAGEN DNeasy Kit (Acheré et al. 2004, Gros-Louis et al. 2005, Pluess 2011)
- NucleoSpin Plant II (Macherey Nagel, used in INRA lab, unpublished)

Examples for amplification protocols (PCR-RFLP)

- 94°C for 6 min followed by 35 cycles of 94°C for 45 s, 55°C for 45 s, 70°C for 3 min, 30 s (Acheré et al. 2004).
- 94°C for 5 min followed by 35 cycles of 94°C for 30 s, 65°C for 45 s (UBC460), 50°C for nad4-3/4, 55°C for nad5-1/2, atpA1-R, elongation 3 min for UBC460 and 2 min for the rest of primers at 72° C, final elongation at 72° C for 10 min (Polezhaeva et al. 2010).

Examples for amplification protocols (cpSSR):

- 94°C for 5 min followed by 35 cycles of 94°C for 30 s, 55°C for 45 s, 72°C for 30s; final elongation at 72° C for 10 min (Polezhaeva et al. 2010).
- 94°C for 5 min followed by 35 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 30s; final elongation at 72° C for 6 min (Semerikov and Lascoux 2003).

Example for amplification protocols (cpDNA sequencing)

- 94°C for 3 min followed by 35 cycles of 94°C for 1 min, 56°C for 1 min, 72°C for 1 min and 20 s; final elongation at 72° C for 10 min (Gros-Louis et al. 2005).

Example for amplification protocols (mtDNA sequencing)

- 94°C for 2 min followed by 35 cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 1 min; final elongation at 72° C for 10 min (Gros-Louis et al. 2005).

Important results

- Four cpDNA (matK, trnL-intron, trnT-trnL trnL-trnF) and five mtDNA markers (cox1-1, matR-1, nad1-b/c, nad3-1 and nad5-1) were developed to distinguish unambiguously four larch species (*Larix laricina, Larix decidua, Larix kaempferi,* and *Larix sibirica*) used in intensive forestry in Western Europe or eastern North America and trace forest reproductive material (Acheré et al. 2004, Gros-Louis 2005).

- By combining the mitochondrial PCR-RFLP marker *f13* and the chloroplast PCR-RFLP marker *rbcL-TaqI*, *Larix decidua* and *Larix kaempferi* could be discriminated (Acheré et al. 2004). The two markers are sufficient to identify first-generation hybrid individuals.
- Japanese larch is found to be closely related to populations of *Larix kamtschatica* inhabiting the Kuril Islands and South Sakhalin (Polezhaeva et al. 2010).
- Despite the restricted natural distribution of Japanese larch, the mtDNA showed geographic structure (San Jose-Maldia et al. 2009).

Table 2: PCR-RFLP markers (cpDNA, mtDNA) used for *Larix kaempferi* and other larix species

Type	Amplified region	Primer sequence 5‘ – 3‘	References	Source of primer pairs
cpDNA	trnT-trnF	CATTACAAATGCGATGCTCT ATTTGAACTGGTGACACGAG	1,3	Taberlet et al. (1991)
	rpl20-trnW	T3 + TTTTCGAACTGCTAACCAACG (T3 = AATTAACCCTCACATAAGGG) T7 + ACCTACGGCATCAGGTTTTG (T7=GTAATACGACTCACTATAGGGC)	1,2	Parducci and Szmidt (1999), modified by Semerikov et al. (2006)
	trnL-trnV	CTGCTTCCTAAGAGCAGCGT TTGACATGGTGGAAGTCATCA	1	Parducci and Szmidt (1999)
	psbC-trnS	GGTCGTGACCAAGAAACCAC GGTTCGAATCCCTCTCTCTC	1, 3	Parducci and Szmidt (1999)
	psbD-16S	CCACAAAAACGAAACGGTCT ACTAACTAATCAGACGCGAGCC	1	Parducci and Szmidt (1999)
	rbcL	ATGTCACCACAAACAGAAACTAAAGCAAGTA CTTCACAAGCAGGAGCTAGTTCAGGACTCC	3	Petit et al. (1998)
	trnK	GGGTTGCCCGGGACTCGAAC CAACGGTAGAGTACTCGGCTTTTA	3	Demesure et al. (1995)
	trnK-trnQ	TAAAAGCCGAGTACTCTACCGTTG CTATTCGGAGGTTCGAATCCTTCC	3	Dumolin-Lapégue et al. (1997)
	trnQ-trnR	GGGACGGAAGGATTCGAACC ATTGCGTCCAATAGGATTTGAA	3	Dumolin-Lapégue et al. (1997)
	trnS-trnfM	GAGAGAGAGGGATTCGAACC CATAACCTTGAGGTCACGGG	3	Demesure et al. (1995)
	trnS-trnT	CGAGGGTTCGAATCCCTCTC AGAGCATCGCATTTGTAATG	3	Demesure et al. (1995)
	atpF-rps2	Primer sequence not published	3	Acheré et al. (2004)
	trnR-atpF	Primer sequence not published	3	Acheré et al. (2004)

Type	Amplified region	Primer sequence 5‘ – 3‘	References	Source of primer pairs
mtDNA	nad5-1/2	TTTTTTCGGACGTTTTCTAG TTTGGCCAAGTATCCTACAA	1	Wu et al. (1998)
	nad4-3c/4r	GGAGCTTTCCAAAGAAATAG GCCATGTTGCACTAAGTTAAC	1	Dumolin-Lapégue et al. (1997)
	F13	CTGTTGGTAACTTGGGG GCGCCTCTTTCGGAATAG	3	Acheré et al. (2004)
	UBC460	AACCTAGAGCCAACAGCAGCACCT CCCAACTTCCTCGAAAGCAGATG	4,5	Semerikov et al. (2006)
	C8	GGATCGTAGCGTGGGAACTA AGGGAACTTGTGAACGTTGG	4	Semerikov et al. (2006)
	B11	TACCCGCCTTAACCGTAAGA GACCCGTAGTTTGGCTGAGA	4	Semerikov et al. (2006)
	R11	CATCCCGTCGCTTGTTTAAT CCGGTTGGCACCTTAAATAGA		Semerikov et al. (2006)
	Cox2	TTTTCTTCCTCATTCTKATTT CCACTCTATTGTCCACTTCTA	3	Dumolin-Lapégue et al. (1997)
	nad1-2/3	GCATTACGATCTGCAGCTCA GGAGCTCGATTAGTTTCTGC	3,4	Demesure et al. (1995)
	Nad3-rps12	AATTGTCGGCCTACGAATGTG GCTCG(A=I)GTACGGTC(C=I)GTGCG	3	Wu et al. (1998)
	Nad4-1/2	CAGTGGGTTGGTCTGGTATG TCATATGGGCTACTGAGGAG	3,4	Demesure et al. (1995)
	Nad4-2/3	CTCCTCAGTAGCCCATATGA AACCAGTCCATGACTTAACA	3	Dumolin-Lapégue et al. (1997)
	Nad4-3/4	GGAGCTTTCCAAAGAAATAG GCCATGTTGCACTAAGTTAC	5	Dumolin-Lapégue et al. (1997)
	Nad4-2/4	TGTTTCCCGAAGCGACACTT GGAACACTTTGGGGTGAACA	4	Demesure et al. (1995)
	Nad5-1/2	GAAATGTTTGATGCTTCTTGGG ACCAACATTGGCATAAAAAAAGT	3,4,5	Wu et al. (1998)
	Rps14-cob	CACGGGTCGCCCTCGTTCCG GTGTGGAGGATATAGGTTGT	3	Demesure et al. (1995)
	Mh02	TTTTAGGGCCATTTGCCTGC TCTATGGACAAGAGCCCGACCT	4	Jeandroz et al. (2002)
	Mh09‘	CCATCCAGCCATGTCTCATC AGGGCTTCACATAGAGCATC	4	Jeandroz et al. (2002)
	Mh27	TGCTTTCCAATTTACCACGAG GATACGCTTTCCTGGCATAC	4	Jeandroz et al. (2002)
	Mh50	AGAATGGCAGCAACTAATAAGC ACTATGCACTTCCCTCCCTCA	4	Jeandroz et al. (2002)
	Atp1-R	GCTGGCAAATTCAACCATTT GCAATTAGGCTGGCTTTCC	5	Polezhaeva et al. (2010)

