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

Jean-Charles Bastien, Vanina Guerin, Ana-Maria Szasz-Len, Monika Konnert

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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 *Phytophthora ramorum* has led to a severe decline of the species in the British Isles. For this reason, the species is no more planted there.

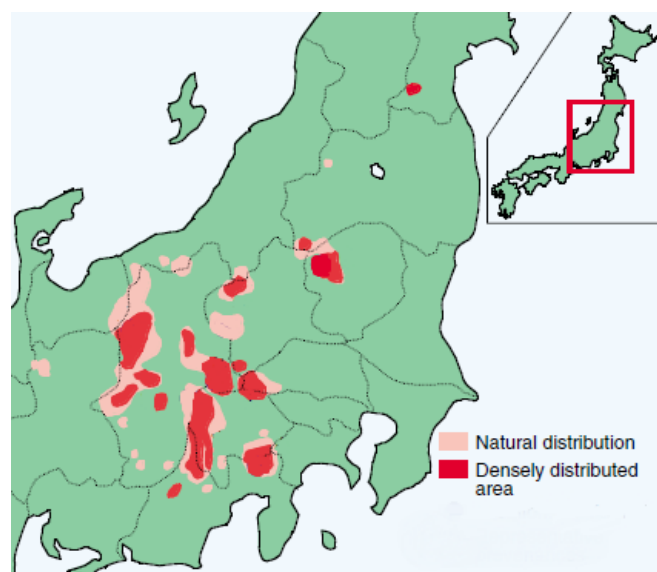


Figure 1. Natural distribution of Japanese Larch in Japan (in Hoshi 2004)

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 described 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-phosphate 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 – *TaqI*, *HapII*, *HhaI*, *HaeIII* and *BclI*. 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 *AluI*, *HaeIII*, *HinfI*, *HpaII*, *MboI*, *RsaI*, *SfiI*.

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 *fl3* 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 larch 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	ATGTCACCACAAACAGAACTAAAGCAAGTA CTTCACAAGCAGGAGCTAGTTCAGGACTCC	3	Petit et al. (1998)
	trnK	GGGTTGCCCGGGACTCGAAC CAACGGTAGAGTACTCGGCTTTTA	3	Demesure et al. (1995)
	trnK-trnQ	TAAAAGCCGAGTACTCTACCGTTG CTATTCCGGAGGTTCGAATCCTTCC	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	CGAGGGTTTCGAATCCCTCTC AGAGCATCGCATTGTGAATG	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	GGATCGTAGCGTGGGAATA AGGGAACCTTGTGAACGTTGG	4	Semerikov et al. (2006)
	B11	TACCCGCCTTAACCGTAAGA GACCCGTAGTTTGGCTGAGA	4	Semerikov et al. (2006)
	R11	CATCCCGTCGCTTGTTTAAT CCGTTGGCACCTTAAATAGA		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 ACCAACATTTGGCATAAAAAAAGT	3,4,5	Wu et al. (1998)
	Rps14-cob	CACGGGTCGCCCTCGTTCGG GTGTGGAGGATATAGGTTGT	3	Demesure et al. (1995)
	Mh02	TTTTAGGGCCATTTGCCTGC TCTATGGACAAGAGCCCGACCT	4	Jeandroz et al. (2002)
	Mh09'	CCATCCAGCCATGTCTCATC AGGGCTTCACATAGAGCATC	4	Jeandroz et al. (2002)
	Mh27	TGCTTTCCAATTTACCACGAG GATACGCTTTCCTGGCATAAC	4	Jeandroz et al. (2002)
	Mh50	AGAATGGCAGCAACTAATAAGC ACTATGCACTTCCCTCCCTCA	4	Jeandroz et al. (2002)
Atp1-R	GCTGGCAAATTC AACCATTT 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:TGGTTTGATTTCATTCGTTTCAT	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:GGGATAGAGGGACTTGAAC	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:CAGAAGTCGTTTTCGATATACG R:ATTGATTTCGATGTAGGCATCG	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 *EcoRI* and *MseI*. Three selective nucleotides were used in the case of the *EcoRI* primer and four for the *MseI* primer. The *EcoRI* primer was labeled by $g^{33}P$ -ATP.

The following primer combinations were used:

- *EcoI*+ ACG x *MseI*+CCCA, *MseI*+CCAC, *MseI*+CCAG (Semerikov and Lascoux 2003)
- *EcoI*+ ACG x *MseI*+CCTC, *MseI*+CCCA, *MseI*+CCAC, *MseI*+CCAG, *MseI*+CCTG, *MseI*+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 than 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 Bosquet 1998).

