International Seed Federation
Seed is Life

Guideline on indication of the proportion of hybrid seeds in hybrid larch (Larix x eurolepis) seed lots

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PREPARED BY THE
ISF Tree and Shrub Group

SCOPE

The scope of the guide was meant to cover all controlled hybrid tree species. Currently hybrid larch is the most relevant controlled hybrid species that subject for international seed trade however other species may follow in the future.

PURPOSE AND DISCLAIMER

This guide is designed to assist stakeholders – tree seed companies and seed producers who are engaged in tree seed production and sales – in providing the best service to their customers through transparency and information sharing.

The Guide is not exhaustive. It is the responsibility of any user of this Guide to consider the user's specific circumstances when developing a process specific to its organization, and in meeting any applicable legal requirements.

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INTRODUCTION

Hybrid larch is an important tree species in Europe. Currently there is no harmonization within Europe on the minimum proportion of hybrid seeds in hybrid larch seed lots. There are member states who already set up a minimum threshold while in others this area remains totally unregulated. This creates confusion among tree seed companies, nurseries and the forest industry when seeds, reproductive materials or young plants are crossing borders. Harmonization of minimum proportions of hybrid seeds in Europe seems challenging especially in the short term. One of the alternative solutions is to increase transparency and information sharing of the traded seeds that could give clarity for all respective stakeholders in the value chain.

RECOMMENDATIONS

The ISF Tree and Shrub Group recommends to its members to implement the following measures:

- Include information in the seed supplier document on the proportion of hybrid seeds in hybrid larch seed lot. (Annex 1)
- Upon demand, supply the test result that indicates the proportion of hybrids. The test should be performed by an accredited (official) laboratory using an internationally recognized (validated) testing method. (Annex 2)
- Inform non ISF member tree seed companies and other stakeholders in the value chain (nurseries, foresters etc.) about these proposed measures.

ANNEX 1

The OECD FRM Suppliers Document is the preferred format to indicate the proportion of hybrids seeds in hybrid seed lots.

COMMERCIAL DATA (1)	
SUPPLIER'S DOCUMENT NUMBER (1.1)	OECD/MASTER CERTIFICATE NUMBER (1.2):
BOTANICAL NAME (1.3):	
COMMON NAME (1.4):	
BATCH IDENTITY (1.5):	DELIVERED QUANTITY (1.6):
SUPPLIER (1.7):	SUPPLIED TO (1.8)

GENETIC CHARACTERS (2)			
CATEGORIES (2.1):			
SOURCE IDENTIFIED (2.1.1) ☐	SELECTED (2.1.2) ☐	QUALIFIED (2.1.3) ☐	TESTED (2.1.4) ☐ Conditionally approved (2.1.4.1): ☐

NATURE OF FOREST REPRODUCTIVE MATERIAL (2.2):	TYPE OF BASIC MATERIAL: (2.3):
SEED (2.2.1) ☐	SEED SOURCE (2.3.1) ☐
	STAND (2.3.2) ☐
PARTS OF PLANT (2.2.2) ☐ ..... Type (2.2.2.1)	SEED ORCHARD (2.3.3) ☐
PLANT (2.2.3) ☐ ..... Type (2.2.3.1)	PARENTS OF FAMILY (IES) (2.3.4) ☐
	CLONE (2.3.5) ☐
	CLONAL MIXTURE (2.3.6) ☐

PURPOSE (2.4):
REGISTER REFERENCE (2.5):

COUNTRY AND REGION OF PROVENANCE OR LOCATION OF BASIC MATERIAL (2.6):		
COUNTRY OF BASIC MATERIAL (2.6.1):	REGION OF PROVENANCE (2.6.2):	LOCATION COORDINATES (2.6.3)

ELEVATION / ALTITUDE (IN METERS) (2.6.4):

ORIGIN OF BASIC MATERIAL: (2.7):		
AUTOCHTONOUS (2.7.1) ☐	NON AUTOCHTONOUS (2.7.2) ☐	UNKNOWN (2.7.3) ☐

NUMBER OF HARVESTED TREES (WHERE APPROPRIATE, BEST ESTIMATE) (2.8):

QUALITY CHARACTERS (3)	
A) SEED:	
PURITY % (3.1):	INERT MATTER % (3.1.1) OTHER SEED % (3.1.2)
MOISTURE CONTENT % (3.2):	GERMINATION (%) (3.3): SUBSTRATE (3.3.1):
VIABILITY (%) (3.4): Method (3.4.1):	THOUSAND SEED WEIGHT (g) (3.5):
NUMBER OF GERMINABLE (VIABLE) SEEDS /KG (3.6):	DATE OF LAST ANALYSIS (3.7)
YEAR OF RIPENING (3.8):	HYBRID (%) (if appropriate): (3.9):
B) PLANTS:	
LENGTH OF TIME IN NURSERY/ AGE OF THE PLANT (3.10)	HAS THERE BEEN SUBSEQUENT VEGETATIVE PROPAGATION OF MATERIAL DERIVED FROM SEED (3.11)?
ADDITIONAL INFORMATION AS REQUIRED BY THE NATIONAL DESIGNATED AUTHORITY (4):	

ANNEX 2

The proposed method for sampling and testing the proportion of hybrid is the method developed by Institut National de la Recherche Agronomique (INRA).