1-Semerikov and Lascoux 2003, 2-Semerikov et al. 2003, 3-Acheré et al. 2004, 4-San Jose-Maldia et al. 2009, 5-Polezhaeva et al. 2010

Table 3: Primer information for amplification of chloroplast microsatellites (cpSSRs) and variable fragments for sequencing (cpDNA and mtDNA) in genetic analysis of *Larix* species (including *Larix kaempferi*) (Ta= annealing temperature)

Locus	Type	Primer sequence [5'-3'] F= forward, R = reverse	T a (°C)	Size (bp)	Ref.	Source of primer pairs
Pt9383	cpSSR	F: AGAATAAACTGACGTAGATGCCA R: AATTTTCAATTCCTTTCTTTCTCC	48	118	1	Vendramin et al. (1996)
Pt9393		F:GACGTAGATGCTATGGGTACG R:GAGAGCGGTATGAGGGAAGA	55	135	2	Polezhaeva et al. (2010)
Pt9833		F:GACGATGGACGCTCTTTCTC R:GATCGGGCGGGATAATGTA	55	84	2	Polezhaeva et al. (2010)
Pt30		F:TCAATCCTAACCATATCAGGTG R:TCATAGCGGAAGATCCTCTTT	55	139	2	Polezhaeva et al. (2010)
Pt26081		F:CCCGTATCCAGATATACTTCCA R:TGGTTTGATTCATTCGTTCAT	55	112	1	Vendramin et al. (1996)
TrnLV		F:AAATACCACGGGCCTCCTA R:TTGACATGGTGGAAGTCATCAT	55	86	2	Polezhaeva et al. (2010)
Pt30204		TCATAGCGGAAGATCCTCTTT CGGATTGATCCTAACCATACC	55	145	1	Vendramin et al. (1996)
matK	cpDNA amplification and sequencing	F:GAACTCGTCGGATGGAGTG R:GAGAAATCTTTTTCATTACTACAGTG	56		3	Wang et al. (1999)
trnL Intron		F:CGAAATCGGTAGACGCTACG R:GGGGATAGAGGGACTTGAAC	56		3	Taberlet et al. (1991)
trnT-trnL		F:CATTACAAATGCGATGCTCT R:CGAAATCGGTAGACGCTACG	56		3	Taberlet et al. (1991)
trnL-trnF		F:CGAAATCGGTAGACGCTACG R:ATTTGAACTGGTGACACGAG	56		3	Taberlet et al. (1991)
trnT-trnF		CATTACAAATGCGATGCTCT ATTTGAACTGGTGACACGAG	48		4	Taberlet et al. (1991)
Cox1-1	mtDNA amplification and sequencing	F:TTATTATCACTTCCGGTACT R:AGCATCTGGATAATCTGG	60		3	Lu et al. (1998)
matR-1		F:CGACAGAAGCACGAAATTCC R:ACCCGACGATAACTAGCTTC	60		3	Qiu et al. (1999)
nad1-b/c		F:GCATTACGATCTGCAGCTCA R:GGAGCTCGATTAGTTTCTGC	60		3	Demesure et al. (1995)
nad3-1		F:CAGAAGTCGTTTCGATATACG R:ATTGATTCGATGTAGGCATCG	60		3	Soranzo et al. (1999)
nad5-1		F:AGTCCAATAGGGACAGCACAC R:GCTTTGATAGCTGCTTTATCTGC	60		3	Jaramillo-Correa et al. (2003)

1-Semerikov and Lascoux 2003, 2- Polezhaeva et al. (2010), 3- Gros-Louis et al. 2005, 4-Wei and Wang 2003

4. Randomly amplified polymorphic DNA (RAPD) markers

Primer used and important results

Scheepers et al. (2000) analyzed the following 11 markers that differentiated *Larix decidua* and *Larix kaempferi.* Two of these markers were mitochondrial (maternally inherited) (DeVerno et al. 1993).

- OPH-11 – 2,2 kb – 100 % presence in *Larix decidua*
- OPD-15 – 1,4 kb - 100 % presence in *Larix decidua* (mtDNA)
- OPE-17 – 0.8 kb - 100 % presence in *Larix decidua*
- OPF-05 – 2.25 kb -100 % presence in *Larix decidua*
- OPG-12 – 1.3 kb - 100 % presence in *Larix kaempferi*
- OPH-14 – 1.45 kb -100 % presence in *Larix kaempferi*
- OPC-16 – 1.38 kb - 100 % presence in *Larix kaempferi*
- OPC-06 – 0.93 kb - 100 % presence in *Larix kaempferi*
- OPR-08 – 1.2 kb - 100 % presence in *Larix kaempferi* (mtDNA)
- OPD-10 – 1.2 kb - 100 % presence in *Larix kaempferi*
- OPF-13 – 1.0 kb - 100 % presence in *Larix kaempferi*

The following four markers were sufficient to estimate the F1 hybrid (*Larix* X *eurolepis*) fraction in a seed lot: OPH-14, OPC-06, OPH-11, OPF-05.

For DNA-amplification the following PCR-protocol was used:

- 1 cycle of 3 min at 93°C, 1 min at 37°C and 2 min at 72°C, 35 cycles of 1 min at 93°C, 1 min at 37°C and 2 min at 72°C, followed by a final cycle of 10 min at 72°C.

Semerikov et al. (2003) used 4 RAPD primers to develop PCR-based mitochondrial DNA markers useful for phylogenetic studies in larch species. The following four RAPD primers produced fragments considered for further analysis:

UBC460 - 5‘-ACTGACCGGC-3‘
OPB11 - 5‘-GTAGACCCGT-3‘
OPC8 - 5‘-TGGACCGGTG-3‘
OPR11 - 5‘-GTAGCCGTCT-3‘

The RAPD fragments were cut out of a 1% agarose gel, purified using a gel extraction kit (Qiagen), cloned into pGEM-T easy plasmid (Promega) and sequenced.

Gros-Louis et al. (2005) tested 130 RAPD-primers using the following kits from Operon Biotechnologies, Alameda, CA:

- OPL–OPQ,
- OPC-6,
- OPD-10, OPD-15,
- OPE-17,
- OPF05, OPF-13,
- OPG-12,
- OPH-11, OPH-14,
- OPR-18.

For DNA-amplification the following PCR-protocol was used:

- 1 cycle of 1 min at 94°C, 20 cycles of 15 s at 94°C, 15 s at 35°C, and 1 min 30 s at 72°C followed by 25 cycles of 15 s at 94°C, 15 s at 35°C, and 1 min 30 s at 72°C, with a ramp at this extension step of 5 s per cycle, final extension of 10 min at 72°C.

Amplification products (6 µl) were separated into a 0.5% Synergel (Gordon Technologies, Mississauga, Ontario) plus 1.0% agarose gels using 0.75× Tris–phosphate–EDTA (TPE) running buffer. Amplification products were stained with ethidium bromide and visualized under UV light.

5. Nuclear DNA markers (AFLPs, nSSRs, EST-SSRs, SNPs)

a) AFLPs (Amplified Fragment Length Polymorphism)

Semerikov and Lascoux (2003) and Semerikov et al. (2003) used besides other markers the AFLP technique (Vos et al. 1995) for analyzing larch species differentiation at the nuclear level.

DNA was digested with *Eco*RI and *Mse*I. Three selective nucleotides were used in the case of the *Eco*RI primer and four for the *Mse*I primer. The *Eco*RI primer was labeled by g^{33}P-ATP.