Locus	Motif	Primer sequence		Size (bp)	T_a	N_a	Ref.	Genebank accession number
		Forward	Reverse					
bcLK033	(TC) ₁₄	M13-GGAAATGTAGATGAGCAATAA	AGGTGCGGTAGTACAAAAGTGA	197–251	63–53	9	1	AB234185
bcLK056	(AG) ₂₀	M13-ATGGGCTAAGGTATGTTTTACG	TTGCCAACATCTATACCAGTCT	174–200	63–53	12	1	AB234186
bcLK066	(TG) ₁₂	M13-GCAACCCGACAATGATTACATAG	CCTAAAACCTGAACCTTTGCTCAAT	155–172	63–53	5	1	AB234187
bcLK093a	(AG) ₁₇	M13-TTCCCCCGATGTATATTCACCT	TGACCCGTGGTATTTGGGATGTA	136–176	63–53	17	1	AB234188
bcLK187	(AG) ₁₃	M13-AGGACGGAGAGTCATTCGTG	AACCCTAGTGATTTTAAGGAGAGAGA	160–186	63–53	12	1	AB234189
bcLK189	(AG) ₁₇ AT(AG) ₆	M13-ACCATACGCATACCCAATAGA	AGTTTTCCCTTTCCCCACACAAT	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-CCATTCCTCCATAGGTTTCATTG	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-GGAGAGGGCCACTACTATATATAC	ATGCGTTCCCTTCATTCCTCTCT	152-168	63-53	9	1	AB234193	
bcLK225	(GA) ₂₀	M13-CGTGGTTCCCATCCTCTAAA	TGGCAGCTAAAGGATTAAGAA	180-213	63-53	12		AB234194	
bcLK228	(AG) ₁₈	M13-CCCCTAACCCCTAGAATCCCAATAA	GAGGAAGGGGACAAAGTCATT	183-234	63-53	17	1,4,5	AB234195	
bcLK229	(GA) ₂₁	M13-ATGCCCAAAAACGAAAAAGT	TTTGCACCTGCCAGATTCAGA	108-134	63-53	12	1,4	AB234196	
bcLK232	(AG) ₁₀	M13-TGTTGCTGGGTTGTGTTAGA	GGTAATAGTTCAGTCTTTG	142-178	63-53	10	1	AB234197	
bcLK235	(TC) ₉ (AC) ₂ AG(AC) ₁₄	M13-TTCACCTTGTGATCCTAGAGTTA-GA	AACCCCTAACCATATAATATCCA	177-220	63-53	9	1,4	AB234198	
bcLK241	(AG) ₁₂	M13-TGAGGTTAGGAGCATCTCGT	GTCCCTTCATCGCCTCTTCTTT	164-176	63-53	5	1	AB234199	
bcLK253	(AG) ₁₇	M13-AACACCATAGTCAATGTGC	TCCTCTTGTGATGCCACTT	217-243	63-53	14	1,4	AB234200	
bcLK258	(TC) ₂₉ TT(TC) ₈	M13-AAGGTGCTCGTATAAICTCTGG	AGAGTGCCTTCGATCATCAT	107-179	63-53	26	1	AB234201	
bcLK260	(TG) ₁₄ (AG) ₉	M13-CTCCATAAGGGGCATCACAT	TGGGCTCAAAGTTTGGACATTA	115-126	63-53	5	1,4	AB234202	
bcLK263	(TC) ₂₀	M13-CGATTTGGTATAGTGGTCATTTGT	CCATCATACCTTCTTGAAGAG	205-255	63-53	23	1,4,5	AB234203	
LAReSSR12*	(ATT) ₄ (TGT) ₄ (GTGGCA) ₄	ATTATTGCCCTCTGAGTTTG	ATTACCCCAATCCCATC	131	56	4	2	JG745369	
LAReSSR14*	(TCAGGC) ₅	ACATTTAGCAGATGACCCAC	ATGCGGAGGTTGAGTTGG	146	56	3	2	AB251473	
LAReSSR19*	(CAT) ₄	CCGAAATGAAGTCCGTGAG	GCAGCAGCAAAGTCCCTAAAT	140	55	2	2	JG745370	
LAReSSR27*	(AGTCC) ₄ (GTCCA) ₆	GGCTGAGGTTGCGAAAGA	CAATTACATAAGTGGGACGAGA	142	56	4	2	JG745371	
LAReSSR72*	(AT) ₆	ATGGCTGTGGAAGCGGAATA	AAGGGATCACGAACTGAAGTGG	168	60	4	2	JG745368	
LAReSSR85*	(TAC) ₄	TTTTCGTATGGTCAAGTCTTG	TGCTATCCCCAAGTCAGTCAT	172	52	3	2	JG771979	
Sb14*	-	TACTTCGAGTGTCTCTCATTG	GCTGTCAGAGTTTGTAAACATC	-	55	1	7		
Sb34*	-	TATCCATCGCCTGCTTCTCAC	TGTAGTCAGTCCGAAATGTACC	-	55	1	7		
Sb41*	-	GCTGAGGGGAAGGATTGATAC	GCTTCGACAGGCATATTAACAG	-	55	1	7		
Sb46*	-	GGCTGTCAATACAAAGTCATTC	TCACGTTGTTATTGTTGTAC	-	55	1	7		
Sb51*	-	TGAAACAGACTTCTCGTACTG	TTCTTACGTAGCTGCTCTAAC	-	55	1	7		
Sb60*	-	TGGGAGAAATGACTAGATTTGTG	AAGCCTTGACAATAAGTAAGTG	-	55	1	7		
Sb62*	-	GTATTACCCAGCTCAAGTTCC	ACAGTACGCCCGCAGACAAATG	-	55	1	7		
UAKLla1	(TCT) ₄	ATCTCCTTCATCGTCCAC	CCCCAACTAATACCTAATCTAC	175-178		1	3	X54464	
UAKLly2	(CA) ₅	CGAAAGCGAAAGAGAGTATCG	GTTCCCAAGGAGAAACCCCTA	250-276		1	3	LLY2 (EL)	
UAKLly7	(TG) ₈	GATTACATCGTGGGTAGGAC	AAGTGATTTGGTGTGGGTGAC	182-190		2	3	LLY7 (EL)	
UAKLly10a	(CA) ₅ AA(CA) ₇	TGGTCCGATTTGAGTGAAG	ACCCATCCCATGATAGGAG	274-330		2	3	LLY10 (EL)	
UAKLly13	(AT) ₅ (GT) ₃₀ (GA) ₆ (A) ₇	TCTGTTTACCATCCATAAATC	CCACAACCCATTTAATATC	154-186		1	3	LLY13 (EL)	