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ORIGINAL PAPER

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Chloroplast and mitochondrial molecular tests identify European×Japanese larch hybrids

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Abstract Hybrids between European and Japanese larches combine the properties of both parental species (drought resistance, canker resistance, stem straightness) and exhibit a fast growth rate. They are produced in seed orchards, generally by natural pollination. Seeds are collected and used for afforestation as interspecific hybrids. However, there are no convenient tests to assess the interspecific hybrid proportion. In the present study, we developed diagnostic molecular markers suitable for the individual identification of hybrids, whatever their developmental stage. Our strategy involved testing a combination of maternally inherited markers from the mitochondrial genome (mtDNA) and paternally inherited markers from the chloroplast genome (cpDNA). Hybrids were then identified by the presence of a mitochondrial sequence inherited from one parental species and a chloroplast sequence inherited from the other parental species. To achieve this aim, markers discriminating both parental species were first sought. Amplifications of mitochondrial and chloroplast sequences were performed using specific PCR primers. After testing 33 primer pairs in combination with nine restriction enzymes, we detected one mitochondrial marker, *f*13 which was amplified in Japanese larch and absent in European larch, and one chloroplast marker, *ll-Taq*I which showed different restriction patterns depending on the species. A restriction fragment of 601 bp was obtained in Japanese larch while two fragments of 120 bp and 481 bp were observed in European larch. These patterns were found in all 197 individuals tested from the two pure species. These markers were then used for the evaluation of the hybrid proportion in a seed lot produced from seed orchards; this was assessed as between 43% and 53% depending on the parental species. The male and female parental species could be determined for each progeny.

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Introduction

Various crosses between species of the *Larix* genus have been reported throughout the world, but the most economically important ones concern hybrids between European larch (*Larix decidua* Mill.) and Japanese larch [*L. kaempferi* (Lamb.) Carr.]. The interspecific hybrid *L.×eurolepis* Henry, combines interesting traits of both parental species, i.e., relative canker resistance and fast juvenile growth from *L. kaempferi* together with stem straightness and wood quality from *L. decidua* (Bastien and Keller 1980; Pâques 1989). Selected varieties are commercially produced in hybridisation seed orchards, composed of an intimate mixture of *L. decidua* and *L. kaempferi* clones (Mitchell 1958). While two-clone orchards would optimise hybrid vigour, orchards are most commonly established with a couple or several clones of each parental species in order to enhance genetic diversity. Seeds are generally produced by open pollination. Therefore, the seed orchard crop consists of a variable proportion of hybrid seeds, with seeds from one or both target parental

species as well from pollen contamination. While morphological traits allow identification of species at the mature stage, so far no such traits exist to distinguish either hybrid seeds or juvenile plants. The hybrid fraction is not known, except for seed lots from a few orchards where isozymes can provide a reliable assessment. However, isozymes are generally not efficient for hybrid identification since several alleles are shared by both species at most loci (Häcker and Bergmann 1991; Ennos and Qian 1994). Consequently, they can be used in few seed orchards only. There is a need for the development of molecular markers to distinguish *L. decidua* and *L. kaempferi* and their hybrids. Such studies have already been developed for other tree species for which hybrids are difficult to detect on the basis of morphology, such as in *Paulownia* (Wang et al. 1994) or *Fraxinus* (Jeandroz et al. 1996). Scheepers et al. (2000) have developed RAPD markers to identify larch hybrids from their parental species. They obtained several markers suitable for the identification of parental species and of their hybrids. In spite of their success for hybrid larch identification, the application of these makers has not yet been extended. Recognition of F_1 interspecific hybrids can be achieved most readily using uniparentally inherited genetic markers possessing important and unique characteristics. In Pinaceae, the mitochondrial genome is maternally inherited whereas the chloroplast genome is paternally inherited (Neale and Sederoff 1989; Sutton et al. 1991; Hipkins et al. 1994; Chen et al. 2002). These features were confirmed for *Larix* (Szmidt et al. 1987; De Verno et al. 1993). Thus, specific chloroplast and mitochondrial DNA markers would be ideal for the identification of interspecific hybrids since these will provide information from the two parents. Introgression in natural stands of Pinaceae species have been shown by polymorphism analysis of cytoplasmic genomes (Govindaraju et al. 1988; Sutton et al. 1991; Wang and Szmidt 1994). Polymorphisms of cytoplasmic genomes are thus well adapted for hybrid identification in *Larix*.

In this study, we looked for variations in the cpDNA and the mtDNA in order to detect taxon-specific polymorphisms between *L. decidua* and *L. kaempferi.* A representative sample of both *Larix* species was analysed. Sequences of cpDNA and mtDNA were amplified by polymerase chain reaction using specific primers. In the absence of size polymorphisms in the amplified fragments, restriction enzymes were tested to obtain fragment length polymorphisms. Diagnostic markers from both cpDNA and mtDNA could then be identified. The usefulness of these markers for hybrid identification was tested on a seed sample from a seed orchard. Association of cpDNA and mtDNA markers enabled effective hybrid identification irrespective of the plant developmental stage.

Materials and methods

Plant material

For the identification of diagnostic polymorphisms for hybrid recognition, 26 native provenances of *L. decidua* and 12 of *L. kaempferi* were used. The INRA Tree Breeding Research Station in Orléans (France) supplied the plant material, a total of 197 individuals. These individuals represent almost the whole natural range of the species. The geographic origins of *L. decidua* individuals were: the Alps (4 isolates), the Carpathian mountains (2 isolates), central Poland (3 isolates), Sudeten region (15 isolates) and the Tatra mountains (2 isolates). Some non-autochthonous individuals were also added, leading to a total of 109 individuals. For *L. kaempferi*, 53 individuals originating directly from Japanese sources (Hondo Island: Akanagi, Asamayana, Ina, Kamikochi, Mizunoto, Mt Fuji, Nagakurayama, Nishidake, Okunikko, Takasegawa, Yashubara, Yatsugatake) and 35 non-autochthonous individuals were available, leading to a total of 88 individuals.