The following primer combinations were used:

- *Eco*1+ ACG x *Mse*1+CCCA, *Mse*1+CCAC, *Mse1*+CCAG (Semerikov and Lascoux 2003)
- *Eco*1+ ACG x *Mse*1+CCTC, *Mse*1+CCCA, *Mse*1+CCAC, *Mse1*+CCAG, *Mse*1+CCTG, *Mse*1+CCAG (Semerikov et al. 2003)

Arcade et al. (2000) analysed 114 AFLPs resulting from 5 AFLP primer combinations and constructed a single-tree genetic linkage map of European and Japanese larch.

b) nSSRs (putatively neutral microsatellites) and EST-SSRs (expressed sequence tag derived microsatellites)

Loci and primers used

More then 200 nuclear microsatellites were developed for *Larix kaempferi*: 19 polymorphic simple sequence repeats (SSR) markers in Isoda and Watanabe (2006), 165 SSR marker in Chen et al. (2015, Supplementary Material), six ***expressed sequence tags*** (EST-SSRs) in Yang et al. (2011).

28 microsatellite markers were amplified in cross-species transferability tests for *Larix kaempferi* (6 SSR marker in Khasa et al. 2000, 13 SSR marker in Wagner et al. 2012 and 9 SSR marker in Zhang et al. 2015) (Table 4). Wagner et al. (2012) designed multiplexes for Larch SSRs. Gros-Louis et al. (2005) tested the transferability of EST-SSRs developed by Perry and Bosquet (1998) to *Larix* species, among them also *Larix kaempferi.*

Table 4: Primer sequences, annealing temperatures (T_a), allele length in base pairs (bp), number of alleles scored (N_a) and references for nSSR markers available for genetic analyses in *Larix kaempferi* (* = EST-SSR developed by Perry and Bousquet 1998).

Locus	Motif	Primer sequence		Size (bp)	T_a	N_A	Ref.	Genebank accession number
		Forward	Reverse					
bcLK033	(TC)14	M13-GGAAATGTAGAGATGAGCAATAA	AGGTGCGGTAGTACAAAGTGA	197–251	63-53	9	1	AB234185
bcLK056	$(AG)_{20}$	M13-ATGGGCTAAGGTATGTTTTACG	TTGCCAACATCTATACCAAGTCT	174–200	63-53	12	1	AB234186
bcLK066	$(TG)_{12}$	M13-GCAACCGACAATGATTACATAG	CCTAAAACTGAACTTTGCTCAAT	155–172	63-53	5	1	AB234187
bcLK093a	$(AG)_{17}$	M13-TTCCCCGATGTATATTCACCT	TGACCGTGGTATTTGGATGTA	136–176	63-53	17	1	AB234188
bcLK187	$(AG)_{13}$	M13-AGGACGGAGAGAGTCATTCTG	AACCCTAGTGATTTTAAGGAGAGA	160–186	63-53	12	1	AB234189
bcLK189	$(AG)_{17}AT(AG)_6$	M13-ACCATACGCATACCCAATAGA	AGTTTTCCTTTCCCACACAAT	122–196	63-53	12	1,4, 5	AB234190
bcLK194	$(AG)_{17}$	M13-AAGAGCAAGAATGGGAGTAAG	CATCCAATATCTCCTCTATAAACC	116–136	63-53	7	1	AB234191
bcLK211	$(CT)_{16}$	M13-CCATTCTCCATAGGTTCATTG	ATGCTCCTTACTAAGTCAGATACAC	207–232	63-53	12	1,4, 5	AB234192

Locus	Motif	Primer sequence		Size (bp)	T_a	N_A	Ref.	Genebank accession number
		Forward	Reverse					
bcLK224	$(AG)_{17}$	M13-GGAGAGGCCACTACTATTATTAC	ATGCGTTCCTTCATTCCTCT	152–168	63-53	9	1	AB234193
bcLK225	$(GA)_{20}$	M13-CGTGGTTCCCATCCTCTAAA	TGGCAGCTAAAGGATTAAAGAA	180–213	63-53	12		AB234194
bcLK228	$(AG)_{18}$	M13-CCCTAACCCTAGAATCCAATAA	GAGGAAGGCGACAAGTCATT	183–234	63-53	17	1,4, 5	AB234195
bcLK229	$(GA)_{21}$	M13-ATGCCCAAAAACGAAAAAGT	TTTGCACTGCCAGATTCAGA	108–134	63-53	12	1,4	AB234196
bcLK232	$(AG)_{19}$	M13-TGTTGCTGGGTTGTTGTTAGA	GGGTAATAGTTCCAGTCTTTG	142–178	63-53	10	1	AB234197
bcLK235	$(TC)_9(AC)_2AG(AC)_{14}$	M13-TTCACTTGTGATCCTAGAGTTA-GA	AACCCCTAACCATATAATATCCA	177–220	63-53	9	1,4	AB234198
bcLK241	$(AG)_{12}$	M13-TGAGGTTAGGAGCATCTCGT	GTCCTTCATCGCCTCTTCTT	164–176	63-53	5	1	AB234199
bcLK253	$(AG)_{17}$	M13-AACACCATAGTGCAATGTGC	TCCTCTTGTTGATGCCACTT	217–243	63-53	14	1,4	AB234200
bcLK258	$(TC)_{29}TT(TC)_8$	M13-AAGGTGCTCGTATAATCTCTGG	AGAGTGCCTTCGATCATCAT	107–179	63-53	26	1	AB234201
bcLK260	$(TG)_{14}(AG)_9$	M13-CTCCATAAGGGGCATCACAT	TGGGCTCAAGTTTGGACATTA	115–126	63-53	5	1,4	AB234202
bcLK263	$(TC)_{20}$	M13-CGATTGGTATAGTGGTCATTGT	CCATCATACCTTCTTGAAGAG	205–255	63-53	23	1,4, 5	AB234203
LAReSSR12*	$(ATT)_4(TGT)_4(GTGGCA)_4$	ATTATTGCCCTCTGAGTTTG	ATTCACCCCAATCCCATC	131	56	4	2	JG745369
LAReSSR14*	$(TCAGGC)_5$	ACATTGAGCAGATGACCCAC	ATGCGGAGGTTGAGTTGG	146	56	3	2	AB251473
LAReSSR19*	$(CAT)_4$	CCGAAATGAAGTCCGTGAG	GCAGCAGCAAGTCCTAAAT	140	55	2	2	JG745370
LAReSSR27*	$(AGTCC)_4(GTCCA)_6$	GGCTGAGGTTGCGAAAGA	CAATTACATAAGTGGGACGAGA	142	56	4	2	JG745371
LAReSSR72*	$(AT)_6$	ATGGCTGTGGAAGCGAATA	AAGGGATCACGAACTGAACTGG	168	60	4	2	JG745368
LAReSSR85*	$(TAC)_4$	TTTCGTATGGTCAAGTTCTG	TGTCTATCCCAAGTCAGTCAT	172	52	3	2	JG771979
Sb14*	-	TACTTCGAGTGTCTCTCATTG	GCTGTCAGAGTTTGTAACATC	-	55	1	7	
Sb34*	-	TATCCATCGCCTGCTTCTCAC	TGTAGTCAGTCCGAATGTACC	-	55	1	7	
Sb41*	-	GCTGAGGGGAAGGATTGATAC	GCTTCGACAGGCATATTACAG	-	55	1	7	
Sb46*	-	GGCTGTCAATACAAGTCATTC	TCACGTTGTTATTGTTGTCAC	-	55	1	7	
Sb51*	-	TGAAACAGACTTCTCGTACTG	TTCTTACGTAGCTGCTCTAAC	-	55	1	7	
Sb60*	-	TGGGAGAATGACTAGATTGTG	AAGCCTTGACAATAGTAAGTG	-	55	1	7	
Sb62*	-	GTATTACCCAGCTCAAGTTCC	ACAGTACGCCGCAGACAAATG	-	55	1	7	
UAKLla1	$(TCT)_4$	ATCTCCTTCATCGTCCAC	CCCCAACTAATACCTAATCTAC	175–178		1	3	X54464
UAKLly2	$(CA)_5$	CGAAAGCGAAGAGAGTATCG	GTTCCCAAGGAGAAACCCTA	250–276		1	3	LLY2 (EL)
UAKLLy7	$(TG)_8$	GATTACATCGTGGGTAGGAC	AAGTGATTTGGTGTGGTGAC	182–190		2	3	LLY7 (EL)
UAKLly10a	$(CA)_5AA(CA)_7$	TGGTCGGATTGAGTGAAG	ACCCATCCCATGATAGGAG	274–330		2	3	LLY10 (EL)
UAKLly13	$(AT)_5(GT)_{20}(GA)_6(A)_7$	TCTGTTTACCATCCATAAATC	CCACAACCCATTTTAATATC	154–186		1	3	LLY13 (EL)