Locus	Motif	Primer sequence		T _a	N _A	Ref.	Genebank accession number
		Forward	Reverse				
UAKLly6	(GT) ₁₇	AGTTGTACTGTGTGGTC	CTGCCCTCAACCACCTTCTTC	214-264	1	3,4	LLY6 (EL)
lardec012611(Ld31)	(AC) ₁₈	TTGAACTAGGGAGATCCGGC	AATAAAATAGCATTCATGTGTAGC	104-147	8	5	-
lardec022835(Ld50)	(CA) ₁₈	GAAGGGGACTTTACATGCC	TCCATCTTTATGTCTCTTCCATGC	157-205	12	5	-
lardec023929(Ld42)	(TG) ₁₄	TCGTATGCAATGTCCAAATTTCC	TCCAAGTGAGGTCACACGAG	167-191	6	5	-
lardec025807(Ld101)	(AC) ₁₂	ACACCAAGGACTCTCTGACTAC	GGTGATTCAGAAAGCAGGTG	179-215	7	5	-
lardec023228(Ld56)	(AC) ₁₆	AGCCATCGTGTCTTCTTTG	CTTGTAACCTGTGCACCCACC	219-247	9	5	-
Lg01	(AGC) ₄	CAGTGGTGTCCCGTGGTGTGA	GACCTCCTCCACACCTAAT	141-160	3	6	XP_006375-910.1
Lg02	(AGG) ₄	CTCTGTGACCAAGAAACCAA	CATGAAGACGAAGAATGCACT	120-140	2	6	XP_002306-980.2
Lg06	(AGA) ₅	CAAGGATGGAGCAGACGAT	AGCCTCGCACTTTGACAGA	135-150	2	6	-
Lg14	(TC) ₆	GGGGATTGCAGAGTAGAAA	AAACAGCCATCGAAATGAG	140-150	1	6	XP_002319-953.1
Lg25	(AAG) ₄	GTGAGAGGTCAAACCCCAA	AGAAGAGTCTGGTCCACCGCT	105-125	2	6	XP_003608-708.1
Lg32	(AT) ₆	CTCTGTGGCACCAGCATG	TTGTCTTCCGGTATTTCACA	105-115	1	6	XP_002307-364.1
Lg36	(GA) ₅	TGCCCATCCTCTTTTGTTTA	AGCACCTGATTCACACATTCT	175-190	1	6	-
Lg37	(CT) ₆	ACAATGGCTTCCCTTCAACA	TATGAGGTGGTTAGGGAGA	175-190	1	6	XP_002299-125.2
Lg41	(AGA) ₄	ACTTCCACTAAGGTTGACA	ATCCACTGCCCTTCTGGTCAT	147-180	3	6	XP_002313-280.1
LARKeSSRH002	(AGC) ₆	AGGAGGCGGTTTCAGTTCAG	GACCTCCTGGGATTTGGATT	117-156	7	8	KP863070
LARKeSSRH008	(ACTGGGC) ₄	GAGATGTACACAGTCCGCC	CCTGTTCGGATCCACAGAAT	400-414	3	8	KP863071
LARKeSSRH028	(AAAATGTGAC) ₂	TGCCCATTTGAATCCTTAACA	TCGTTGTAGAAGAATGGGGC	198	1	8	KP863072
LARKeSSRH029	(AAAGGACCTO) ₂	TGGAGTTGCACACTACGAGG	GTGATCGGGAGTTTCATCGAC	263	1	8	KP863073
LARKeSSRH034	(AAACTCTTC) ₃	AACACACCTGGCCCTGTAAG	GGCTGTATTGTATTGATAAGGC	93-111	3	8	KP863074
LARKeSSRH042	(AAATAG) ₃	GGACACTTTTCTGCTTCCCA	CAGGTGGCAGAGTACCCACT	334-346	2	8	KP863075
LARKeSSRH045	(AAATATATAT) ₂	CGCCACCTTCCCTAATTTACA	CCCCAACCCTAAGACACAGA	274	1	8	KP863076
LARKeSSRH046	(AAAATCTTTT) ₂	ATGTTTTTGGGTTTTTGGAGC	CAGGTTTATAGCTTTGGTTTGGGA	154-174	3	8	KP863077
LARKeSSRH052	(AATG) ₆	AGGGATGGTTGCTGTGGTAG	CATTTCTCCGAGTGGGTTGT	333-349	3	8	KP863078
LARKeSSRH057	(AT) ₁₁	GGACGTCTTAAGCATGCCA	AAAGTTCGAAGTGAAGCGGA	110-130	7	8	KP863079