Hybrid larch seed lots were collected from a Belgian seed orchard in Halle. This orchard is of a multiclonal type: i.e., 19 *L. decidua* clones (281 trees) and 16 *L. kaempferi* clones (294 trees) are intermixed on a tree-by-tree basis. The seed crop used in this study (1998) was collected from 12 *L. decidua* clones and three *L. kaempferi* clones separately. Seeds were germinated on filter paper in Petri dishes, at 25°C in the dark. At radicle emergence, seeds were transferred into the greenhouse and grown in fine vermiculite blocks under a 16 h day photoperiod. Germination rates were 53% and 51% for seeds collected from *L. decidua* and *L. kaempferi* respectively. These germination rates were similar to those obtained on the other larch seed lots. One month later, needles were sampled on the plantlets and were frozen at −80°C until DNA extraction. In total, 97 plants from female parent *L. decidua* and 108 plants from female parent *L. kaempferi* were analysed to determine the hybrid fraction in the seed crop from this orchard. We also sampled the needles of different parental clones in the Halle seed orchard.

DNA extraction

Total DNA was isolated from 100 mg of needle tissue per sample using a Qiagen DNeasy Kit and then used for PCR reactions.

PCR amplification and polymorphism analyses

Non-coding regions were amplified in order to reveal polymorphisms. Intergenic regions in the chloroplast genome, introns in the mitochondrial genome and some coding sequences (e.g. *rbc*L, *cox*2) were investigated (Table 1). As there are few sequences of chloroplast and mitochondrial genomes available in *Larix*, consensus primer pairs were designed from conserved regions. Moreover, we also defined some primer pairs to amplify a cpDNA region that has been reported to display polymorphisms in *Larix*. In a phylogenetic analysis of *Larix* Qian et al. (1995) found one polymorphic site differentiating *L. decidua* and *L. kaempferi* in the chloroplast genome using *Pinus contorta* probe K140 (Ennos, personal communication). This single probe corresponds to the region located between the *psb*A and *rps2* genes according to Lidholm and Gustafsson (1991). We designed two cpDNA primer pairs to amplify the regions *trn*R-*atp*F and *atp*F-*rps*2 in order to complete the region corresponding to the probe K140. Primer sequences were based on the complete nucleotide sequence of the chloroplast genome of *Pinus thunbergii* (Wakasugi et al. 1994). D. Scheepers (personal communication) provided a primer pair to amplify a fragment, called *f*-13, with maternal inheritance (5′-CTGTTGGTAACTTGGGG-3′ and 5′-GCGCCTCTTTCGGAATAG-3′). This is considered to be of mitochondrial origin.

Twenty-two chloroplast and 11 mitochondrial fragments were investigated to characterise *L. decidua* and *L. kaempferi* (Table 1). Amplifications were performed in 25 μl volumes using an MJ

Table 1 Chloroplast and mitochondrial DNA sequences investigated

Chloroplast genome			Mitochondrial genome		
Amplified region		Source of primer pairs used	Amplified region		Source of primer pairs used
f2	*atp*F-*rps*2	This study	*cx* 1	*cox* 1	Wu et al. (1998)
as	*psa*A-*trn*S	Demesure et al. (1995)	*cx 2*	*cox* 2 exon1- *cox* 2 exon2	Dumolin-Lapègue et al. (1997b)
cs	*psb*C-*trn*S	Demesure et al. (1995)	*f* 13	*f* -13	Scheepers (pers. comm.)
lf	*rbc*L- *orf* 512	Petit et al. (1998)	*nd* 1	*nad* 1 exon2- *nad* 1 exon3	Demesure et al. (1995)
li	*rbc*L-*psa*I	Petit et al. (1998)	*nd - rp*	*nad* 3- *rps* 12	Wu et al. (1998)
ll	*rbc*L	Petit et al. (1998)	*nd* 4/1	*nad* 4 exon1- *nad* 4 exon2	Demesure et al. 1995)
c2c1	*rpo*C2-*rpo*C1	Petit et al. (1998)	*nd* 4/2	*nad* 4 exon2- *nad* 4 exon3	Dumolin-Lapègue et al. (1997b)
cd	*trn*C-*trn*D	Demesure et al. (1995)	*nd* 5	*nad* 5 exon1- *nad* 5 exon2	Wu et al. (1998)
dt	*trn*D-*trn*T	Demesure et al. (1995)	*nd* 7	*nad* 7 exon3- *nad* 7 exon4	Dumolin-Lapègue et al. (1997b)
fv	*trn*F-*trn*V	Dumolin-Lapègue et al. (1997b)	*rp - cob*	*rps* 14- *cob*	Demesure et al. (1995)
hk	*trn*H-*trn*K	Demesure et al. (1995)	18–5S	18S rrna-5S rrna	Petit et al. (1998)
kk	*trn*K	Demesure et al. (1995)	–	–	–
kq	*trn*K-*trn*Q	Dumolin-Lapègue et al. (1997b)	–	–	–
ml	*trn*M-*rbc*L	Demesure et al. (1995)	–	–	–
qr	*trn*Q-*trn*R	Dumolin-Lapègue et al. (1997b)	–	–	–
rf	*trn*R-*atp*F	This study	–	–	–
sm	*trn*S-*trn*fM	Demesure et al. (1995)	–	–	–
st	*trn*S—*trn*T	Demesure et al. (1995)	–	–	–
tc	*trn*T-*psb*C	Dumolin-Lapègue et al. (1997b)	–	–	–
tf	*trn*T-t*rn*F	Taberlet et al. (1991)	–	–	–
va	*trn*V-16S rrna	Petit et al. (1998)	–	–	–
vl	*trn*V-*rbc*L	Dumolin-Lapègue et al. (1997b)	–	–	–

Research PTC-100 thermal cycler. The reaction mixture contained 20–40 ng genomic DNA, 1 ng BSA, 2 mM $MgCl_2$, 20 mM Tris HCl, pH 8.0, 1 mM EDTA, 1 mM dithiothreitol, 0.3 mM of each dNTP, 0.3 μM of each primer and 1.5 U *Taq* DNA polymerase (Appligene-Oncor, France). The amplification consisted of the following program: 6 min at 94°C followed by 35 cycles, each of 45 s at 94°C, 45 s at 55°C and 3 min 30 s at 70°C. A final elongation step (72°C) of 10 min was then performed. Amplification products were separated by electrophoresis in 1% or 1.5% (w/v) agarose gels run in 1×TAE buffer at 15 V/cm for 1 h. Gels were stained with ethidium bromide (0.5 μg/ml staining solution) and visualised under UV light.