Locus	Motif	Primer sequence		Size (bp)	T_a	N_A	Ref.	Genebank accession number
		Forward	Reverse					
UAKLly6	$(GT)_{17}$	AGTTGTACTGTGTGTTGGTC	CTGCCTCAACCACTTCTTC	214–264		1	3,4	LLY6 (EL)
lardec012611(Ld31)	$(AC)_{18}$	TTGAACTAGGGAGATCCGGC	AATAAAATAGCATTCCATGTGTAGC	104–147		8	5	-
lardec022835(Ld50)	$(CA)_{18}$	GAAGGCGACTTTACATGCCC	TCCATCTTTATGTCTCTTCCATGC	157–205		12	5	-
lardec023929(Ld42)	$(TG)_{14}$	TCGTATGCATTGTCCAAATTTCC	TCCAAGTGAGGTCACACGAG	167–191		6	5	-
lardec025807(Ld101)	$(AC)_{12}$	ACACCAAGGACTCTCTGACTAC	GGTGATTCCAGAAGCAGGTG	179–215		7	5	-
lardec023228(Ld56)	$(AC)_{16}$	AGCCATCGTGGTTCTTCTTTG	CTTGTAACTGTGCACCCACC	219–247		9	5	-
Lg01	$(AGC)_{4}$	CAGTGGTGTCCGTGGTGTA	GACCTCCTCCACACCTAAT	141–160	51.3	3	6	XP_006375-910.1
Lg02	$(AGG)_{4}$	CTCTGTGACCAAGAAACCAA	CATGAAGACGAAGAATGCACT	120–140	51.8	2	6	XP_002306-980.2
Lg06	$(AGA)_{5}$	CAAGGATGGAGCAGACGAT	AGCCTCGCACTTTGACAGA	135–150	50.4	2	6	-
Lg14	$(TC)_{6}$	GGGGATTGCAGAGTAGAAA	AAACAGCCATCGAAATGAG	140–150	49.5	1	6	XP_002319-953.1
Lg25	$(AAG)_{4}$	GTGAGAGGTCAAACCCCAA	AGAAGAGTCTGGTCCACGCT	105–125	53.8	2	6	XP_003608-708.1
Lg32	$(AT)_{6}$	CTCTGTCGCACCAGCATTG	TTGTCTTCCGGTATTCACA	105–115	47.5	1	6	XP_002307-364.1
Lg36	$(GA)_{5}$	TGCCCATCCTCTTTGTTTA	AGCACCTGATTCCACATTCT	175–190	50.6	1	6	-
Lg37	$(CT)_{6}$	ACAATGGCTTCCTTCAACA	TATGAGGTGGTTAGGGAGA	175–190	47.3	1	6	XP_002299-125.2
Lg41	$(AGA)_{4}$	ACTTCCACTAAGGTTGACA	ATCCACTGCCTTCTGGTCAT	147–180	49.4	3	6	XP_002313-280.1
LARKeSSRH002	$(AGC)_{6}$	AGGAGGCGGTTCAGTTCAG	GACCTCCTGGGATTTGGATT	117-156	56	7	8	KP863070
LARKeSSRH008	(ACTGGGC)4	GAGATGTACACAGTCCGCCC	CCTGTTCGGATCCACAGAAT	400-414	56	3	8	KP863071
LARKeSSRH028	(AAAATGTGAC)2	TGCCCATTTGAATCCTTAACA	TCGTTGTAGAAGAATGGGGC	198	56	1	8	KP863072
LARKeSSRH029	(AAAGGACCTC)2	TGGAGTTGCACACTACGAGG	GTGATCGGGAGTTCATCGAC	263	56	1	8	KP863073
LARKeSSRH034	(AAACTCTTC)3	AACACACCTGGCCCTGTAAG	GCGCTGTATTGTATTGATAAGGC	93-111	56	3	8	KP863074
LARKeSSRH042	(AAATAG)3	GGACACTTTTCTGCTTCCCA	CAGGTGGCAGAGTACCCACT	334-346	56	2	8	KP863075
LARKeSSRH045	(AAATATATAT)2	CGCCACCTTCCCTATTTACA	CCCCAACCCTAAGACACAGA	274	56	1	8	KP863076
LARKeSSRH046	(AAAATTCTTT)2	ATGTTTTTGGGTTTTGGAGC	CAGGTTTATAGCTTTGGTTTGGA	154-174	56	3	8	KP863077
LARKeSSRH052	(AATG)6	AGGGATGGTTGCTGTGGTAG	CATTTCTCCGAGTGGGTTGT	333-349	56	3	8	KP863078
LARKeSSRH057	(AT)11	GGACGTCTTAAGCATGCCA	AAAGTTCGAAGTGAAGCGGA	110-130	56	7	8	KP863079