Locus	Motif	Primer sequence		T _a	Size (bp)	N _A	Ref.	Genebank accession number
		Forward	Reverse					
LARkeSSRH094	(ACATAGTAGG)2	CTGATGGCACATAGCTGCAC	CTTGACAAAGGAGCCAAAAGC	56	253-265	4	8	KP863080
LARkeSSRH106	(AGCAT)4	AGCAGCTGTGTGTGTGG	TGCAAAATCGTCTTCACAAAGC	56	247-257	2	8	KP863081
LARkeSSRH122	(ACCCCTC)6	TGCTTCCGCAGATATAGCCT	CTAAGTTTGTGCGCCGAGAT	56	240-264	5	8	KP863082
LARkeSSRH125	(AT)11	TCTCCCAACCACCCAAAGTTA	TCAGGTTCTGGGTTTGGTTTC	56	237-251	6	8	KP863083
LARkeSSRH128	(AAAT'TGGCCT)2	TGGCCAATTTTGAGTTCAAAGT	AGAGGTCCTCGTAAACGGCAGA	56	239-249	2	8	KP863084
LARkeSSRH131	(AGATG)5	GAAAGATCAACAACAAGGGGG	TGTCCAGGCAACTGAAACAG	56	287	0	8	KP863085
LARkeSSRH136	(AACC AACCCAG)2	GGGACGTACTGAGACCGTGT	TCATTAAGTGGGCATGTGGA	56	373-397	2	8	KP863086
LARkeSSRH137	(AAATAAGC)2	ATACATAATTCCTTCCGGCCC	TTGGAAAAGACTCCAGGATGG	56	170	1	8	KP863087
LARkeSSRH140	(AAAGCC)3	GGAGTAGTGCATATGGGCGT	TATGCTTTTCCAGCCAAC	56	294-336	6	8	KP863088
LARkeSSRH147	(AGC)8	AAATGAAGAACCAGCAACAG	AGCTCTCGATTCATGGCTGT	56	181-193	2	8	KP863089
LARkeSSRH149	(ACGGCACTCC)2	CAAGGAGAACTGAAGGCTGG	TTTTCTCGTCAACTGAGGGCT	56	251-291	3	8	KP863090
LARkeSSRH168	(AGCAGG)5	ACTTCAGTATCACCCGCCAC	CGATCTTTCGGCTCTTATCG	56	145-169	5	8	KP863091
LARkeSSRH177	(AAATAGCTTC)2	TGGCTTTTGGCAACAAGTGAC	GGCCATCCTCTGTTCATGATT	56	394-414	3	8	KP863092
LARkeSSRH179	(AAAGAAAGTTC)2	AACACCAAAGT'TGCTGGGAC	GGCTGAGGAT'TATGATCGGA	56	335	1	8	KP863093
LARkeSSRH180	(AAAGATAACC)2	ACATCTCCCTTGGTCTCT	CTTGTCTCCTGGCGAAGTAAC	56	169-178	2	8	KP863094
LARkeSSRH182	(AAACCC)3	CTGATCAGGGTGAGATGGGT	GCTGCTGT'TGTTGTTGCTGT	56	314	1	8	KP863095
LARkeSSRH187	(AACAGC)5	AGATTTGGAAGCAGCAGGAA	AAGTTGTTACGCCCAICTCG	56	123-141	3	8	KP863096
LARkeSSRH189	(ACTGGC)6	GTAAGGAGGAGGAT'TGGGT	AGTTCATCCTTCTGGCTGGA	56	255-273	4	8	KP863097
LARkeSSRH191	(AACCCCTCCC)2	TTGAAATTCGTCTCCTGGGTCTC	GTCTGAAACGACGAAAGAAGCC	56	145-163	3	8	KP863098
LARkeSSRH197	(AAACGGACGG)2	TTAGCAAAAAGTCTTCGCCGT	ACGAAACTACCGGGATGAAC	56	327-337	2	8	KP863099
LARkeSSRH206	(AACAAATAT)2	TGCAGTTCGTGT'TGCTAACC	CCACCTGGCGAAGTATGAT	56	312-362	3	8	KP863100
LARESSRH217	(ACGCC)3	ATCCCAAGAACCAGATATCCC	TGACCGAATTTTCTCTCGCTT	56	418-436	3	8	KP863101
LARkeSSRH221	(AGCATC)3	AGATTCGGTTTTCATGGACG	GCAAGCGAGAGAAAAGCAGTT	56	376-394	4	8	KP863102
LARkeSSRH224	(AACGTCC)3	GCTGCCCAGGTGAAGAATAC	TCCCAATTTCACAATCATAGGAG	56	177-184	2	8	KP863103
LARkeSSRH233	(ATCCCC)4	AGGGGCAGGCTTAATCACTT	GATTCGAAGAAAAT'TGCCCA	56	444-456	3	8	KP863104
LARkeSSRH236	(AGC)8	GAATGCCAAT'TGGAACAGCTT	TGCCCTGTGCTCGTTTCATAAG	56	300-321	6	8	KP863105
LARkeSSRH239	(AATCCAGTG)2	AATAGTTTGGGGAACCCGACC	CCCTGGTTCAT'TGACCGCAT	56	333-342	2	8	KP863106
LARkeSSRH251	(AACAGC)3	GTTGTTCAGCCCAAT'TCGAT	AGATTTGGAAGCAGCAGGAA	56	125-143	3	8	KP863107
LARkeSSRH253	(AGGATC)3	AACGGGT'TATCAAGCACTG	ATGCGTTTCAITTCGATCCCTC	56	342-366	2	8	KP863108
LARkeSSRH256	(AGCCCC)4	TATCCGGCACCCCTGTAATA	GGT'TTGATGGGAAAAC'TGCAT	56	113-125	3	8	KP863109
LARkeSSRH264	(AGATGG)3	CCGACGCTAT'TCCCAACTAA	CTTGGAAAGGCTATGGCTAGG	56	96-132	6	8	KP863110
LARkeSSRH274	(AGCCC)5	CGGACGAATAGATCCCGAA	ATGAGGCAGGGTTCGTGTTAG	56	252-272	5	8	KP863111

