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SHORT COMMUNICATIONS

DNA from Various Tissues of Far Eastern Larches and Its Applicability for RAPD Assay

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Abstract—We studied applicability of RAPD assay for investigating genetic variability of far eastern larches. Techniques to isolate DNA from larch vegetative organs (needles, young shoots and stems) and megagametophytes were selected, and conditions of polymerase chain reaction were optimized. Fifty out of 84 random primers proved efficient, and 22 and 12 primers initiated weak and no synthesis, respectively. The tested primers worked efficiently on the DNA isolated from both diploid and haploid tissues.

Larches (*Larix spp.* Mill.) grow and are cultivated in many territories of Russia and Russian Far East. The biology and ecology of the Far Eastern larches has been reviewed in a number of publications (Il'chenko, 1973; Gukov, 1974; Pozdnyakov, 1975; Bobrov, 1978; Dylis, 1981; Potenko and Razumov, 1996); however, their taxonomy remains controversial. This particularly applies to larches of Primorski Krai. According to one viewpoint (Sukachev, 1931; Kolesnikov, 1946; Gukov, 1976), larches are represented in the Primorski Krai by several species (up to six); other scientists (Bobrov, 1972; Koropachinskii, 1989) question this statement and assign all observed variants except the Korean larch (*L. olgensis* A. Henry) to the complex which is the result of successive introgressive hybridization (Bobrov, 1972) of Dahurian (*L. dahurica* Turccz.), Kamchatka (*L. kamtschatica* (Rupr.) Carr.), and later Korean larches. Resolution of these taxonomic problems was based on morphology, anatomy, and karyology data (Gukov, 1972; Il'chenko, 1973; Potenko and Razumov, 1996); however, it was not enough for a ultimate conclusion.

Koropachinskii (1989) believes that the potentialities of traditional methods are exhausted, and principally new approaches may provide additional resources to discriminate the studied taxa or confirm the genetic homogeneity of the Far Eastern larch complex.

Random amplified polymorphic DNA (RAPD) assay is one such modern approach in plant taxonomy (Welsh and McClelland, 1990; Williams *et al.*, 1990). This method allows a comparison of separate elements of DNA structure (DNA amplification patterns) and is used for the genetic grouping of plants (Kazan *et al.*, 1993; Sivolap and Kalendar', 1995; Zhuravlev *et al.*, 1998). The existence of haploid megagametophyte in conifers lifts the restrictions of this method that result from the dominant nature of RAPD markers (Williams *et al.*, 1990; Isabel *et al.*, 1993). Hence, one can expect new data to resolve the complex taxonomy of far eastern larches with RAPD assay. The adaptation of this method to a new object is an important step.

In this work, we compared methods of DNA isolation from various larch tissues and estimated the isolated DNA applicability for a genetic description of the far eastern representatives of the *Larix* genus using RAPD assay.

MATERIALS AND METHODS

The experiments were carried out on needles and megagametophytes from the Korean larch (*Larix olgensis* A. Henry) collected in natural populations of Ol'ginskii, Kavalerovskii, and Dal'negorskii Districts. In addition, we used material from Vladivostok plantations. DNA was isolated from young (at the auxiblast termini) and old (at the brachyblasts and trunk) needles, young unlignified stems (without needles), and shoots (stems with needles). The material from the plantations was extracted without storage. Needles from the natural larch populations were kept in liquid nitrogen or lyophilized. We also tested the following ways to store the needles in the field conditions: in herbarium, in a polyethylene sack, and in CTAB solution saturated with NaCl (after Rogstad, 1992). The cones for the experiments with megagametophytes were collected in August and kept at room temperature.

DNA was isolated from 500 mg fresh or 100 mg lyophilized needles using the methods proposed for other plants (table). The obtained DNA pellet was dissolved in 100 μl 0.01 M Tris-HCl, pH 8.0, and 0.001 M EDTA. DNA from the megagametophytes was isolated according to the method described by Isabel *et al.* (1993) for *Picea mariana* (Mill.), with modifications concerning volume of the extraction sample (depending on the size of the larch megagametophyte) and homogenizing mode (performed with a special pestle directly in the tube for DNA isolation). DNA concen-