Locus	Motif	Primer sequence		Size (bp)	T_a	N_A	Ref.	Genebank accession number
		Forward	Reverse					
LARKeSSRH094	(ACATAGTAGG)2	CTGATGGCACATAGCTGCAC	CTTGACAAAGGAGCCAAAGC	253-265	56	4	8	KP863080
LARKeSSRH106	(AGCAT)4	AGCAGCTGTTGTTGTTGTGG	TGCAAATCGTCTTCACAAGC	247-257	56	2	8	KP863081
LARKeSSRH122	(ACCCTC)6	TGCTTCCGCAGATATAGCCT	CTAAGTTTGTGCGCCGAGAT	240-264	56	5	8	KP863082
LARKeSSRH125	(AT)11	TCTCCCAACCACCCAAGTTA	TCAGGTTCTGGGTTTGGTTC	237-251	56	6	8	KP863083
LARKeSSRH128	(AAATTGGCCT)2	TGGCCAATTTTGAGTTCAAGT	AGAGGTCTCGTAACGGCAGA	239-249	56	2	8	KP863084
LARKeSSRH131	(AGATG)5	GAAGATCACAACAAAGGGCG	TGTCCAGGCAACTGAAACAG	287	56	0	8	KP863085
LARKeSSRH136	(AACCAACCAG)2	GGGACGTACTGAGACCGTGT	TCATTAACTGGGCATGTGGA	373-397	56	2	8	KP863086
LARKeSSRH137	(AAATAAGC)2	ATACATATTCCTTCCGGCCC	TTGGAAAGACTCCAGGATGG	170	56	1	8	KP863087
LARKeSSRH140	(AAAGCC)3	GGAGTAGTGCATATGGGCGT	TATGCTTTTTCCCAGCCAAC	294-336	56	6	8	KP863088
LARKeSSRH147	(AGC)8	AAATGAAGAACCCGAACACG	AGCTCTCGATTCATGGCTGT	181-193	56	2	8	KP863089
LARKeSSRH149	(ACGGCACTCC)2	CAAGGAGAACTGAAGGCTGG	TTTCTCGTCAACTGAGGGCT	251-291	56	3	8	KP863090
LARKeSSRH168	(AGCAGG)5	ACTTCAGTATCACCCGCCAC	CGATCTTTCGGCTCTTATCG	145-169	56	5	8	KP863091
LARKeSSRH177	(AAATAGCTTC)2	TGGCTTTTGCAACAAGTGAC	GGCCATCCTCTGTCATGATT	394-414	56	3	8	KP863092
LARKeSSRH179	(AAAGAAGTTC)2	AACACCAAAGTTGCTGGGAC	GGCTGAGGATTATGATCGGA	335	56	1	8	KP863093
LARKeSSRH180	(AAAGATACC)2	ACATCCTCCCCTTGGTCTCT	CTTGCTCCTGGCGAAGTAAC	169-178	56	2	8	KP863094
LARKeSSRH182	(AAACCC)3	CTGATCAGGGTGAGATGGGT	GCTGCTGTTGTTGTTGCTGT	314	56	1	8	KP863095
LARKeSSRH187	(AACAGC)5	AGATTTGGAAGCAGCAGGAA	AAGTTGTTCAGCCCATCTCG	123-141	56	3	8	KP863096
LARKeSSRH189	(ACTGGC)6	GTAAGGAGGAGGATTGCGGT	AGTTCATCCTTCTGGCTGGA	255-273	56	4	8	KP863097
LARKeSSRH191	(AACCCTCCC)2	TTGAATTCGTCCTGGGTCTC	GTCTGAACGACGAAGAAGGC	145-163	56	3	8	KP863098
LARKeSSRH197	(AAACGGACGG)2	TTAGCAAAAGTCTTCGCCGT	ACGAAACTACGCGGATGAAC	327-337	56	2	8	KP863099
LARKeSSRH206	(AACAATAATT)2	TGCAGTTCGTGTTGCTAACC	CCACCTGGCGAAGTATTGAT	312-362	56	3	8	KP863100
LAREeSSRH217	(ACGCCC)3	ATCCCAAGAACCGATATCCC	TGACCGATTTTCTCTCGCTT	418-436	56	3	8	KP863101
LARKeSSRH221	(AGCATC)3	AGATTCGGTTTTCATGGACG	GCAAGCGAGAGAAAGCAGTT	376-394	56	4	8	KP863102
LARKeSSRH224	(AACGTCC)3	GCTGCCCAGGTGAAGAATAC	TCCCAATTTCACAATCATAGGAG	177-184	56	2	8	KP863103
LARKeSSRH233	(ATCCCC)4	AGGGGCAGGCTTAATCACTT	GATTCGAAGAAAATTGCCCA	444-456	56	3	8	KP863104
LARKeSSRH236	(AGC)8	GAATGCCATTGGAACAGCTT	TGCCTGTGCTCGTTCATAAG	300-321	56	6	8	KP863105
LARKeSSRH239	(AATCCAGTG)2	AATAGTTTGGGGAACCGACC	CCCTGGTTCTATTGACGCAT	333-342	56	2	8	KP863106
LARKeSSRH251	(AACAGC)3	GTTGTTCAGCCCATTTCGAT	AGATTTGGAAGCAGCAGGAA	125-143	56	3	8	KP863107
LARKeSSRH253	(AGGATC)3	AACGGGGTTATCAAGCACTG	ATGCGTTTCATTCGATCCTC	342-366	56	2	8	KP863108
LARKeSSRH256	(AGCCCC)4	TATCCGGCACCCCTGTAATA	GGTTTGATGGGAAACTGCAT	113-125	56	3	8	KP863109
LARKeSSRH264	(AGATGG)3	CCGACGCTATTCCCAACTAA	CTTGGAAGGCTATGGCTACG	96-132	56	6	8	KP863110
LARKeSSRH274	(AGCCC)5	CGGACGAATAGATCCCAGAA	ATGAGGCAGGGTCGTGTTAG	252-272	56	5	8	KP863111

Locus	Motif	Primer sequence		Size (bp)	T_a	N_A	Ref.	Genebank accession number
		Forward	Reverse					
LARKeSSRH276	(AACCGG)3	GAACCAAACCCAGAACCTGA	CTGGGGATATAAATGGGGCT	154-189	56	5	8	KP863112
LARKeSSRH279	(AATCGATGC)2	AATTCAGGGGACATTGCTTG	TTTCTGGGTCTCAGGAATGG	160-187	56	4	8	KP863113
LARKeSSRH283	(AAAGATGAC)2	TCTAGCCATGTGCATTGTCC	ATTCTGTGTTTTGTCGCACG	331-367	56	4	8	KP863114
LARKeSSRH299	(AAGGAG)3	CGATCTTTCGGCTCTTATCG	ACTTCAGTATCACCCGCCAC	149-173	56	5	8	KP863115
LARKeSSRH301	(AATGGC)4	CCAAGGAAACCAGTGCATTT	CATTGGTTGAGGTGGAGGAG	256-280	56	4	8	KP863116
LARKeSSRH309	(ACCTCC)3	AATGGGCTCTCAATGCAATC	AGGTGACAAATGGGACCAAG	466	56	1	8	KP863117
LARKeSSRH339	(AGC)7	AATTCGTTGGCCTTCAGATG	CGATCTCGGGCATTATGAGT	316-319	56	2	8	KP863118
LAREeSSRHL003	(AAGAT)4	TGTGGTCATTGGTGGACATT	GAGTCCCACATTTGCAGGTT	304-324	56	5	8	KP863119
LAREeSSRHL004	(AACCTC)7	AGATGAGCTCCTGTTGGGAA	TTGCTTTGCAGCTTACCAGA	200-224	56	5	8	KP863120
LAREeSSRHL006	(AAT)10	TGCGTTCTGTGTGTCTCTCC	GGGTAGGCCTGAAGAAGGCT	99-117	56	4	8	KP863121
LAREeSSRHL007	(ACAGC)5	GGACGAGACCAATCCAAACT	CAAAAGCCGGGAGAAATGTA	238-278	56	4	8	KP863122
LAREeSSRHL009	(AGGATG)4	GGTCTTAGTCACAGCCCAGC	TTCGATCCTTCTGAATTGGC	151-175	56	6	8	KP863123
LAREeSSRHL021	(AACAGTCTAG)2	GGTCACATGGGAATGAGCTT	TGACTTGTTATTTCTGAATTTTGGA	160-170	56	2	8	KP863124
LAREeSSRHL034	(AAG)7	CCTTCCGTTGCAATCTTCAT	CTTTCCACACTGCCAAACCT	92-116	56	8	8	KP863125
LAREeSSRHL042	(ACGTCC)3	GAATCTGAGAGCTCCGGGTA	ATCCATGTTTTTGCCTCGAC	87-117	56	6	8	KP863126
LAREeSSRHL046	(AAGCTGTGTC)2	ATCCAACTGGATCCATCAGC	CCGGATAAAGTCCAGCAAGA	380	56	1	8	KP863127
LAREeSSRHL062	(AATGCATACT)2	CGGATCTCCTCCTGAATGAA	GTTGAGCTGTCGGATCACAA	222-242	56	3	8	KP863128
LAREeSSRHL079	(ATCCCC)3	GATTCGAAGAAAATTGCCCA	TACCCGTTTCCATTTCCATC	172-202	56	5	8	KP863129
LAREeSSRHL083	(AAAATCAAG)2	CCAAACCTCAACAACAGCAA	GTGCTGCGGATGAGTACAGA	142	56	1	8	KP863130
LAREeSSRHL085	(AACATTG)2	TTTGGCAGTTTTGACAGTCG	CGAGCCATTTGTGTCTTTGA	123-141	56	4	8	KP863131
LAREeSSRHL101	(AGGCGG)4	ATCAAGATCGCCGGTGTTAC	GATTGCCAAAGCCAATGC	232-250	56	4	8	KP863132
LAREeSSRHL104	(ATC)8	CGGATACGGCAAATTTTCAA	CCTTTGTCTTGGTGCTGGAT	283-313	56	7	8	KP863133
LAREeSSRHL114	(AGC)7	AGGAGGCGGTTCAGTTCAG	CAACGCCAGATTAGGAGAGC	187-232	56	7	8	KP863134
LAREeSSRHL120	(AAAG)8	GAAAAGGGTGGAAATGCAAA	GGCACTACCTAACCAAAGTAGGA	133-145	56	3	8	KP863135
LAREeSSRHL129	(AAAAGCATC)2	ATCTTCCCCTGCTGTTTGTG	GGGAGCGTTGAATGGATAGA	254	56	0	8	KP863136
LAREeSSRHL137	(AGC)7	GAGGATTGTGCACACCTTGA	ATGGGTTTGACAGCGGATAA	100-112	56	4	8	KP863137
LAREeSSRHL138	(AATCATCAT)3	AAGGAGTGGGTTTATTGGGG	AGGTGATGATGATGATGTACAATG	243-252	56	2	8	KP863138
LAREeSSRHL159	(AGAGCC)5	CACAGACCTCATGACGATGG	TTCTGATTCTGCCTCTGGCT	224-236	56	3	8	KP863139
LAREeSSRHL161	(AGATGG)5	CGTTTCCAAAATGCCTCAGT	ACACCCAGGGAAGCTCCTAT	302-332	56	6	8	KP863140
LAREeSSRHL162	(AC)10	GGGTCACGTCTACGAGGTTT	GCTAGGACTGCCACTGGATT	85-119	56	9	8	KP863141
LAREeSSRHL163	(AAGGCC)3	AATGGAAGCGTGAGGACATC	TGGTTAAGGGCAACCAAAAG	263-287	56	4	8	KP863142
LAREeSSRHL165	(AAAGGATGAT)2	TATCCTCCTGCACCATCCTC	TCCTCAGTTGCCCTTTGTTT	382	56	1	8	KP863143