Locus	Motif	Primer sequence		Size (bp)	T _a	N _A	Ref.	Genebank accession number
		Forward	Reverse					
LARKeSSRH276	(AACCGG)3	GAACCAAAACCAGAACCTGA	CTGGGGATATAAATGGGGCT	154-189	56	5	8	KP863112
LARKeSSRH279	(AATCGATG)2	AATTCAGGGGACATTTGCTTG	TTTTCTGGGTCTCAGGAATGG	160-187	56	4	8	KP863113
LARKeSSRH283	(AAAGATGAC)2	TCTAGCCATGTGCATTTGTCC	ATTCTGTGTTTTTGTCCGACG	331-367	56	4	8	KP863114
LARKeSSRH299	(AAGGAG)3	CGATCTTTTCGGCTCTTTATCG	ACTTCAGTATACACCCGCCAC	149-173	56	5	8	KP863115
LARKeSSRH301	(AATGGC)4	CCAAGGAAACCAGTGCATTT	CATTGGTTGAGGTGGAGGAG	256-280	56	4	8	KP863116
LARKeSSRH309	(ACCTCC)3	AATGGGCTCTCAATGCAATC	AGGTGACAAATGGACCAAG	466	56	1	8	KP863117
LARKeSSRH339	(AGO)7	AATTCGTTGGCCCTTCAGATG	CGATCTCGGGCATTTATGAGT	316-319	56	2	8	KP863118
LAREeSSRHL003	(AAGAT)4	TGTGGTCAATGGTGGACATT	GAGTCCACATTTGCAGGTT	304-324	56	5	8	KP863119
LAREeSSRHL004	(AACCTC)7	AGATGAGCTCCTGTTGGGAA	TTGCTTTGCAGCTTACCAGA	200-224	56	5	8	KP863120
LAREeSSRHL006	(AAT)10	TGCGTTCTGTGTCTCTCC	GGGTAGGCCCTGAAGAAAGGCT	99-117	56	4	8	KP863121
LAREeSSRHL007	(ACAGC)5	GGACGAGACCAATCCAAAGT	CAAAAGCCGGGAGAAAATGTA	238-278	56	4	8	KP863122
LAREeSSRHL009	(AGGATG)4	GGTCTTAGTACACAGCCGAGC	TTTCGATCCCTTCTGAATTTGGC	151-175	56	6	8	KP863123
LAREeSSRHL021	(AACAGTCTAG)2	GGTCACATGGGAATGAGCTT	TGACTTGTATTCTGAAATTTTGGGA	160-170	56	2	8	KP863124
LAREeSSRHL034	(AAG)7	CCTTCCGTTGCAATCTTCAT	CTTTCCACACTGCCAAACCT	92-116	56	8	8	KP863125
LAREeSSRHL042	(ACGTCC)3	GAATCTGAGAGCTCCGGGTA	ATCCATGTTTTTTGCCCTCGAC	87-117	56	6	8	KP863126
LAREeSSRHL046	(AAGCTGTGTO)2	ATCCAACTGGATCCATCAGC	CCGGATAAAGTCCAGCAAGA	380	56	1	8	KP863127
LAREeSSRHL062	(AATGCATACT)2	CGGATCTCCTCCTGAATGAA	GTTGAGCTGTGGGATCACAA	222-242	56	3	8	KP863128
LAREeSSRHL079	(ATCCCC)3	GATTCGAAGAAAATTTGCCCA	TACCCGTTTTCCATTTCCCATC	172-202	56	5	8	KP863129
LAREeSSRHL083	(AAAATCAAG)2	CCAAAACCTCAACAAACAGCAA	GTGCTGGGGATGAGTACAGA	142	56	1	8	KP863130
LAREeSSRHL085	(AACATTG)2	TTTTGGCAGTTTTTGACAGTCC	CGAGCCATTTGTGTCCTTTGA	123-141	56	4	8	KP863131
LAREeSSRHL101	(AGGCGG)4	ATCAAGATCGCCGGTGTAC	GATTTGCCAAAGCCCAATGC	232-250	56	4	8	KP863132
LAREeSSRHL104	(ATO)8	CGGATACGGCAAATTTTCAA	CCCTTGTCTTGGTGTGGAT	283-313	56	7	8	KP863133
LAREeSSRHL114	(AGO)7	AGGAGGGGTTTCAGTTTCAG	CAACGCCAGATTAGGAGAGC	187-232	56	7	8	KP863134
LAREeSSRHL120	(AAAAG)8	GAAAAAGGGTGGAAATGCAAA	GGCACTACCTAACCAAAAGTAGGA	133-145	56	3	8	KP863135
LAREeSSRHL129	(AAAAGCATC)2	ATCTTCCCCTGCTGTTTGTG	GGGAGCGTTGAATGGATAGA	254	56	0	8	KP863136
LAREeSSRHL137	(AGO)7	GAGGATTTGTCACACCTTGA	ATGGGTTTGACAGCCGGATAA	100-112	56	4	8	KP863137
LAREeSSRHL138	(AATCATCAT)3	AAGGAGTGGGTTTTATTGGGG	AGGTGATGATGATGATGTACAATG	243-252	56	2	8	KP863138
LAREeSSRHL159	(AGAGCC)5	CACAGACCTCATGACCGATGG	TTCTGATTTCTGCCCTCTGGCT	224-236	56	3	8	KP863139
LAREeSSRHL161	(AGATGG)5	CGTTTCCAAAATGCCCTCAGT	ACACCCAGGGGAAGCTCCTAT	302-332	56	6	8	KP863140
LAREeSSRHL162	(AO)10	GGGTCACGTTCTACGAGGTTT	GCTAGGACTGCCACTGGATTT	85-119	56	9	8	KP863141
