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D. Scheepers · M.-C. Eloy · M. Briquet

Identification of larch species (*Larix decidua*, *Larix kaempferi* and *Larix X eurolepis*) and estimation of hybrid fraction in seed lots by RAPD fingerprints

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Abstract Species-specific RAPD markers were used to identify the different larch *species (Larix decidua* and *Larix kaempferi*) and their interspecific hybrid (*Larix X eurolepis*). Although morphological differences between pure species and the hybrids exist, differentiation is not always possible, especially at an early stage (seed or plantlet). Eleven RAPD markers differentiated the two larch species, and 4 species-specific markers were sufficient to estimate the F_1 hybrid fraction in a seed lot. The species-specific markers were tested on individual trees of European and Japanese larches of diverse geographic origins and on several seed lots of different origins (F_1, F_2 hybrids and pure species). The 4 specific markers found for the European larch and the Japanese larch were monomorphic and present in all provenances and in all F_1 hybrid trees tested. Polymorphic SCAR fragments were obtained for 3 of the 11 fragments originally selected for the RAPD screening phase. For 2 of them, the sequence had some homology with the mitochondrial genome of other organisms and is thus mitochondrial. The two mitochondrial fragments and the OPF-13_{1000} fragment exhibited one polymorphic band, thereby maintaining its species-specific identity: OPF-13_{1000} is specific to the European larch. The 4 RAPD primers selected in this study offer a reliable, quick and cheap tool for the identification of different larch species (*Larix decidua* and *Larix kaempferi*) and their interspecific hybrid (*Larix X eurolepis*).

Key words Species-specific markers · PCR · Hybrid · Conifers

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D. Scheepers · M.-C. Eloy · M. Briquet (✉)
Université catholique de Louvain,
Unité de Biochimie Physiologique, Place Croix du Sud, 2/20,
B-1348 Louvain-la-Neuve, Belgium
e-mail: briquet@fysa.ucl.ac.be
Fax: 32–10–473872

Introduction

The genus Larix may be broadly divided into six phylogenetic taxa which have evolved at various levels from a single common ancestor. In the colder regions of the Northern hemisphere, there are about ten species, three of which occur in North America, six in Asia and one in Europe. Interspecific hybrids can be obtained by means of spontaneous or controlled hybridization.

The European larch is distributed throughout the Alps, the Carpathians, Suderter and in southern and central Poland, forming separate ranges and races. The Japanese larch occurs naturally in central Japan and has a restricted natural distribution throughout Japan. The hybrid of these two species is mainly of superior quality. However, the morphological differences between parents and hybrids are not clear at an early stage (seed or plantlet). In the Belgian Forestry Research Centre, the parent larches (European and Japanese) are planted in an open-pollinated orchard in a conformation to encourage the cross between them, but the percentage of hybrids actually produced by these crosses is unknown. The problem is to find a quick and sure method of estimating the hybrid fraction in these seed lots. The randomly amplified polymorphic DNA (RAPD) fingerprint technique could be used as a tool for typing the different species (European, Japanese and hybrid larches) and to find specific markers for evaluating the hybrid fraction in a seed lot.

We have focused our research on the European larch (*Larix decidua*) (Mill.)), the Japanese larch (*Larix kaempferi* Sarg.) and their interspecific hybrid (*Larix X eurolepis*). The aim of the investigation reported here was to identify different species by a systematic search of discriminant primers and not to predict the performance of the hybrids. Two approaches were explored: (1) analysis of crude RAPD profiles and (2) selection of characteristic bands for a species and its isolation to obtain sequence-characterized-amplified regions (SCAR)-RAPD markers.

Materials and methods

Plant material

All plant material (*Larix decidua, Larix kaempferi, Larix X eurolepis*) was supplied by the Station de Recherches Forestières du Ministère de la Région Wallonne at Gembloux, Belgium (Nanson et al. 1992a, b; Jacques 1991).

Needles or buds from 20 European larches and 20 Japanese larches were collected in orchard no. 554DE and from 60 hybrid larches in nursery no. B7022 in Fenffe (Belgium) for the RAPD analysis. Hybrids are the F_1 generation from the European and Japanese parents.