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Methods of DNA isolation

Procedure	Variant					
	1	2	3	4	5	6
Homogenizing, buffer composition	50 mM Tris-HCl, pH 8.0, 0.7 M NaCl, 10 mM EDTA, 1% CTAB, and 1% ME	50 mM Tris-HCl, pH 8.0, 0.7 M NaCl, 10 mM EDTA, 1% CTAB, and 1% ME	100 mM Tris-HCl, pH 8.0, 5.0 M NaCl, 0.5 M EDTA, 2% SDS, and 0.1 mg/ml proteinase *K*	100 mM Tris-HCl, pH 8.0, 5.0 M NaCl; 0.5 M EDTA; 2% SDS, 0.1% ME, and 1% PVP		100 mM Tris-HCl, pH 9.5, 1.4 M NaCl, 20 mM EDTA, 2% CTAB, and 0.2% ME
Incubation	60°C, 90 min	60°C, 90 or 180 min	37°C, 120 min	65°C, 20 min	65°C, 30 min	65°C, 30 min
Deproteinization, mixture composition	chloroform : octanol (24 : 1), centrifugation at room temperature	chloroform : octanol (24 : 1), centrifugation at room temperature	phenol–chloroform : octanol (24 : 1), centrifugation at 4°C	potassium acetate buffer, pH 5.0, on ice, centrifugation at 4°C	chloroform : octanol (24 : 1), centrifugation at room temperature	chloroform : octanol (24 : 1), centrifugation at room temperature
Sedimentation of the CTAB–DNA complex	1 volume CTAB, 1 M NaCl (to 56°C)	–	–	–	–	–
DNA sedimentation	2 volumes of ethanol, centrifugation at room temperature	2/3 volumes of isopropanol, centrifugation at room temperature	2/3 volumes of isopropanol, centrifugation at 4°C	2/3 volumes of isopropanol, centrifugation at 4°C	2/3 volumes of isopropanol, centrifugation at room temperature	1/10 volume of 3 M sodium acetate, 1 volume of isopropanol, centrifugation at room temperature

Note: References: 1, Murray and Tompson, 1990; 2,Milligan, 1992; 3, *Gennaya* …., 1991, p. 245; 4, *Gennaya* …, 1991, p. 249; 5, modified protocol; 6, Isabel et al., 1993; EDTA, ethylenediaminetetraacetic acid; CTAB, cetyltrimethylammonium bromide; PVP, polyvinylpyrrolidone; SDS, sodium dodecylsulfate; ME, mercaptoethanol.

tration in the probe was estimated by comparing it with the λ phage DNA of known concentration after 1.4% agarose gel electrophoresis with ethidium bromide (Maniatis *et al.*, 1984).

PCR was carried out on a MJ Research minicycler (USA). The reaction conditions were described elsewhere (Reunova *et al.*, 1996); they included 40 amplification cycles: 92°C, 45 s; 37°C, 30 s; 45°C, 15 s; and 72°C, 2 min. In the total, we used 84 primers (Operon Technologies, USA, kits *A*, *B*, *C*, *D*, and *E*). The amplification products were analyzed with 1.4% agarose gel electrophoresis (see above) using λ phage DNA hydrolyzed with *Eco* RI and *Hind* III (Fermentas, Lithuania) as a size marker. A sample with the amplification mix without DNA was used as a control.

RESULTS AND DISCUSSION

Selection of the method for the rapid and simple isolation of sufficient DNA template for PCR is an important step for using RAPD assay in population studies. Hence, we compared certain known methods (table) differing chiefly by the used detergent (CTAB and SDS) and deproteinization method (treatment with chloroform, phenol, proteinase K, and potassium acetate). DNA was isolated from fresh and lyophilized needles. According to electrophoresis (Fig. 1), the efficiency of the methods used significantly depends on the conditions of the storage of the needles. In the first and second variants of isolation (table) from dry material, the DNA was either not isolated at all or in a small quantity after increasing the duration of the material incubation to 180 min (Fig. 1, lanes *11* and *12*). The DNA yield was 0.375 μg per 100 mg dry tissue. The methods with STAB as detergent allowed us to isolate more native DNA from fresh needles as compared to the lyophilized material. Depending on the method, DNA yield ranged from 8 to 30 μg per 500 mg wet tissue (Fig. 1, lanes *9* and *10*).