LAREeSSRHL163	(AAGGCC)3	AATGGAAGCGGTGAGGACATC	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	(AGCGGG)3	TAAATACGGCACAAGCCACA	GGAGGAGCAAATGGATCAAA	203-209	56	2	8	KP863145
LAREeSSRHL217	(ACTCATATG)2	AATCCAACAGAAAGGCCAAGA	GCCGGCAAATAGGTTGATATT	204-249	56	3	8	KP863146
LAREeSSRHL246	(AAT)9	TGGATGGTAAAGAACGCACAG	ACTTTTACCCGTTGTGGTGG	188-218	56	5	8	KP863147
LAREeSSRHL272	(AATATATAT)2	GCAACAACATCGAACAGCAA	TGTTTATAGGCCAAGCCACC	354-372	56	3	8	KP863148
LAREeSSRHL275	(AAACAAT)2	CTACCTAAGTCGGCCACAAA	TATCCTCGGAAACCATGAGG	354-362	56	2	8	KP863149
LAREeSSRHL283	(AATGGCAGAC)2	CCATTTCCCAAACTAAAACGC	GATGATGAGGCCCTTCAAAA	188-198	56	2	8	KP863150
LAREeSSRHL299	(AAACCTACG)2	GAAACGATACAATGGGGCT	CGTCCGGAACAAGAATGATA	446-456	56	2	8	KP863151
LAREeSSRHL308	(AAACATTT)2	TGCATTTGCTTGTGCTGCTAT	ATTGCACTGAATGCACAAGC	286	56	1	8	KP863152
LAREeSSRHL346	(ACCAGC)4	TAGAAAAGGGCAAAAGGCACATG	GGTGCAATTTCTCTCCACTCC	99-111	56	3	8	KP863153
LAREeSSRHL357	(AACAGC)3	AGGTCCAGCCATTGATGAAG	TCAATGCAATCCTGGGGTAT	162-168	56	2	8	KP863154
LAREeSSRHL358	(AATAATCTC)2	CTCCCACCTTACCACGAAAG	TGTGTAGCATTCCGTGCTC	135-145	56	2	8	KP863155
LAREeSSRHL361	(ACTC)5	GTATGCTGCCAAAAGGTGGTT	CATTTCCGGGCTTGTATTTG	275-311	56	3	8	KP863156
LAREeSSRHL366	(ACGGAT)3	TCCGTATCTGGATCTCGGGTT	AAAGAGGCAAGCGGTACTCA	244-256	56	3	8	KP863157
LAREeSSRHL372	(AAACCCG)3	GATTCGGAAATGGGGAATA	AGTTCAAAAATTTGGCGTTG	114-128	56	3	8	KP863158
LAREeSSRHL374	(AC)4	AGTTGAACCAACCCTCATCG	CTGTGGGGTGGAGATCCTTA	246-268	56	9	8	KP863159
LAREeSSRHL380	(AACGGC)3	GGCTGGTACATTTACAGGCAT	AGCCTCTCCTCCTCCTCAAC	184-202	56	3	8	KP863160
LAREeSSRHL391	(ACTGGC)4	AGCGTATGAATGGTCCAGG	ACGAAGATAGCTCGAACGGA	224-230	56	2	8	KP863161
LAREeSSRHL392	(AAACAAAACAG)2	GCGGTACAGGCTTTATCTCAG	ACCTGATGACCACGGGATAG	306-324	56	4	8	KP863162
LAREeSSRHL393	(CCG)8	GCCAGAACCACCGTTAAAAG	AGAGGCGATTATGGGAGCTT	296-302	56	3	8	KP863163
LAREeSSRHL394	(AAAGGC)4	GGGGAGGTGTTTGACAGAGA	AATCAACCCTTGGGAAATGAG	255-261	56	2	8	KP863164
LAREeSSRHL395	(ACCAGG)5	TTTGTCTTTAAGCTGGGCAGT	CAAAGCTTTCCGAAAGGGAAT	272-308	56	6	8	KP863165
LAREeSSRHL396	(AAGAGC)5	CTTTTGCCCTTTTCCCTTCC	TTGTGGGTGTCGTTTCACAAT	308-332	56	5	8	KP863166
LAREeSSRHL397	(AT)14	CAATGATCGAACTGTGGTTCA	GCTCATCTTCAACTTCAATGTGG	223-273	56	6	8	KP863167
LAREeSSRHL398	(AGCCTG)4	AGTCGGGGATGAAATCTGTG	TGTTTCTTTTGGGCATACACC	293-299	56	2	8	KP863168
LAREeSSRHL399	(AAAATC)5	CTTTGTGTGTCGGGATTTCTC	TTCCCTTTTCCCTTGGTCTTT	275-305	56	4	8	KP863169
LAREeSSRHL400	(AGCGGG)5	GAGACCTCCTGGCTTTGAT	TTAGAGCTGTGTGCGGCTGT	272-326	56	4	8	KP863170
LAREeSSRHL401	(ACCGCC)3	AGCAGAATAACGAGCCGAAG	CCCGCCACTACTCTGCTTAG	302-320	56	2	8	KP863171
LAREeSSRHL402	(AO)13	CACATATCTGTGTGTCCTGTG	TTAGGTTGCCAAAACCTGCAA	242-270	56	9	8	KP863172
LAREeSSRHL403	(AT)11	TCCATATTCATAACGCTCCT	GCTCCTTCATGTTGTAAGCAAA	286-290	56	3	8	KP863173
LARKeSSRHL404	(AAGCCC)4	TCTTGTGACATTCGCCTCTG	TCGATGTTGATCTTCACCTG	299	56	1	8	KP863174
LAREeSSRQ001	(CA)10	GCAAACTCATGTAGACTCGCC	CATTGGTGGAAACATTTGCTTG	182-210	56	6	8	JR170819