European larches from 17 different European locations and one of Japanese origin were collected in the international tree experiment (the "Schober" experiment) planted by Galoux of the Station de Recherches Forestières in Nassogne (Belgium) (Table 1). Needles from 8 individual trees per provenance were collected.

DNA extraction

DNA was extracted from needle, buds or embryos using the Doyle and Doyle (1990) protocol (Focus BRL). Needles were ground in liquid nitrogen using a pestle and mortar; buds and embryos were ground directly in a 1.5-ml tube in 50 µl of CTAB buffer using a small pestle. The amount of purified DNA was measured by fluorometry. For the embryo, the DNA was not quantified, and the pellet was resuspended in 1 ml of water.

RAPD procedure

RAPD reactions were performed in a 25-µl volume containing 100 ng of template DNA (2 µl of the 1 ml final solution for the embryo), 10 pmoles of primer DNA (0.4 µ*M*), 100 µ*M* of each dNTP, 1 U *Taq* polymerase (Pharmacia) and 2.5 µl of *Taq* polymerase 10× buffer (Pharmacia; 500 m*M* KCl, 15 m*M* MgCl2, 100 m*M* TRIS-HCl pH 9,0). The sample was covered with 25 µl of mineral oil. DNA amplification, waperformed in a Perkin-Elmer Cetus DNA 480 Thermal Cycler using a polymerase chain reaction (PCR) profile consisting of 1 cycle of 3 min at 93°C, 1 min at 37°Cand 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. For the seed analysis, the second step consisted of 40 cycles. A total of 140 primers (Operon Technologies, Alameda, Calif., kits OPA–OPH) were used.

Table 1 Origins of provenances at the Schober International plantation experiment in Nassogne (Belgium)

Provenances	Country
1) Briançon-Villard	France
2) Pragelato	Italy
3) Dobris	Slovakia
4) Schönwies	Austria
5) Sterzing/Flains	Austria
6)Bruneck Ahrntal	Austria
7) Pergine/Selvot	Italy
8) Cavedine	Italy
9) Tenna	Italy
10) Semmering	Austria
11) Langau 59	Austria
12) Langau 38	Austria
13) Zabreh Dubricko	Slovakia
14) Ruda Nad Moravou	Slovakia
15) Schlitz	Germany
16) Neumunster	Germany
17) Ina	Japan
18) Cavalese	Italy

SCAR

The informative RAPD fragments were partially sequenced at their extremities, and new primers (15–20 bp in length) were designed based on the DNA sequence. The new primer sites may or may not partially overlap with the RAPD primer site. In general, the SCAR primers consisted of a few tens of base pairs separate from RAPD primer sites.

Results

Species-specific markers

The first stage of the study was to obtain species-specific RAPD markers for the parent species: the European larch and the Japanese larch. Hence, 180 primers were assayed on the 20 European and the 20 Japanese larches from orchard no. 554DE. Fifteen fragments differentiated the European and the Japanese larch, and 10 were selected not only for their clear and distinct presence in all the individuals of one species but also for their absence in all the individuals of the other species (4 were specific to the Japanese and 6 to the European larch). From these 10 species-specific bands, 2 were mitochondrial (OPD-15_{1400} and OPR-08_{1200}), which means that they can be used for pure species determination but not for hybrid analysis because of their inheritance from only the maternal parent. Only the monomorphic primers for a species were selected . Because of the dominant character of the RAPD markers, homozygous markers were preferred for the analysis [differentiation between a homozygous (AA) and an heterozygous marker (Aa) is not possible with RAPD markers]. In conifers, the megagametophyte tissue, which is haploid and represents the female gamete of the tree, can be analysed. So the distribution of the marker can be checked by analysing its frequency in the megagametophyte. Because seeds from each individual tree were not available in our case, for the analysis of the megagametophyte, 60 F_1 hybrids from crosses between European and Japanese larches produced from the seed orchard no. 554DE were used. The frequency of presence and absence of the species-specific markers in European, Japanese and hybrid larches are presented in Table 2. In these hybrids the proportion presence for the species-specific markers was higher than 85% and for some markers equivalent to 100% (OPC-16_{1380} and OPC-06_{930}) or close to 100% (98% for OPH-14_{1450}, OPD-10_{1200}, OPF-13_{1000} and 96,3% for OPH-11_{2200}).