The expected patterns exhibited by the polymorphisms were either absence/presence of a fragment, variation in fragment size or monomorphic bands. We looked for restriction fragment length polymorphisms in monomorphic bands using 9 four-base recognition restriction endonucleases: *Alu*I, *Hae*III, *Hha*I, *Hpa*II, *Mbo*I, *Msp*I, *Nde*II, *Rsa*I, and *Taq*I. In addition, *Bcl*I restriction endonuclease was also tested for the chloroplast region between *psb*A and *rps2* since Qian et al. (1995) found differences between *L. decidua* and *L. kaempferi* using this enzyme with the probe K140 (Ennos, personal communication). PCR products (5 μl) were digested with each restriction endonuclease (2 U), following the recommendations of the manufacturer (Appligene-Oncor, France). Digested DNA fragments were then separated by electrophoresis on 8% polyacrylamide gesl. Gels were stained with ethidium bromide and scanned using FM-BIO II (Hitachi, Multi-View).

An initial screen for the extent of polymorphism was tested on 20 individuals of each species. When a polymorphism that could discriminate the species was observed with a marker, it was then analysed in the complete set of 197 plants of parental species. Only markers discriminating species with few or no exceptions were selected. The polymorphic amplified fragments were directly sequenced in both directions to clarify the precise differences between species.

Considering that sampling within species is representative of their natural variation, mis-identification of species with presumed monomorphic molecular markers can occur as a consequence of polymorphisms not detected in the sample tested. The maximal frequency of mis-identification correspond to the assessed frequency f of an eventual other allele according to the sample size N at a given statistical threshold: $(1\text{-}f)^N<0.05$.

Results

Chloroplast genome polymorphisms

Ten out of the 22 cpDNA primer pairs used gave clear amplification products in the two species *L. decidua* and *L. kaempferi*. These primer pairs amplified three different regions of the chloroplast genome: *trn*K to *rps*2 (*kk*, *kq*, *qr*, *rf*, *f2*), *psb*C to *trn*fM (*cs*, *sm*), from *trn*S to *trn*F (*st*, *tf*) and the *rbc*L gene (*ll*). The fragment sizes corresponded to those expected according to the cpDNA sequence of *Pinus thunbergii* (Wakasugi et al. 1994). No length polymorphisms could be detected by electrophoresis on agarose gels.

Amplification products were cut with restriction enzymes. Among the 95 cpDNA fragment/enzyme combinations tested, only six revealed polymorphisms. Four polymorphic combinations consisted of intraspecific variations in *L. decidua* (combinations *sm*/*Taq*I, *kk*/*Taq*I, *f2*/*Hpa*II and *ll*/*Hha*I) and one in *L. kaempferi* (combination *ll*/*Hae*III). The *Bcl*I restriction endonuclease did not reveal any polymorphisms in the *psb*A to *rps*2 region. Only one combination (*ll* digested by *Taq*I, marker *ll-Taq*I) revealed an interspecific polymorphism (Fig. 1). This marker discriminated all *L. decidua* and *L. kaempferi* individuals tested (Table 2). All the *L. decidua* individuals displayed the specific fragments of 481 and 120 bp and all the *L. kaempferi* individuals the specific fragment of 601 bp (sizes determined from the complete sequence of the amplified fragment). This polymorphism is due to a single nucleotide substitution in the restriction site: TCGA, recognised by *Taq* I in *L. decidua* and TCAA in *L. kaempferi*. All individuals shared other fragments (309, 261, 147 and 63 bp). No intraspecific polymorphism of

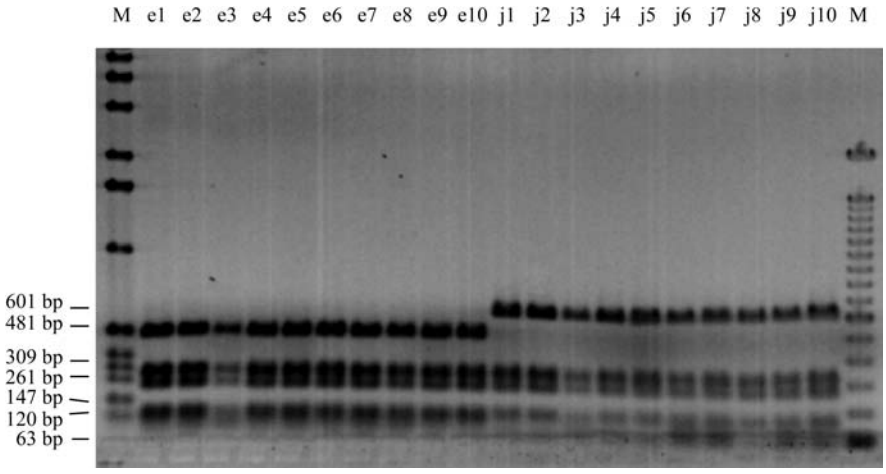

Fig. 1 Polymorphisms of the chloroplast *ll-Taq*I marker in both *Larix* species revealed on a 1.5% agarose gel. *Lanes e1 to e10* individuals of *L. decidua*; *j1 to j10* individuals of *L. kaempferi*, *M* molecular weight markers (1 kb and 100 bp)