Locus	Motif	Primer sequence		Size (bp)	T _a	N _A	Ref.	Genebank accession number
		Forward	Reverse					
LAREsSRQ005	(GA)8	TTCCCTATTCTCATCCACGG	GTCGCCAGTAAATGGCCCTTA	246-252	56	4	8	JR171181
LAREsSRQ006	(AT)6	CCAAGAAGACCAAAAACATCAGA	TCGTCCCTGTTCACAACCA	131-175	56	6	8	JR171219
LAREsSRQ010	(TC)7	CCCAGAATGCAATACGGACT	TTCCCAAGGAAAATCTGGTGTG	216, 222	56	2	8	JR171974
LAREsSRQ017	(CAG)5	CCACCTCAAATCTTCTCCCA	CCATGCATATGAGTCTGCTGC	127-139	56	5	8	JR173000
LAREsSRQ020	(AG)6	TGATCGGCTTAAGTAAACCAA	TTGTGAGTGTTTGTGTGCGCA	219-231	56	3	8	JR173379
LAREsSRQ032	(TTG)6	CCCCCTGCACACCAATTTT	CAAGAATGCCGATACCGAAT	152-170	56	2	8	JR175164
LAREsSRQ035	(AT)7	CCTCGAACACTCACTAAACTTGC	ATGCCCTCTTGTGCAATCTT	108-118	56	5	8	JR175381
LAREsSRQ036	(TGC)6	TACTTCCCTGTGCTGGGTTT	GAAAAAGACTCCCAAGGGG	207-219	56	6	8	JR175557
LAREsSRQ048	(GAA)5	TGAAGAAGAAGCGGAAGAGG	AGGCTATACGCTTCCCTGCAA	434-461	56	2	8	JR176325
LAREsSRQ051	(TA)8G(TA)6	CGACTCAGCCACCTCGTAAT	ATTGCCAGAACCCCTTTTCT	234-268	56	13	8	JR176852
LAREsSRQ053	(AT)6	TGTCGCCCTTCACTCTGTGAG	ATCAATGCGGTGAAGATTCC	167-181	56	6	8	JR177135
LAREsSRQ066	(CA)14	GCTCTTGTGTGAGCCACCTTC	ATGGTTTGGATGCACATGAA	142-156	56	3	8	JR178582
LAREsSRQ067	(TC)8	ATCTCCTTGGAAATGTGTGCC	GGGGCGATTACCCTAAATGT	221-233	56	6	8	JR178682
LAREsSRQ070	(TA)6	GCTCCTCTTGCACAGTCTCC	TGCTCCATTTGTGGGTGTTA	164-198	56	12	8	JR178932
LAREsSRQ074	(AT)8	GTATGAAGAGCACCCCAAGG	GCAATAGTTGCAAGGCATGT	124-146	56	11	8	JR179414
LAREsSRQ104	(CA)7	ATCACTGCTCATGAGTCGCA	GTATGCGTTTGGGTGTGTGT	205-233	56	6	8	JR183015
LAREsSRQ113	(AC)10	TCCAATGGAGGACGTAAAGG	TCATGCATCATAACATTTGAATAACA	184-204	56	8	8	JR184160
LAREsSRQ114	(CA)7	GAAAACGGATATGGGAATGGA	TTGATGAATGGTAATCTGACCTATG	129-147	56	6	8	JR185111
LAREsSRQ115	(CTG)6	AATTAATGCGCTCACCTCG	GCAGATAAGCAGCCCTTCTT	317-332	56	3	8	JR185400
LAREsSRQ120	(ACTCT)5	ATTCCCCAATTCACGAAGC	TACTCCGAGAGGAGGCAGAA	110-115	56	2	8	JR186302
LAREsSRQ125	(AT)10	AAGGAAAATAAAGCCCTCG	TGCTCTCAGGTTGCAATGAG	100-144	56	11	8	JR186594
LAREsSRQ127	(ATG)5	GGTTTCCATTACAACCTCAAGG	GGATTCAGCTTCGGCTTTCAC	371-377	56	3	8	JR186781
LAREsSRQ137	(TG)7	GTGCCCTTGTTGGGTGTGCTTT	AAGAGTTGCCACCCATAAAGC	272-278	56	3	8	JR188117
LAREsSRQ141	(TC)9	CACACATGCAAAAGCAAACAA	TGTGTGTGAATGTGAGAGGGA	133-141	56	5	8	JR188688
LAREsSRQ183	(CTC)7	TGTTTGACGGTGACTGAAGG	TAGAGGAGCAGCGAGAGGAG	126-141	56	4	8	JR193542
LAREsSRQ187	(TG)8	TGAGGATTTCTTTCCCAATGC	CATTTGGATCCCAAAAGGGTAG	178-190	56	5	8	JR193964
LAREsSRQ195	(AGA)5	GCAGATTGTAGAAAGGGCTGC	CATCGCCTTCTCACACAGA	220-223	56	2	8	JR194843
LAREsSRQ206	(GTT)5	GCAGACCAATTTTCGTGAT	CGCATCTCAGAGGGAGAGAG	446-470	56	5	8	JR139531
LAREsSRQ209	(GGA)5	CCACGGAGTTTGGACTGAAT	CTAAACAGAGCCCAAGCGTC	182-188	56	3	8	JR139801
LAREsSRQ210	(TA)9	GTTCGATTTTGGCCCACTA	GATCAATTTTGGTTGCTGTCA	165-191	56	8	8	JR139804
LAREsSRQ213	(TTC)5	TTTTTGCTTTGTGAATGTGGC	TGGGATCCTGAGGGACTATG	300	56	1	8	JR140280
LAREsSRQ216	(AT)8	ATTTCTGCGGCAAGAGTTG	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	AATTAGTGGTGTCTTCGGTGG	TGGCACTTCTTGTAAATAAAAATCAA	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	TCTGCATCCTAGTGTGTGG	CCCCGGATCTTCTGAAACA	116-131	56	4	8	JR146140
