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Research Article

Preliminary Comparative Analysis of Phenological Varieties of *Quercus robur* by ISSR-Markers

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Quercus robur L. is a valuable wood species having long ontogeny and promising to create long-living artificial plantings of recreational and ameliorative purposes in the steppes zone of Russia and other countries. In this work we have performed the genotyping of varieties of *Quercus robur* L. obtained from collection of Botanical Garden of Southern Federal University using intersimple sequence repeat (ISSR) molecular markers. The most polymorphic ISSR-marker (GA) 8YC was found in the collection. The polymorphic DNA markers identified in the present study can be used for the future breeding works to obtain valuable genotypes of *Quercus* genus. In addition we have performed DNA fingerprinting of the prospective sample of the variety *Q. robur* var. *tardiflora* Czern.

1. Introduction

Efficient creation of artificial forests should be based on the use of a genetically diversified planting material of high-quality. Among such a material the English oak (*Quercus robur* L.) is an important component of artificial forest plantations [1].

Q. robur is a widespread species in the natural forest associations [2] of the middle latitudes and mountainous zones of the northern hemisphere and Asia Minor (an area corresponding to the western two-thirds of Turkey), North Africa, the Caucasus, and Europe [3]. The actual area occupied by *Q. robur* in Russia is located within the subzone of broad-leaved forests and forest-steppe of the European part of the country [4].

Now *Q. robur* cannot be found in the natural associations of West Siberia flora, although oak forests are an essential part of the forest-steppe zone of Western Siberia.

Wide distribution of *Q. robur* in the area of the continental climate is characterized by its freeze-thaw resistance [5, 6]. *Q. robur* refers to salt-tolerant woody plants growing satisfactorily in saline soils [7]. However, some authors have an opposite view [8, 9]. There are different, conflicting data related to *Q. robur*. Pogrebnyak showed that *Q. robur* is a xeromesophyte [10]. Accordingly to other data *Q. robur* is very tolerant to soil conditions and the continental climate but it prefers fertile and well-watered soils [7, 11]. However, it is capable of growing in conditions of high humidity [12]. Drought resistance of *Q. robur* is primarily determined by its strong root system, so this species can grow on dry poor, rocky soils.

Currently, this type of *Q. robur* habitat becomes mobile and reduced in size due to its substitution by other species [3].

Significant reduction in the duration of the ontogeny is a serious problem of artificial plantations of *Q. robur* [13, 14].

In this connection the following varieties of *Q. robur* L. (firstly described by Chernyaev [15] in 1858) are of interest: *Q. robur* var. *praecox* Czern. and *Q. robur* var. *tardiflora* Czern., which differ in time of occurrence of their phenological phases.

Two varieties of *Quercus robur* were studied: *Q. robur* var. *tardifolia* and *Q. robur* var. *praecox*, which are cultivated in the Botanical Garden of Southern Federal University.

The main diagnostic signs to distinguish *Quercus robur* var. *tardiflora* Czern. and *Quercus robur* var. *praecox* Czern. are [16] as follows:

(1) Bud, bloom, beginning of shoot growth, and most other phenological phases occur in *Quercus robur* var. *tardiflora* Czern. at 2-3 weeks later than in *Quercus robur* var. *praecox* Czern..

(2) In contrast to *Quercus robur* var. *praecox* Czern, the senescent leaves of *Quercus robur* var. *tardiflora* Czern. do not fall down, remaining on the shoots until the end of winter.

(3) *Q. robur* var. *tardiflora* is less defeated by pests and diseases.

(4) Comparing to *Quercus robur* var. *praecox* Czern, *Q. robur* var. *tardiflora* has a higher and a more regular crown shape, straighter trunk, and a higher quality of wood. It prefers moist habitats.

Q. robur var. *praecox*, which was referred to in an experiment as a typical form, may be found all over the artificial population of *Q. robur* in the Botanical Garden.

Only two samples have been selected and described from the population in conformity with the phenological cycle of *Q. robur* var. *tardiflora*. The first (N 1) was planted in 1888 and the second (N 2) in 1975.

The first sample of *Q. robur* var. *tardiflora* is the most interesting. *Q. robur* var. *tardiflora* Czern. (N 1) has a high decorative quality and exceeds other samples in duration of ontogenesis.