Table 2 Interspecifc polymorphisms observed for the diagnostic markers in pure species

Marker	*ll - Taq* 1		*f* -13	
Species	*L. decidua*	*L. kaempferi*	*L. decidua*	*L. kaempferi*
Sample size tested	109	88	109	88
Individuals with typical *L. decidua* pattern	109	0	109	0
Individuals with typical *L. kaempferi* pattern	0	88	0	88
Maximal frequency of misidentification (P >0.95)	0.027	0.033	0.027	0.033

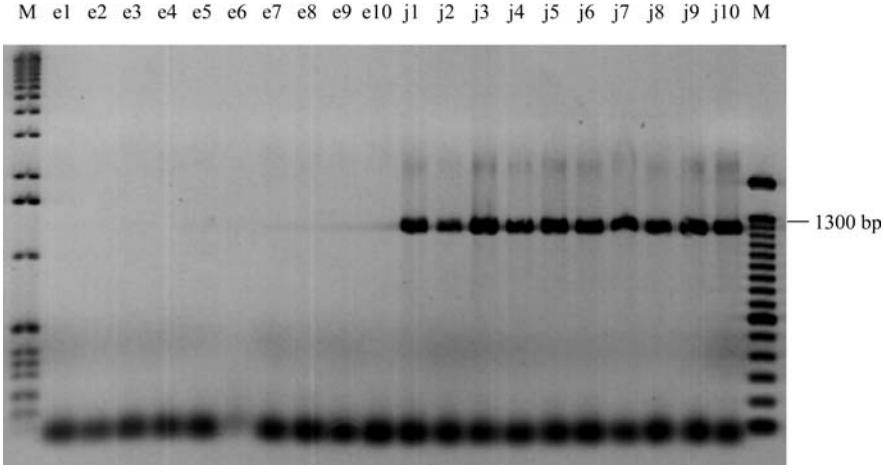

Fig. 2 Polymorphisms of the mitochondrial *f*13 marker in both *Larix* species revealed on a 1.5% agarose gel. *Lanes e1 to e10* individuals of *L. decidua*, *lanes j1 to j10* individuals of *L. kaempferi*, *M* molecular weight markers (1 kb and 100 bp)

the fragment *ll* restricted with *Taq*I has been detected for the different isolates of both *Larix* species (197 representative individuals). Fragment *ll* (1,381 bp) represents a part of the *rbc*L gene (1,428 bp), which encodes the large subunit of ribulose 1,5 bisphosphate carboxylase. The sequence of fragment *ll* was identical with the *rbc*L sequences in *L. kaempferi* (EMBL accession AB045038; Kobayashi et al. 2000), while in *L. decidua* there was a one nucleotide difference from the EMBL accession AB019826 (Wang et al. 1999) in an *Alu*I restriction site (an intraspecific polymorphism not detected in the initial screening). We recorded four different nucleotides in the *ll* sequences of the two species, but only three, including that of the *Taq*I restriction site, in the *rbc*L sequences of EMBL accessions.

Mitochondrial genome polymorphisms

Eight of the 11 mtDNA primer pairs tested gave amplification products (*cx*2, *f*13, *nd*1, *nd*4/1, *nd*4/2, *nd*5, *nd-rp*, *rp-cob*). No polymorphisms could be detected following PCR or PCR-RFLP for *cx*2, *nd*1, *nd*4/1, *nd*4/2, *nd*5, *nd-rp* and *rp-cob*. The sequences of *nd*1 (1,100 bp) were identical in both species. In contrast, the fragment *f*13, of approximately 1,300 bp, was amplified only in *L.*

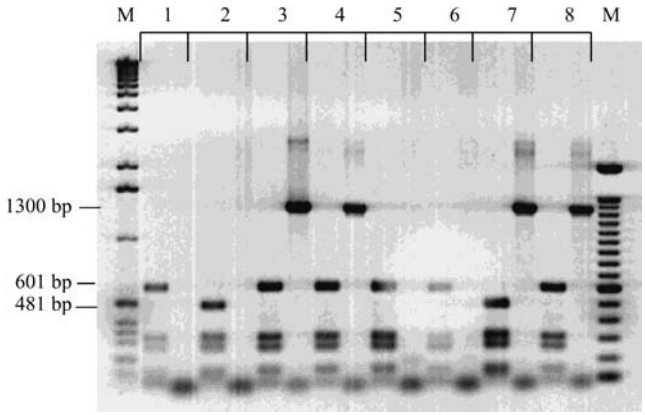

Fig. 3 Combined test of maternity (mitochondrial marker *f*13) and paternity (chloroplast marker *ll-Taq*I) for hybrid larch identification applied to plantlets from the Halle orchard. For each plantlet, the *left lane* corresponded to the chloroplast marker and the *right lane* to the mitochondrial marker. The presence of the mitochondrial fragment of 1,300 bp indicates that the maternal parent is a tree of *L. kaempferi*, the chloroplast fragment of 481 bp indicates that the paternal parent is *L. kaempferi* while that of 601 bp indicates that it is *L. decidua*. Plantlets 1, 5, 6 and 7 were interspecific hybrids

kaempferi, (Fig. 2), as detected by Scheepers (personal communication). The 197 individuals tested were all assigned to a taxon consistent with their documented taxa (Table 2). The sequence of the fragment *f*13 had no homology with recorded sequences in GenBank and its function remains unknown.