Locus	Motif	Primer sequence		Size (bp)	T_a	N_A	Ref.	Genebank accession number
		Forward	Reverse					
LAREeSSRHL166	(AAACCCT)3	CTCAAGAGGTATCAAGCGGC	TAAGGGCTAAGTGGGTGCTC	207	56	1	8	KP863144
LAREeSSRHL215	(AGGCGG)3	TAAATACGCACAAGCCCACA	GGAGGAGCAAATGGATCAAA	203-209	56	2	8	KP863145
LAREeSSRHL217	(ACTCATATG)2	AATCCAACAGAAGGCCAAGA	GCCGGCAAATAGGTTGATATT	204-249	56	3	8	KP863146
LAREeSSRHL246	(AAT)9	TGGATGGTAAGAACGCACAG	ACTTTTACCCGTTGTGGTCG	188-218	56	5	8	KP863147
LAREeSSRHL272	(AATATATAT)2	GCAACAACATCGAACAGCAA	TGTTTATAGGCCAAGCCACC	354-372	56	3	8	KP863148
LAREeSSRHL275	(AAACAAAT)2	CTACCTAAGTCGGCCCACAA	TATCCTCGGAAACCATGAGG	354-362	56	2	8	KP863149
LAREeSSRHL283	(AATGGCAGAC)2	CCATTCCCAAACTAAAACGC	GATGATGAGGCCCTTCAAAA	188-198	56	2	8	KP863150
LAREeSSRHL299	(AAACCCTACG)2	GAACAGCATACAATGGGGCT	CGTCGCGAACAAGAATGATA	446-456	56	2	8	KP863151
LAREeSSRHL308	(AAACATTT)2	TGCATTGTCTTGCTGCCTAT	ATTGCACTGAATGCACAAGC	286	56	1	8	KP863152
LAREeSSRHL346	(ACCAGC)4	TAGAAAGGGCAAAGGCACTG	GGTGCATTTCTCTCCACTCC	99-111	56	3	8	KP863153
LAREeSSRHL357	(AACAGC)3	AGGTCCAGCCATTGATGAAG	TCAATGCAATCCTGGGGTAT	162-168	56	2	8	KP863154
LAREeSSRHL358	(AATAATTCTC)2	CTCCCACCTTACCACGAAAG	TGTGTTAGCATTCCGTGCTC	135-145	56	2	8	KP863155
LAREeSSRHL361	(ACTC)5	GTATGCTGCCAAAGGTGGTT	CATTTCCGGGCTTGTATTTG	275-311	56	3	8	KP863156
LAREeSSRHL366	(ACGGAT)3	TCGTATCTGGATCTCGGGTT	AAAGAGGCAAGCGGTACTCA	244-256	56	3	8	KP863157
LAREeSSRHL372	(AAACCCG)3	GATTCGGAAATGCGGAAATA	AGTTCAAAAATTGGGCGTTG	114-128	56	3	8	KP863158
LAREeSSRHL374	(AC)4	AGTTGAACCAACCCTCATCG	CTGTGGGGTGGAGATCCTTA	246-268	56	9	8	KP863159
LAREeSSRHL380	(AACGGC)3	GGCTGGTACATTTCAGGCAT	AGCCTCTCCTCCTCCTCAAC	184-202	56	3	8	KP863160
LAREeSSRHL391	(ACTGGC)4	AGCGTATGAATTGGTCCAGG	ACGAAGATAGCTCGAACGGA	224-230	56	2	8	KP863161
LAREeSSRHL392	(AAACAAACAG)2	GCGGTCAGGCTTTATCTCAG	ACCTGATGACCACGGGATAG	306-324	56	4	8	KP863162
LAREeSSRHL393	(CCG)8	GCCAGAACCACCGTTAAAAG	AGAGGCGATTATGGGAGCTT	296-302	56	3	8	KP863163
LAREeSSRHL394	(AAAGGC)4	GGGGAGGTGTTTGACAGAGA	AATCAACCGTTGGGAATGAG	255-261	56	2	8	KP863164
LAREeSSRHL395	(ACCAGG)5	TTTGCTTTAAGCTGGGCAGT	CAAAGCTTTCCGAAGGGAAT	272-308	56	6	8	KP863165
LAREeSSRHL396	(AAGAGC)5	CTTTTGCCCTTTTCCTTTCC	TTGTGGGTGTCGTTCACAAT	308-332	56	5	8	KP863166
LAREeSSRHL397	(AT)14	CAATGATCGAACTGTGGTTCA	GCTCATCTTCAACTTCATGTGG	223-273	56	6	8	KP863167
LAREeSSRHL398	(AGCCTG)4	AGTCGGGGATGAAATCTGTG	TGTTTCTTTGCGCATACACC	293-299	56	2	8	KP863168
LAREeSSRHL399	(AAAATC)5	CTTTGTGTTGCGGGATTCTC	TTCCTTTTCCCCTTGGTCTT	275-305	56	4	8	KP863169
LAREeSSRHL400	(AGGCGG)5	GAGGACCTCCTGGCTTTGAT	TTAGAGCTGTGTTGCGCTGT	272-326	56	4	8	KP863170
LAREeSSRHL401	(ACCGCC)3	AGCAGAATAACGAGCCGAAG	CCCGCCACTACTCTGCTTAG	302-320	56	2	8	KP863171
LAREeSSRHL402	(AC)13	CACATATCTGTGTGTGCCTGTG	TTAGGTTGCCAAAACTGCAA	242-270	56	9	8	KP863172
LAREeSSRHL403	(AT)11	TCCATATTGCATAACGCTCCT	GCTCCTTCATGTTGTAAGCAAA	286-290	56	3	8	KP863173
LARKeSSRHL404	(AAGCCC)4	TCTTGTGACATTCGCCTCTG	TCGATGGTGATCTTCACCTG	299	56	1	8	KP863174
LAREeSSRQ001	(CA)10	GCAAACTCATGTAGACTCGCC	CATTGGTGGAACATTGCTTG	182-210	56	6	8	JR170819