LAREeSSRQ285	(GAG)5	CCGAGACATGATGCTGAGAA	TATTTGCAGAAAGCCCAACC	162-192	56	7	8	JR149637
LAREeSSRQ299	(CTG)5	AAACCAATGAAAATGCCTGC	TCCCCAGCCAACTCTCATAC	431-485	56	2	8	JR151216
LAREeSSRQ316	(TC)7	AGCTCTCTGTGCTTTCTCGC	GGAAAAGAGCAATTCAGCAGG	194-206	56	2	8	JR153273
LAREeSSRQ322	(CAG)5	AGGCGTCTGAGCTACCAAAA	CGACGACACCCCAATACCTTT	426-459	56	3	8	JR153722
LAREeSSRQ330	(TC)6	CAGGAAGTTGGGCAGCTTAG	GGTCTTGGCCCTTGTTGTTGT	255-267	56	3	8	JR154204
LAREeSSRQ352	(GCA)5	CCACCTCAAAATCTTCTCCCA	AGATGGAATACTGTTGGCGG	249-288	56	5	8	JR155690
LAREeSSRQ364	(AT)7	GATGAAATGGCGAAAGCAT	ACTGGCAATGTCCAAACTC	280-306	56	11	8	JR157274
LAREeSSRQ375	(TC)6	AGTGGCAGTCAGCATCTCCT	AGAAGATTTTGCAGAGGGCA	212-230	56	3	8	JR158646
LAREeSSRQ377	(TCATCC)5 ^{tcagtcctca} ggt(TCAGTC)5	TCATCATCCTCCTCGTCCTC	AAGATTCAGTGGATGGCGAC	178-208	56	6	8	JR158866
LAREeSSRQ382	(CAG)5	TGGTTCAACTTCTCTCGCCT	GGAAATGTGAACCGAAGACCGGT	299	56	1	8	JR159113
LAREeSSRQ386	(GA)13	TCCATCTTTTATTTGGCAGGC	CCATCAGAGATGGGAGTGCT	128-148	56	9	8	JR159815
LAREeSSRQ393	(AG)6	CCTTGTGAAGGGCACAGTTT	ATGAGGTCTGTGAGGGGTTG	371	56	1	8	JR160488
LAREeSSRQ397	(GA)9	TCTGAATCAATGTATCATGTATCGAA	CTGTCAGTCAATGCTGCGTTT	132-154	56	10	8	JR161052
LAREeSSRQ399	(AAG)5	AGACTCCCTGTTGGAAAAGGCA	AGACTCCCTGTTGGAAAAGGCA	254-263	56	2	8	JR161168
LAREeSSRQ403	(CAT)8	ACACAACATGCTACGATGCC	GCTTCTAGGCGTTCAACGAG	216-246	56	9	8	JR161642
LAREeSSRQ406	(AG)6	TGCATTCTGTAAATGCCAA	TGTTGATGAGCAATGACCCGT	361-385	56	4	8	JR161926
LAREeSSRQ408	(GACTG)7	CAAGCAITCTTCCCCAAAAA	TAAGTCCAGTCCAGTCCCGGT	144-180	56	6	8	JR162009
LAREeSSRQ409	(AT)9	AAAATTCATCCTCGAACACTCA	TGGACAATGTTCCATGCAGT	181-199	56	8	8	JR162187
LAREeSSRQ430	(CGG)5(CTG)2TTGA (TGC)6trtgatcgtgatg g(TGC)8	TTTGTGGTCCGATCAGGAGTC	CAACTTTTGGGTTGGGAGAA	286-313	56	9	8	JR166454
LAREeSSRQ439	(AAT)5	TCTCGCTCGGCTTCTACATT	GAGATTTCTGCTGCTTCCCTG	258	56	1	8	JR168298
LAREeSSRQ444	(TGC)6	GAACGTTCAAACTGCACAG	TTGAGTTCAITTTGGCTGCAAG	406-415	56	2	8	JR168664
LAREeSSRQ449	(AT)8	CCCTTAGCCCTCTTTTGGGA	ACCATCGAAACGTGTCAACAA	272-298	56	5	8	JR169475

I-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. 2010, 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 et al. 2015)

Examples for amplification protocols (nSSR)

- 94°C for 1 min followed by 10 cycles of 94°C for 30 s, 63-53° C (-1° C at each cycle) for 45 s, followed by 25 cycles of 94°C for 30 s, 53° C for 30 s, 72°C for 1 min; final elongation at 72° C for 10 min (Isoda and Watanabe 2006).
- 94°C for 4 min followed by 30 cycles of 94°C for 45 s, 56° C for 45 s, 72°C for 45 s; final elongation at 72° 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, T_a (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, T_a (Table 4) for 45 s, 72°C for 60s; final elongation at 72° 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° 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 principis-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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DNA-markers

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