Characterisation of hybrids

The ability of the two diagnostic markers (*ll-Taq*I and *f*13) to discriminate hybrids and parental species was tested in the Halle seed orchard. These markers allowed us to identify all parental clones as *L. decidua* or *L. kaempferi* clones. *L. decidua* clones presented the typical *ll-Taq*I restriction fragments of 120 bp and 481 bp and did not allow amplification with the *f*13 primer pair while the *L. kaempferi* clones exhibited the *ll-Taq*I restriction fragment of 601 bp and the *f*13 fragment. One clone was in fact mislabelled in the orchard; a morphological analysis confirmed the mistake deduced from molecular marker patterns.

Germinated seeds, collected from female *L. decidua,* showed the lack of fragment *f*13 and segregated for the *ll-Taq*I marker (plantlets 1, 2, 5 and 6 in Fig. 3). Plantlets exhibiting the *ll-Taq*I restriction fragments of 120 bp and 481 bp were produced from pollination by *L. decidua* individuals while those with the *ll-Taq*I restriction fragment of 601 bp were produced from pollination by *L. kaempferi* (plantlets 1, 5 and 6 in Fig. 3) and thus were interspecific hybrids.

In contrast, germinated seeds collected from female *L. kaempferi*, showed the presence of the fragment *f*13, and also segregated for the *ll-Taq*I marker (plantlets 3, 4, 7 and 8 in Fig. 3). Plantlets exhibiting the *ll-Taq*I restriction fragment of 601 bp were produced from pollination by *L. kaempferi* (plantlets 3, 4 and 8 in Fig. 3), while those showing the *ll-Taq*I restriction fragments of 120 bp and 481 bp (plantlet 7 in Fig. 3) were produced from pollination by *L. decidua* and were thus the result of interspecific hybridisation.

Finally, the chloroplast (*ll-Taq*I) and mitochondrial (*f*13) diagnostic markers were used to estimate the hybrid proportion in the two seed lots obtained in 1998; 52.6% and 42.6% of seeds collected from the *L. decidua* clones and from *L. kaempferi* clones respectively. The other seeds originated either from selfing or intraspecific crosses.

Discussion

Species and hybrid identification

In this study, we found two markers that differentiated *L. decidua* from *L. kaempferi*. The mitochondrial *f*13 marker was strictly maternally inherited as confirmed by the analysis of seeds produced in the Halle orchard. The cpDNA *ll-Taq*I marker was transmitted by pollen, since seeds have a genotype different to the maternal one. These observations on a large progeny sample in *Larix* confirmed the maternal inheritance of the mitochondrial genome (De Verno et al. 1993) and the paternal inheritance of the chloroplast genome (Szmidt et al. 1987). Such inheritance of cytoplasmic genomes is usual in the family Pinaceae and can also be found in a few angiosperms like *Actinidia* (Testolin and Cipriani 1997).

According to their inheritance and to their interspecific polymorphisms, the two markers *ll-Taq*I and *f*13 are sufficient to identify first-generation hybrid individuals by the association of the mitochondrial female parent genotype and the chloroplast male parent genotype. Indeed, simultaneous analysis of the diagnostic cpDNA and mtDNA markers will allow the individual identification of hybrids but also the determination of the direction of the interspecific cross (*L. decidua* × *L. kaempferi* or reciprocal).

These markers have been tested with a sample of about 100 independent individuals of each parental species and indicate a complete concordance between the marker phenotypes and taxonomic assignment based on morphological characteristics. This will give a maximum possible error rate of approximately 3% if any undetected randomly distributed polymorphism exists for the markers tested within each species (Table 2). The hybrid fraction in a seed lot can be thus assessed whatever the genetic composition of the orchard under consideration. This form analysis can be applied for to plantlets in the nursery as well as adult trees, whatever their developmental stage or the season.

The test based on the *f*13 and *ll-Taq*I markers is easy to perform. These markers are obtained after a specific amplification step and only one of them requires a further restriction digest. Polymorphisms can then be observed directly on agarose gels (Figs. 1 and 2). For the mitochondrial marker, we recommend the use of a positive

control to avoid ambiguities due to the absence of amplification in the female parent *L. decidua*. When the test is performed on seeds, it can be further simplified; the *ll-Taq*I marker alone, amplified from the complete seed DNA, including embryo and megagametophyte DNA, shows association with both the maternal and paternal patterns. A hybrid can be recognised by the simultaneous presence of the 120 bp, 481 bp and 601 bp restriction fragments. We have successfully tested this possibility (data not shown) and the three fragments were clearly observed.

The hybrid fractions in the seed lots we analysed (43% and 53%) were high compared to those (4–29%) reported by Scheepers et al. (2000) for *Larix* seed orchards which were open pollinated. In contrast, when artificial pollination (Philippe and Baldet 1992) is applied, a higher level of hybridisation has been revealed (Scheepers et al. 2000). In the Halle seed orchard, the high number of clones could favour interspecific hybridisation.

Polymorphisms of parental species

The level of polymorphism between the two *Larix* species was remarkably low. Sequences of the intron between exons 2 and 3 of *nad*1 and of the *rbc*L gene revealed only three point mutations (all in the *rbc*L gene) from 2,500 bp. Only two out of the amplified fragments revealed interspecific differences.

Firstly, a mitochondrial sequence was amplified in *L. kaempferi* and not in *L. decidua*. The absence of amplification was probably due to a deletion/insertion in the amplified region since a new primer pair designed in the amplified fragment, at more than 20 bases away from the original *f*13 primer pair also provided an amplified product in *L. kaempferi* only. Plant mtDNA has a very low rate of gene sequence evolution (Wolfe et al. 1987), suggesting a much lower rate of point mutation in plant mtDNA than in cpDNA (Sederoff 1987; Palmer and Herbon 1988). Such an observation has been particularly demonstrated in *Quercus* (Dumolin-Lapègue et al. 1998) or *Olea* (Besnard et al. 2002). Moreover, Lu et al. (1998) reported the lack of RNA editing for the *cox*I gene in few gymnosperms including *Larix sibirica*. They considered that this could reduce the divergence between species.