The presence of SDS in the extraction mix (table, variants 3 and 4) allowed the DNA to be successfully isolated from both fresh and dry material, although the DNA yield was higher for the fresh material than for the lyophilized one. The DNA yield in the case of phenol–chloroform and proteinase *K* (variant 3) was 72.6 and 1.16 μg for the wet and lyophilized material, respectively (data not shown); while in the case of potassium acetate it was 65 and 2 μg (Fig. 1, lanes *5* and 6). Hence, the use of SDS allows DNA to be isolated even from dry material and provides for a significantly higher DNA yield from wet material.

The variants with proteinase *K* and potassium acetate buffer (table, variants 3 and 4) provide for a higher

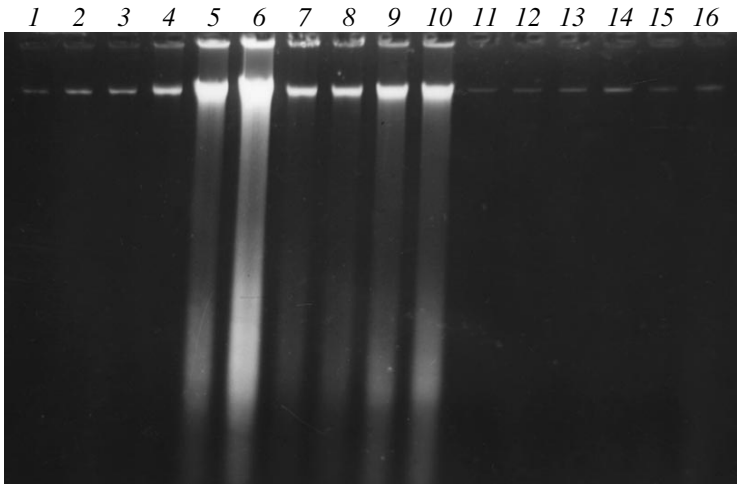

Fig. 1. Samples of DNA isolated from larch needles by various methods (see Table); lanes *1–4*, λ phage DNA, 20, 30, 40, and 50 ng, respectively; lanes *5–10*, fresh needles; *5* and *6*, variant 4; *7* and *8*, variant 5; *9* and *10*, variant 2, incubation 180 min; lanes *11–16*, lyophilized needles; *11* and *12*, variant 2, incubation 180 min; *13* and *14*, variant 4; *15* and *16*, variant 5.

DNA yield; however, complication of the isolation procedure by cooled high-speed centrifugation provides for no significant advantage of these techniques in the studies of larch population and genetics. The variant of DNA isolation from the needles selected for further work with dry material (table, variant 5) includes extraction in a buffer proposed in (*Gennaya*…, 1991), incubation in water bath at 65°C for 30 min, and further deproteinization and DNA sedimentation as in the variant 2 (table). DNA yield from the lyophilized material was 1.25 μg on average in this case (Fig. 1, lanes *15* and *16*).

Plant tissues could vary in their content of substances affecting DNA yield, hence, we tested DNA yield from various plant organs. DNA was isolated from wet tissues after Milligan (1992) (table, variant 2). The samples isolated from young needles and shoots contained an even amount of DNA. The least DNA amount was isolated from the needles at a later developmental stage. A high DNA yield was obtained from stems, 52.8 μg per sample, which is considerably higher compared to other larch tissues for this technique. Apparently, young unlignified stems are the most convenient material to isolate large amounts of DNA from larch by other techniques as well; however, it is advisable to work with needles in the long-term experiments since this developmental stage is very short.

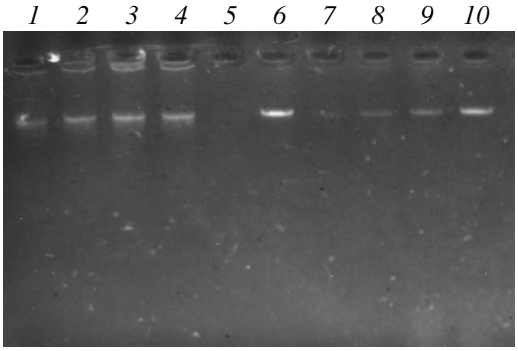

Fig. 2. Samples of DNA isolated from various larch megagametophytes; lanes *1–4*, λ phage DNA, 20, 30, 40, and 50 ng, respectively; lanes *5–10*, DNA from megagametophytes.