To enhance the power of the markers, we tested them on a wide variety of European larches of geographical origins (17 provenances) and one Japanese provenance as a control. These provenances are located in the International Schober experiment in Nassogne (Belgium) (Table 1). Eight trees of each geographical provenance were tested and for the 11 species-specific bands, all the individuals from all the provenances matched the distribution of the European or Japanese markers (all the European larches possessed the European markers and not the Japanese).

Table 2 Frequency of presence of the species-specific markers

Primers	Size of bands	Presence in European larch	Presence in Japanese larch	Presence in Hybrid larch
OPH-11	2.2 kb	100%	0%	96.3%
OPD-15	1.4 kb	100%	0%	Mitochondrial
OPE-17	0.8 kb	100%	0%	85%
OPF-05	2.25 kb	100%	0%	92%
OPG-12	1.3 kb	0%	100%	87%
OPH-14	1.45 kb	0%	100%	98%
OPC-16	1.38 kb	0%	100%	100%
OPC-06	0.93 kb	0%	100%	100%
OPR-08	1.2 kb	0%	100%	Mitochondrial
OPD-10	1.2 kb	0%	100%	98%
OPF-13	1 kb	0%	100%	98%

SCAR-RAPD markers

Species-specific bands were extracted and partially sequenced in order to design new primers of 15–20 mer that were specific to the DNA sequence (SCAR primers). In most cases, a single fragment was amplified in the two species that showed a monomorphic electrophoretic profile similar to that observed by Bodenes *and al*. (1996) in their study on two closely related oak species. Polymorphic SCAR fragments were obtained for 3 fragments ($OPD\text{-}15_{1400}$, $OPR\text{-}08_{1200}$ and $OPF\text{-}13_{1000}$) among the 11 that were originally selected in the RAPD screening phase. For the $OPD\text{-}15_{1400}$ and $OPR\text{-}08_{1200}$ species-specific band, the sequence has some homology with the mitochondrial genome of other organisms and is thus mitochondrial, as would be expected considering their maternal inheritance in the hybrids. The two mitochondrial fragments and the $OPF\text{-}13_{1000}$ fragment exhibited one polymorphic band that maintained its species-specific identity: $OPF\text{-}13_{1000}$ is specific to European larch. Because of the low number of SCAR-RAPD fragments obtained, we could not use them to identify the different larch species; we should have at least 2 SCAR-RAPD specific fragments for each species.

However, in order to be able to implement this technique in other laboratory environments, we tested the specific RAPD markers ($OPH\text{-}14_{1450}$, $OPC\text{-}06_{930}$, $OPH\text{-}11_{2200}$ and $OPF\text{-}05_{2250}$) with three different PCR machines and with three different Taq polymerases: the RAPD profiles did not change and the species-specific bands remained distinctive for each species.

Seed lot analysis

For the seed batch analysis we used a set of 4 RAPD primers: 2 European markers (OPH-14 and OPC-06) and 2 Japanese markers (OPH-11 and OPF-05).The RAPD amplifications were made separately for each primer, and the F_1 hybrids had to display the species-specific band for each primer. Four primers were used because they were not 100% homozygous and for one primer the band might have been absent in some samples. However, we never observed a specific band for one species in another species. While this absence was not be simultaneous for each primer the test was still valid. Nevertheless, more primers could be used to enhance the reliability of the test; currently, 11 species-specific RAPD markers are available for the identification of the European and Japanese larch.

Table 3 Percentage of each species in seed lots

Origin of seed lots	Hybrid	Japanese	European
1) 80/1/DE (Belgium)	4.16%	93.75%	2.08%
2) 93/1/DE (Denmark)	9.315%	0%	90.625%
3) 96/554/DE (Belgium)	29.16%	5.2%	65.625%
4) Verger des Barres (France)	84%	0%	16%

We tested the 4 primers on four different seed lots (considered as F_1 hybrids) from various geographical origins to determine the percentage of hybrids (Table 3). About 96 embryos were analysed in each seed lot. The F_1 hybrid seed lots (1, 2, 3, 4) came from different seed orchards with very different outputs. Lot number 1 came from a orchard where some Japanese trees were very productive, leading to a over-representation of some very productive clones. Lot no. 2 came from Denmark where the harvest was taken only from the European larch, which explains the fact that no pure Japanese seeds were detected. Lot no. 3 came from a common hybrid orchard where the percentage of hybrids was low. Lot no. 4 is a commercial lot with a special type of fertilization which consisted of a pulverization of Japanese pollen on European larch trees. These primers were also checked on various seed lots from F_2 hybrids, pure European and pure Japanese larches.