Locus	Motif	Primer sequence		Size (bp)	T_a	N_A	Ref.	Genebank accession number
		Forward	Reverse					
LAREeSSRQ005	(GA)8	TTCCCTATTCTCATCCACGG	GTCGCCAGTAAATGGCCTTA	246-252	56	4	8	JR171181
LAREeSSRQ006	(AT)6	CCAAGAAGACCAAAACATCAGA	TCTGTCCCTGTTCACAACCA	131-175	56	6	8	JR171219
LAREeSSRQ010	(TC)7	CCCAGAATGCAATACGGACT	TTCCCAAGGAAAATCTGGTG	216, 222	56	2	8	JR171974
LAREeSSRQ017	(CAG)5	CCACCTCAAATCTTCTCCCA	CCTGCATATGAGTCTGCTGC	127-139	56	5	8	JR173000
LAREeSSRQ020	(AG)6	TGATCCGCTTAAGGTAACCAA	TTGTGAGTGTTTGTGTCGCA	219-231	56	3	8	JR173379
LAREeSSRQ032	(TTG)6	CCCCCTGCACACCATTTT	CAAGAATGCCGATACCGAAT	152-170	56	2	8	JR175164
LAREeSSRQ035	(AT)7	CCTCGAACACTCACTAAACTTGC	ATGCCTCCTTGTGCATTCTT	108-118	56	5	8	JR175381
LAREeSSRQ036	(TGC)6	TACTTCCCTGTGCTGGGTTT	GAAAAAGACTCCCAAAGGGG	207-219	56	6	8	JR175557
LAREeSSRQ048	(GAA)5	TGAAGAAGAAGCGGAAGAGG	AGGCTATACGCTTCCTGCAA	434-461	56	2	8	JR176325
LAREeSSRQ051	(TA)8G(TA)6	CGACTCAGCCACCTCGTAAT	ATTGCCAGAACCCCTTTTCT	234-268	56	13	8	JR176852
LAREeSSRQ053	(AT)6	TGTCGCCTTCACTCTGTGAG	ATCAATGCGGTGAAGATTCC	167-181	56	6	8	JR177135
LAREeSSRQ066	(CA)14	GCTCTTGTTGAGCCACCTTC	ATGGTTTGGATGCACATGAA	142-156	56	3	8	JR178582
LAREeSSRQ067	(TC)8	ATCTCCTTGGAATGTGTGCC	GGGGCGATTACCCTAAATGT	221-233	56	6	8	JR178682
LAREeSSRQ070	(TA)6	GCTCCTCTTGCACAGTCTCC	TGCTCCATTTGTGGGTGTTA	164-198	56	12	8	JR178932
LAREeSSRQ074	(AT)8	GTATGAAGAGCACCCCAAGG	GCAAATAGTTGCAAGGCATGT	124-146	56	11	8	JR179414
LAREeSSRQ104	(CA)7	ATCACTGCTCATGAGTCGCA	GTATGCGTTTGCGTGTGTGT	205-233	56	6	8	JR183015
LAREeSSRQ113	(AC)10	TCCAATGGAGGACGTAAAGG	TCATGCATCATAACATTGAATAACA	184-204	56	8	8	JR184160
LAREeSSRQ114	(CA)7	GAAACGGATATGGGAATGGA	TTGATGAATGGTAATCTGACCTATG	129-147	56	6	8	JR185111
LAREeSSRQ115	(CTG)6	AATTAAATGCGCTCACCTCG	GCAGATAACGCAGCCTTCTT	317-332	56	3	8	JR185400
LAREeSSRQ120	(ACTCT)5	ATTCCCCATTTCACGAAGC	TACTCCGAGAGGAGGCAGAA	110-115	56	2	8	JR186302
LAREeSSRQ125	(AT)10	AAGGGAAAATAAAGCCCTCG	TGCTCTCAGGTTGCAATGAG	100-144	56	11	8	JR186594
LAREeSSRQ127	(ATG)5	GGTTTCCATTACAACTCAAGGG	GGATTCAGCTTCGCTTTCAC	371-377	56	3	8	JR186781
LAREeSSRQ137	(TG)7	GTGCCTTGTGGGTTGTCTTT	AAGAGTTGCCACCCATAAGC	272-278	56	3	8	JR188117
LAREeSSRQ141	(TC)9	CACACATGCAAAGCAAACAA	TGTGTGTGAATGTGAGAGGGA	133-141	56	5	8	JR188688
LAREeSSRQ183	(CTC)7	TGTTTGACGGTGACTGAAGG	TAGAGGAGCAGCGAGAGGAG	126-141	56	4	8	JR193542
LAREeSSRQ187	(TG)8	TGAGGATTCTTTCCCAATGC	CATTGGATCCCAAAGGGTAG	178-190	56	5	8	JR193964
LAREeSSRQ195	(AGA)5	GCAGATTGTAGAAGGGCTGC	CATCGCCTTTCTCACACAGA	220-223	56	2	8	JR194843
LAREeSSRQ206	(GTT)5	GCAGACCCATTTTCGTGATT	CGCATCTCAGAGGGAGAGAG	446-470	56	5	8	JR139531
LAREeSSRQ209	(GGA)5	CCACGGAGTTTGGACTGAAT	CTAAACAGAGCCCAAGCGTC	182-188	56	3	8	JR139801
LAREeSSRQ210	(TA)9	GTTCGATTTTGGCCCATCTA	GATCAATTTTGGTTCGTTGTCA	165-191	56	8	8	JR139804
LAREeSSRQ213	(TTC)5	TTTTGCTTTGTGAATGTGGC	TGGGATCCTGAGGGACTATG	300	56	1	8	JR140280
LAREeSSRQ216	(AT)8	ATTTCTGCGGCAAAGAGTTG	AGAGAGGAAGGACTTTCGGC	374-406	56	3	8	JR140886