Natural Primorski Krai populations of larch grow in hard-to-reach regions, so that the collection and delivery of the material may take considerable time. Hence, the methods of material storage during transportation is important. We tried several ways of storing the needles: in herbarium, in polyethylene sacks, and in NaCl-saturated STAB solution (Rogstad, 1992). After 30 days of storage, DNA was isolated by the STAB method (table, variants 2). A sufficient amount of DNA was isolated from the needles stored in herbarium net and polyethylene sack (25 and 30 μg, respectively), while no DNA was isolated from the needles stored in the STAB–NaCl solution. However, the both DNA samples contained a lot of low-molecular fragments. Herbarium storage has the advantage preserving the material for over a year without visible changes, while the needles mold quickly in a polyethylene sack. Thus, the methods applied do not provide for long-term storage of DNA in the needles, suggesting minimizing the transportation time.

The protocol of DNA isolation from *Picea mariana* megagametophytes (table, variant 6) documented by Isabel *et al.* (1993) proved also to be efficient for isolating DNA from larch megagametophytes (Fig. 2, lanes *5–10*). The yield of DNA from single larch megagametophyte was 0.35 μg on the average. This amount is good enough for at least 10 amplification reactions.

DNA samples obtained by various methods were used as a template for PCR amplification. We have

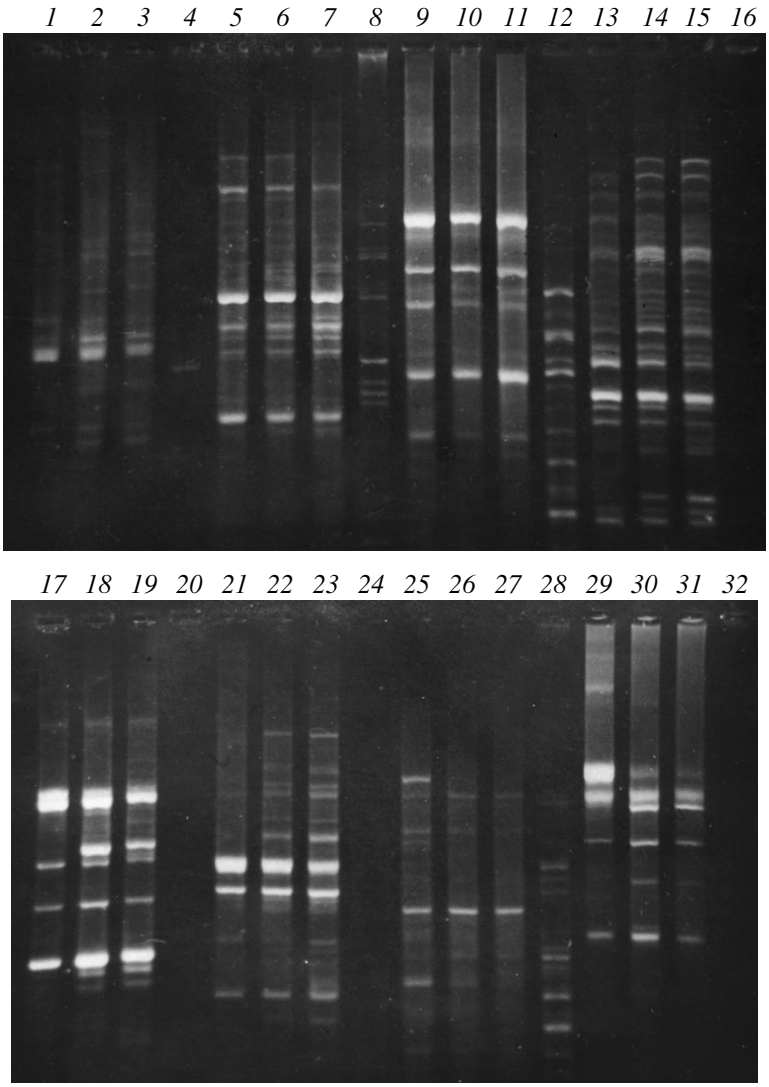

Fig. 3. Products of larch DNA amplification as a function of primers and $MgCl_2$ to concentration; primers OPA-01 (*1–4*); OPA-02 (*5–8*); OPA-03 (*9–12*); OPA-04 (*13–16*); OPA-11 (17–20); OPA-12 (*21–24*); OPA-13 (*25–28*); OPA-14 (*29–32*); 2.0 mM $MgCl_2$, lanes *1*, *5*, *9*, *13*, *17*, *21*, *25*, and *29*; 2.8 mM $MgCl_2$, lanes *2*, *6*, *10*, *14*, 18, *22*, *26*, and *30*; 3.6 mM $MgCl_2$, lanes *3*, *7*, *11*, *15*, *19*, *23*, *27*, and *31*; control (reaction mix without DNA), lanes *4*, *8*, *12*, *16*, *20*, *24*, *28*, and *32*.