Secondly, the chloroplast *ll* sequence (1,381 bp) was polymorphic and showed three point mutations between the two *Larix* species, one of them being involved in a *Taq*I restriction site. We identified a specific polymorphism in the *rbc*L gene enabling the distinction between *L. decidua* and *L. kaempferi*. Moreover a high level of polymorphism exists in this gene in conifers (Tsumura et al. 1995). However, intra-specific variations for the *rbc*L gene were detected in *L. decidua* and *L. kaempferi*. This intraspecific diversity is characterised by a restriction fragment length polymorphism as already shown for various tree species including *Quercus* (Dumolin-Lapègue et al. 1997a). The reliability of the intraspecific variation in the *rbc*L gene for differentiation of trees from different geographical origins has not been investigated. In *L. decidua*, Maier (1992) has defined two geographical groups of isolates according to their patterns at several isoenzyme loci. Chloroplast DNA polymorphisms could also reveal a geographic clone (Vendramin et al. 1998). The chloroplast *trn*T-*trn*F sequence analysed by Wei and Wang (2003) did not show any difference between *L. decidua* and *L. kaempferi* in the 1,368 bp amplified fragment. The chloroplast genomes of both *Larix* species appeared very similar as already shown by Qian et al. (1995). In pines, Wang and Szmidt (1994) revealed a low level of chloroplast polymorphism by restriction fragment polymorphism within pure species while they noticed large variation within hybrid species.

Amplification and primer transferability

Among the 33 chloroplast and mitochondrial primers tested, only 18 (55%) led to an amplification in the *Larix* species. Most of the primers tested were successfully used and validated in various other plant species, including angiosperms and gymnosperms: *Quercus* (Demesure et al. 1995; Dumolin-Lapègue et al. 1997b), *Fagus* (Demesure et al. 1995) and *Pinus* (Wu et al. 1998; Chen et al. 2002). Nevertheless, their application in a single taxonomic unit, as in *Larix* species, was not completely successful. Jaramillo-Correa et al. (2003) have also observed difficulties for applying universal mitochondrial primers in gymnosperms.

Conclusions

This test for hybrid identification in *Larix* using PCR-RFLP methodology is fast, effective and reproducible. It can be advantageously applied to a wide variety of plant material at any stage, including very early stages such as seeds. At the practical level, besides seed purity qualification and certification, the estimation of hybrid proportion in seed lots allows the ability to control annual efficiency of pollination (analysis of crop over years), to compare different seed orchards management (e.g., structure of orchards, pollination techniques), and to analyse each clone's propensity to produce hybrid seeds within a given seed orchard. All these applications will improve the efficiency of hybridisation seed orchards for producing a higher hybrid seed set. They could be of benefit to foresters to allow plantations with higher proportions of hybrids and higher productivity. Nevertheless, the proposed test can only be applied for first generation hybrids, which are exclusively commercialised so far. Second generation hybrids are currently produced in a few of seed orchards only, but will probably be developed in the near future because of the difficulties involved in producing hybrid seeds in first generation hybridisation orchards. For the identification of advanced generation hybrids (e.g. F_2, backcross), additional nuclear DNA markers are required. Markers already available in *Larix* such as

SCARs (Scheepers et al. 2000) and SSRs (Khasa et al. 2000) can be tested.

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References

Bastien JC, Keller R (1980) Intérêts comparés du mélèze hybride (*Larix*×*eurolepis* Henry) avec les deux espèces parentes. Rev For Fr 32:521–530

Besnard G, Khadari B, Baradat P, Bervillé A (2002) Combination of chloroplast and mitochondrial DNA polymorphisms to study cytoplasm genetic differentiation in the olive complex (*Olea europaea* L.). Theor Appl Genet 105:139–144

Chen J, Tauer CG, Huang Y (2002) Paternal chloroplast inheritance patterns in pine hybrids detected with *trnL-trnF* intergenic region polymorphism. Theor Appl Genet 104:1307–1311

Demesure B, Sodzi N, Petit RJ (1995) A set of universal primers for amplification of polymorphic non-coding regions of mitochondrial and chloroplast DNA in plants. Mol Ecol 4:129–131

De Verno LL, Charest PJ, Bonen L (1993) Inheritance of mitochondrial DNA in the conifer *Larix*. Theor Appl Genet 86:383–388

Dumolin-Lapègue S, Demesure B, Fineschi S, Le Corre V, Petit RJ (1997a) Phylogeographic structure of white oaks throughout the European continent. Genetics 146:1475–1487

Dumolin-Lapègue S, Pemonge MH, Petit RJ (1997b) An enlarged set of consensus primers for the study of organelle DNA in plants. Mol Ecol 6:393–397

Dumolin-Lapègue S, Pemonge MH, Petit RJ (1998) Association between chloroplast and mitochondrial lineages in oaks. Mol Biol Evol 15:1321–1331

Ennos RA, Qian T (1994) Monitoring the output of a hybrid larch seed orchard using isozyme markers. Forestry 67:63–74

Govindaraju DR, Wagner DB, Smith GP, Dancik BP (1988) Chloroplast DNA variation within individual trees of a *Pinus banksiana -Pinus contorta* sympatric region. Can J For Res 18:1347–1350

Häcker M, Bergmann F (1991) The proportion of hybrids in seed from a seed orchard composed of two larch species (*L. europaea* and *L. leptolepis*). Ann Sci For 48:631–640

Hipkins VD, Krutovskii KV, Strauss SH (1994) Organelle genomes in conifers: structure, evolution, and diversity. For Genet 1:179–189