Locus	Motif	Primer sequence		Size (bp)	T_a	N_A	Ref.	Genebank accession number
		Forward	Reverse					
LAREeSSRQ218	(AT)6	AATTAGTGGTGCTTCGGTGG	TGGCACTTCTTGTAAATAAAATCAA	245-281	56	4	8	JR140959
LAREeSSRQ235	(CAGCAA)5	CACCATAAGCAACAGCGAAA	GTGCCGATGGATGTCTTTCT	183-195	56	3	8	JR143407
LAREeSSRQ243	(AT)7	TTCGTGTACAGCGTTCAAGC	TCCGGAATATCGTCACAACA	149-185	56	13	8	JR144253
LAREeSSRQ247	(CT)8	CTACGAGAGGCTCGATACGC	CTTCAGTCTGGAGCTGACCC	428-466	56	8	8	JR144913
LAREeSSRQ257	(ATC)5	TCTGCATCCTAGTGCTGTGG	CCCCTGGATCTTCTGAAACA	116-131	56	4	8	JR146140
LAREeSSRQ285	(GAG)5	CCGAGACATGATGCTGAGAA	TATTTGCAGAAGCCCAAACC	162-192	56	7	8	JR149637
LAREeSSRQ299	(CTG)5	AAACCAATGAAAATGCCTGC	TCCCCAGCCAACTCTCATAC	431-485	56	2	8	JR151216
LAREeSSRQ316	(TC)7	AGCTCTCTGTGCTTTCTCGC	GGAAAGAGCAATTCAGCAGG	194-206	56	2	8	JR153273
LAREeSSRQ322	(CAG)5	AGGCGTCTGAGCTACCAAAA	CGACGACACCCAATACCTTT	426-459	56	3	8	JR153722
LAREeSSRQ330	(TC)6	CAGGAAGTTGGGCAGCTTAG	GGTCTTGGCCTTGTTGTTGT	255-267	56	3	8	JR154204
LAREeSSRQ352	(GCA)5	CCACCTCAAATCTTCTCCCA	AGATGGAATACTGTTGGCGG	249-288	56	5	8	JR155690
LAREeSSRQ364	(AT)7	GATGAAATTGCGGAAAGCAT	ACTGGGCAATGTCCAAACTC	280-306	56	11	8	JR157274
LAREeSSRQ375	(TC)6	AGTGGCAGTCAGCATCTCCT	AGAAGATTTTGCAGAGGGCA	212-230	56	3	8	JR158646
LAREeSSRQ377	(TCATCC)5tcagtctca gtt(TCAGTC)5	TCATCATCCTCCTCGTCCTC	AAGATTCAGTGGATGGCGAC	178-208	56	6	8	JR158866
LAREeSSRQ382	(CAG)5	TGGTTCAACTTCTCTCGCCT	GGAATGTGAACGAAGACGGT	299	56	1	8	JR159113
LAREeSSRQ386	(GA)13	TCCATCTTTATTTGGCAGGC	CCATCAGAGATGGGAGTGCT	128-148	56	9	8	JR159815
LAREeSSRQ393	(AG)6	CCTTGTGAAGGGCACAGTTT	ATGAGGTCTGTGAGGGGTTG	371	56	1	8	JR160488
LAREeSSRQ397	(GA)9	TCTGAATCAATGTATCATGTATCGAA	CTGTCAGTCATGCTGCGTTT	132-154	56	10	8	JR161052
LAREeSSRQ399	(AAG)5	AGACTCCTGTTGGAAAGGCA	AGACTCCTGTTGGAAAGGCA	254-263	56	2	8	JR161168
LAREeSSRQ403	(CAT)8	ACACAACATGCTACGATGCC	GCTTCTAGGCGTTCAACGAG	216-246	56	9	8	JR161642
LAREeSSRQ406	(AG)6	TGCATTCTGTTAATGCCCAA	TGTTGATGAGCAATGACCGT	361-385	56	4	8	JR161926
LAREeSSRQ408	(GACTG)7	CAAGCATTCTTCCCCAAAAA	TAAGTCCAGTCCAGTCCGGT	144-180	56	6	8	JR162009
LAREeSSRQ409	(AT)9	AAAATTTCATCCTCGAACACTCA	TGGACAATGTTCCATGCAGT	181-199	56	8	8	JR162187
LAREeSSRQ430	(CGG)5(CTG)2TTGA (TGC)6tgttgatgctgatg g(TGC)8	TTTTGGTCCGATCAGGAGTC	CAACTTTTGGGTTGGGAGAA	286-313	56	9	8	JR166454
LAREeSSRQ439	(AAT)5	TCTCGCTCGGCTTCTACATT	GAGATTCTGCTGCTTCCCTG	258	56	1	8	JR168298
LAREeSSRQ444	(TGC)6	GAACGTTCAAACTGCACACG	TTGAGTTCATTGGCTGCAAG	406-415	56	2	8	JR168664
LAREeSSRQ449	(AT)8	CCCTTAGCCTCTTTTTGAGGA	ACCATCGAACGTGTCAACAA	272-298	56	5	8	JR169475

1-Isoda and Watanabe 2006, M13(–21) -tail was attached to the 5′ end of forward primer of each locus, 2-Yang et al. 2011, 3-Khasa et al. 2000, 4-Pluess 2011, 5-Wagner et al. 2012, 6-Zhang et al. 2015, 7- Gros-Louis et al. 2005, 8-Chen et al. 2015

Material for extraction

Plant tissue used for extraction included seed, buds, leaves (in general frozen needles), e.g. Isoda and Watanabe (2006), Wagner et al. (2012), Nishimura and Setoguchi (2011), Gros-Louis et al. (2005), Chen et al. (2015), phloem (Wagner et al. (2012) and cambium (Khasa et al. 2000).

DNA-extraction and amplification protocols

Total DNA was extracted from the mentioned tissue using:

- NucleoSpin Plant II (Macherey Nagel, used in INRA lab)
- the QIAGEN DNeasy Plant Mini Kit (Gros-Louis et al. 2005, Pluess 2011, Zhang et al. 2015)
- a CTAB protocol after Shiraishi and Watanabe (1995) (Isoda and Watanabe (2006) and after Doyle and Doyle (1990) (Yang et al. 2011, Chen at al. 2015)

Examples for amplification protocols (nSSR)

- 94°C for 1 min followed by 10 cycles of 94°C for 30 s, 63-53^0 C (-1^0 C at each cycle) for 45 s, followed by 25 cycles of 94°C for 30 s, 53^0 C for 30 s, 72°C for 1 min; final elongation at 72^o C for 10 min (Isoda and Watanabe 2006).
- 94°C for 4 min followed by 30 cycles of 94°C for 45 s, 56^0 C for 45 s, 72°C for 45 s; final elongation at 72^o C for 7 min (Chen et al. 2015).

Examples for amplification protocols (EST-SSR):

- 94°C for 3 min followed by 40 cycles of 94°C for 30 s, Ta (Table 4) for 45 ,s 72°C for 1 min; final elongation at 72°C for min (Yang et al. 2011).
- 95°C for 5 min followed by 25 cycles of 95°C for 30 s, Ta (Table 4) for 45 s, 72°C for 60s; final elongation at 72^o C for 20 min (Zhang et al. 2015).
- 94°C for 4 min followed by 40 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 1 min; final elongation at 72^o C for 10 min (Gros-Louis et al. 2005).

SSR – Multiplexing (Wagner et al. 2012)

- Multiplex 1 – Ld31, bcLK211, Ld30, bcLK228, Ld50, bcLK189, bcLK253
- Multiplex 2 – Ld58, Ld45, Ld42, bcLK263, Ld101, Ld56
- Amplification protocol for multiplexes 1 and 2:
- 95°C for 15 min followed by 35 (multiplex 1)/30 (multiplex 2) cycles of 94°C for 30 s, T_a (Table 4) 56°C for 1 min, 72°C for 1 min; final elongation at 60°C for 30 min

Important results

- New EST-SSR markers were developed for Larix *kaempferi* (Gros-Louis et al. 2005, Yang et al. 2015, Zang et al. 2015). The markers are transferable also to other *Larix species.*
- New SSR markers were developed and identified as highly polymorphic in *Larix kaempferi*. Most of them could be amplified in related *Larix* species (*Larix olgensis, Larix gmelinii, Larix principi-rupprechtii*) (165 nSSR between them 145 polymorphic developed by Chen et al. 2015, 20 primer pairs between them 19 polymorphic developed by Isoda and Watanabe 2006).
- In a *Larix kaempferi* Danish seed orchards, SSR markers were used to evaluate the selfing rate, the paternal contribution to the progenies and the pollution rate from external larch sources (Hansen 2008).
- A 34% introgression rate by spontaneous hybridization between *L. kaempferi* and *L. laricina* was observed in Québec (Canada), suggesting to take into consideration the proximity of this exotic species in the management of natural genetic resources (Meirmans et al. 2014).

c) SNPs (single-nucleotide polymorphisms)

- Gros-Louis et al. (2005) used SNP in a study aiming at distinguishing larch species (*Larix decidua, Larix sibirica, Larix kaempferi* and *Larix laricina*). The results were the identification of three gene loci (Sb14, Sb48, Sb51) with fixed interspecific polymorphisms implicating 17 SNPs and 2 indels.
- Li et al. (2014) identified many single-nucleotide polymorphisms (SNPs) in a genome-wide marker development for *Larix kaempferi*. Among these SNPs, 364227 (78.6%) were determined from transcripts with annotation information, and they were distributed in 32453 known genes.

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7. Selected references referring to genetic investigations on related *Larix* species

Isozymes

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