tested 84 arbitrary 10-nucleotide primers with various sequences and 60–70% (G+C) content. Fifty primers proved to be efficient, while 22 and 12 primers initiated weak and no synthesis, respectively. Figure 3 presents electrophoretic gels of the amplification products of larch DNA isolated after Milligan (variant 2) from young unlignified stems with eight efficient primers. A concentration of Mg^{2+} ions in the reaction mix was selected empirically (from 2 to 3.6 mM) to provide the highest stability of the primer–DNA complex. The amplification patterns obtained with each primer are specific and differ by the number of fragments, their size, and the proportion of each fragment in the RAPD spectrum (majority). The number of amplicones formed during larch DNA amplification varies from 2 to 20, while their size ranges from 200 to 2000 bp. Usually no amplification products were found in the control sample; however, certain primers initiated synthesis in the absence of DNA (Fig. 3, lanes *8*, *12*, *28*) as observed elsewhere (Williams *et al.*, 1990).

DNA isolated from the needles and young shoots by different methods (Table, variants 2 and 4) also proved to be an efficient template for amplification with arbitrary primers.

The primers efficient in PCR of the DNA isolated from larch vegetative organs were also efficient with the DNA isolated from megagametophytes. Figure 4 presents the amplification products of DNA isolated from the megagametophytes (with primers OPA-04, OPA-11, and OPA-12). The amount of DNA in the reaction mix, providing for efficient amplification, was

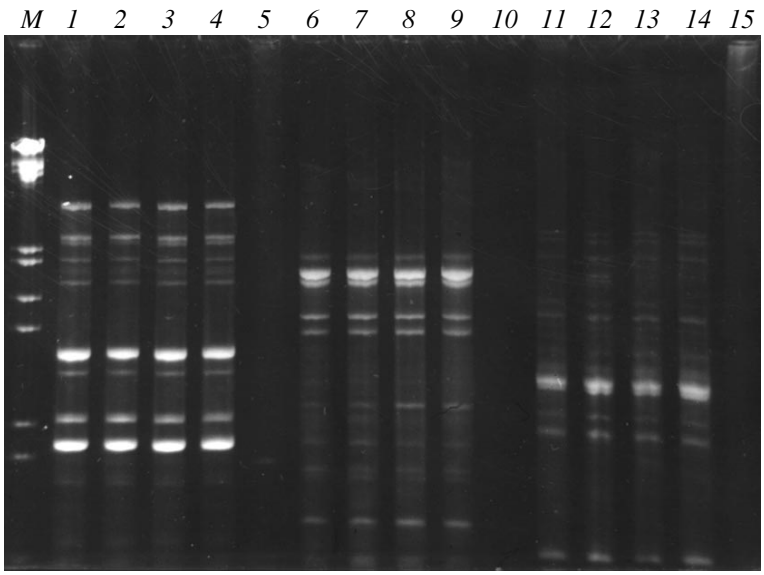

Fig. 4. Products of megagametophyte DNA amplification as a function of primers and DNA concentration; *M*, λ phage DNA hydrolyze by *Eco* RI and *Hind* III; primers OPA-04 (lanes *1–5*), OPA-11 (*6–10*), OPA-12 (*11–15*); 10 ng DNA, lanes *1*, *6* and *11*; 15 ng DNA, lanes *2*, *7*, and *12*; 20 ng DNA, lanes *3*, *8*, and *13*; 25 ng DNA, lanes *4*, *9*, and *14*; control (reaction mix without DNA), lanes *5*, *10* and *15*.

selected empirically. Usually 25 ng DNA is used for plant PCR (Williams *et al.*, 1990). Selected amplification conditions of DNA from larch gametophytes are efficient with lower DNA concentrations.

Thus, the performed experiments allowed us to isolate the DNA that adapted to studies of population genetics from vegetative and generative organs of the larch and to select efficient primers and optimal primer–template ratios for RAPD assay.

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