Jaramillo-Correa JP, Bousquet J, Beaulieu J, Isabel N, Perron M, Bouillé M (2003) Cross-species amplification of mitochondrial DNA sequence-tagged-site markers in conifers: the nature of polymorphism and variation within and among species in *Picea*. Theor Appl Genet 106:1353–1367

Jeandroz S, Frascaria-Lacoste N, Bousquet J (1996) Molecular recognition of the closely related *Fraxinus excelsior* and *F. oxyphylla* (Oleaceae) by RAPD markers. For Genet 3:237–242

Khasa PD, Newton CH, Rahman MH, Jaquish B, Dancik BP (2000) Isolation, characterization, and inheritance of microsatellite loci in alpine larch and western larch. Genome 43:439–448

Kobayashi K, Yoshikawa J, Suzuki M (2000) DNA identifiation of *Picea* species of the last glacial age in northern Japan. Jpn J Hist Bot 8:67–80

Lidholm J, Gustafsson P (1991) The chloroplast genome of the Gymnosperm *Pinus contorta*: a physical map and a complete collection of overlapping clones. Curr Genet 20:161–166

Lu MZ, Szmidt AE, Wang XR (1998) RNA editing in gymnosperms and its impact on the evolution of the mitochondrial *coxI* gene. Plant Mol Biol 37:225–234

Maier J (1992) Genetic variation in European larch (*Larix decidua* Mill.). Ann Sci For 49:39–47

Mitchell AF (1958) Establishment of a seed orchard for the production of hybrid larch seed. For Comm 138–147

Neale BB, Sederoff RR (1989) Paternal inheritance of chloroplast DNA and maternal inheritance of mitochondrial DNA in loblolly pine. Theor Appl Genet 77:212–216

Palmer JD, Herbon LA (1988) Plant mitochondrial DNA evolves rapidly in structure, but slowly in sequence. J Mol Evol 28:87–97

Pâques LE (1989) A critical review of larch hybridization and its incidence on breeding strategies. Ann Sci For 46:141–153

Petit RJ, Demesure B, Dumolin S (1998) cpDNA and mtDNA primers in plants. In: Karp A, Isaac PG, Ingram D (eds) Molecular tools for screening biodiversity: plants and animals. Chapman and Hall, London, pp 256–261

Philippe G, Baldet P (1992) Mechanized pollen harvesting with a view to hybrid larch seed production. Ann Sci For 49:297–303

Qian T, Ennos RA, Helgason T (1995) Genetic relationships among larch species based on analysis of restriction fragment variation for chloroplast DNA. Can J For Res 25:1197–1202

Scheepers D, Eloy MC, Briquet M (2000) Identification of larch species (*Larix decidua*, *Larix kaempferi* and *Larix*×*eurolepis*) and estimation of hybrid fraction in seed lots by RAPD fingerprints. Theor Appl Genet 100:71–74

Sederoff RR (1987) Molecular mechanisms of mitochondrial-genome evolution in higher plants. Am Nat 130:S30–S45

Sutton BCS, Flanagan DJ, Gawley JR, Newton CH, Lester DT, El-Kassaby YA (1991) Inheritance of chloroplast and mitochondrial DNA in *Picea* and composition of hybrids from introgression zones. Theor Appl Genet 82:242–248

Szmidt AE, Aldén T, Hällgren JE (1987) Paternal inheritance of chloroplast DNA in *Larix*. Plant Mol Biol 9:59–64

Taberlet P, Gielly L, Pautou G, Bouvet J (1991) Universal primers for the amplification of the non-coding regions of chloroplast DNA. Plant Mol Biol 17:1105–1109

Testolin R, Cipriani G (1997) Paternal inheritance of chloroplast DNA and maternal inheritance of mitochondrial DNA in the genus *Actinidia*. Theor Appl Genet 94:897–903

Tsumura Y, Yoshimura K, Tomaru N, Ohba K (1995) Molecular phylogeny of conifers using RFLP analysis of PCR-amplified specific chloroplast genes. Theor Appl Genet 91:1222–1236

Vendramin GG, Anzidei M, Madaghiele A, Bucci G (1998) Distribution of genetic diversity in *Pinus pinaster* Ait. as revealed by chloroplast microsatellites. Theor Appl Genet 97:456–463

Wakasugi T, Tsudzuki J, Ito S, Shidata M, Sugiura M (1994) A physical map and clone bank of the black pine (*Pinus thunbergii*) chloroplast genome. Plant Mol Biol Rep 12:227–241

Wang WY, Pai RC, Lai CC, Tin TP (1994) Molecular evidence for the hybrid origin of *Paulownia taiwaniana* based on RAPD markers and RFLP of chloroplast DNA. Theor Appl Genet 89:217–275

Wang XR, Szmidt AE (1994) Hybridization and chloroplast DNA variation in a *Pinus* species complex from Asia. Evolution 48:1020–1031

Wang XR, Tsumura Y, Yoshimaru H, Nagasaka K, Szmidt AE (1999) Phylogenetic relationships of Eurasian pines (*Pinus pinaceae*) based on chloroplast *rbc*L, *mat*K, *rpl*20-*rps*18 spacer, and *trn*V intron sequences. Am J Bot 86:1742–1753

Wei XX, Wang XQ (2003) Phylogenetic split of *Larix*: evidence from paternally inherited cpDNA t*rn*T-*trn*F region. Plant Syst Evol 239:67–77

Wolfe KH, Wen-Hsiung LI, Sharp PM (1987) Rates of nucleotide substitution vary greatly among plant mitochondrial, chloroplast, nuclear DNAs. Proc Natl Acad Sci USA 84:9054–9058

Wu J, Krutovskii KV, Strauss SH (1998) Abundant mitochondrial genome diversity population differentiation and convergent evolution in pines. Genetics 150:1605–1614