Discussion

In this study, we found 11 markers that differentiated the two species, the European larch (*Larix decidua*) and the Japanese larch (*Larix kaempferi*). Two of these markers were mitochondrial markers and were maternally inherited (DeVerno *et al*. 1993). Four species-specific markers (2 European: OPH-14 and OPC-06; 2 Japanese: OPH-11 and OPF-05) were sufficient to estimate the F_1 hybrid (*Larix X eurolepis*) fraction in a seed lot.

Although morphological differences between pure species and the hybrids exist, differentiation is not always possible. Weissman and Reck (1987), analyzing needle oils, found that a monocyclic diterpene alcohol (thunbergol) is present only in oleoresins from *Larix kaempferi* and hybrids but could not be identified in *Larix decidua*. This chemical marker can only distinguish between the European larch and the hybrids but not between the hybrids and the Japanese larch. Bergmann and Ruetz (1987), using the enzyme system of shikimate dehydrogenase, were able to distinguish the interspecific hybrid seeds (embryos) from a single maternal clone. Ennos and Qian (1994) developed a general method for estimating the proportion of hybrid seed from hybrid larch seed orchards of any design (one or many maternal clone), making use of genetic variation at the SKDH isozyme locus. The presence of few isozyme loci (Hacker and Bergmann 1991), cytological data and a high sequence homology between the two species (Nkongolo and Klimaszewska 1995) suggest that they are closely related. Nevertheless, genetic distances are greater between species than within species and could be used to genetically differentiate the two species (Arcade *et al*. 1996). RAPD markers have been used previously for the identification of hybrid trees (Perron *et al*. 1995). We used them to identify European larch, the Japanese larch and their inter-specific hybrid. The 4 specific markers found for the European larch and the Japanese larch are monomorphic and present in all provenances and in all F_1 hybrids trees tested. Because RAPD markers are dominant, the homozygous character must be checked. In general, the distribution of a marker can simply be calculated by its frequency in the megagametophyte, which is haploid and represents the female gamete of the tree. However, in our case, seeds (and thus megagametophytes) from each tree individually were available only for 3 trees of the European larch. The analysis was therefore made on 60 megagametophytes from each of these 3 trees, and the 4 primers were found to be homozygous. To compensate for the lack of seeds from individual trees, we tested the species-specific markers by analysing the segregation of species-specific bands in 60 F_1 hybrid trees. The presence of the species-specific bands was higher than 85% and mostly close to 100%. Thus, this presence ratio is sufficient for a reliable test to identify the different larch species.

The 4 RAPD primers selected in this study offer a reliable, quick and cheap tool for the identification of different larch species (*Larix decidua* and *Larix kaempferi*) and their interspecific hybrid (*Larix X eurolepis*) because: (1) the test can be applied to a wide variety of plant material (embryo, bud, needle, cambium); (2) the species-specific markers have been tested on individual European larch trees from diverse geographic origins, and Japanese species-specific fragments were never observed in any European larch of the Schober experiment; the European species-specific bands are monomorphic for all the European individuals of the Schober experiment; (3) high numbers of seed lots of different origins (Denmark, Belgium, France)were tested with our set of 4 primers; (4) a current criticism made against the RAPD technology is that it is not exportable; to circumvent this criticism, the reproducibility of the reactions was also tested not only by repeating the same reaction but also with three different *Taq* polymerases and on three different PCR machines, with the RAPD profile being the same and the species-specific bands are present in all cases.

This RAPD methodology is also used routinely to certify the clonal identity of clonal edges of larches propagated by cuttings and to identify some old stands when there is any doubt about the real origin of the trees.

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