In contrast to the specimens of *Q. robur* var. *praecox*, planted in similar conditions in the Botanical Garden in 1930, it has no signs of ageing.

It differs from *Q. robur* var. *tardiflora* in that it sheds leaves at the time characteristic for deciduous species that significantly enhances its decorative properties.

This sample currently has a height of 26 meters and trunk diameter of 108 centimeters.

The phenological characteristics of *Q. robur* var. *tardiflora* Czern. and *Q. robur* var. *praecox* Czern were showed in Tables 1 and 2.

The annual time scale of phenological cycle is shown in Table 2.

Samples of *Q. robur* var. *praecox* were selected from three different generations of specimens (see Table 2): N 1 and N 2 planted in 1930, N 3 in 1963, and N 4 and N 5 in 1975.

Several authors have noted that these varieties have significant differences in the environmental stress resistance, growth, and development [17–23].

Q. robur var. *tardiflora* is a promising and prospective variety, which, however, is already widely used for the artificial formation of green areas in European countries [24]. From such a point of view, this variety has been shown as the most promising that was the result of studies conducted during last few years at the Botanical Garden of the Southern Federal University [13].

Obviously, a clear genetic identification *Q. robur* var. *tardiflora* will allow identifying its other, closely allied varieties, as well as identifying the individual, most genetically valuable specimens for the further propagation via *in vitro* culture and the subsequent bulk producing of planting material.

Table 1: Time scale of phenological phases of *Q. robur* varieties.

Phenological phases	Calendar date	
	Q. r. var. *praecox*	*Q. r.* var. *tardiflora*
Bud	14.IV ± 2.1	21.IV ± 3.4
Beginning of shoot growth	15.IV ± 2.3	24.IV ± 2.7
Leafing	18.IV ± 2.1	1.V ± 2.2
Efflorescence	24.IV ± 2.7	5.V ± 2.7
Full leafy	28.IV ± 2.1	9.V ± 2.1
End of flowering	2.V ± 2.7	7.V ± 2.2
End of shoot growth	20.V ± 4.5	29.V ± 8.2
Secondary shoot growth	15.VI ± 8.2	30.VI ± 14.6
Full maturation of shoots	24.VII ± 6.5	7.VIII ± 8.2
Mass staining	5.X ± 3.4	13.X ± 8.5
Massive maturation of seeds	4.IX ± 5.7	16.IX ± 2.6
Massive fruit abscission	13.IX ± 8.9	21.IX ± 6.4
Massive defoliation	12.X ± 1.3	28.X ± 4.1

A number of works provide the detailed information on the application of ISSR- and SSR-markers for the study of genetic divergence in the cortical oaks [25, 26] and assessment of phylogenetic relationships [27], as well as on the use of intermicrosatellite DNA markers for genotyping of the family *Fagaceae* [2, 24, 27–29].

The aim of this work is to study the genetic affinity of *Q. robur* var. *tardiflora* Czern. and *Q. robur* var. *praecox* Czern. using ISSR-markers, as well as the applicability of different ISSR-markers for genetic analysis of kinship relationships within the genus *Quercus*.

2. Materials and Methods

2.1. Plant Material. Samples of *Q. robur* var. *praecox* were selected from three different generations of specimens (see Table 2): N 1 and N 2 planted in 1930; N 3 in 1963; and N 4 and N 5 in 1975. Samples of *Q. robur* var. *tardiflora* were selected from three different generations of specimens. N 1 was planted in 1888 and N 2 in 1975.

In addition, in order to predetermine the position of the selected varieties of *Q. robur* in the phylogenetic tree of the genus, the following samples were added: one sample of *Q. robur* var. "*fastigiata*" obtained by seeds and two samples of *Q. petraea* (Matt.) Liebl. (Russia, the Republic of Adygea) which is a species phylogenetically closest to *Q. robur*. Furthermore, one sample of *Q. libani* Oliv., *Q. macrocarpa* Michx, and *Q. rubra* L., respectively, was taken in the experiment as phylogenetically distant forms (Table 3).

2.2. DNA Extraction. Plant samples were obtained in a period from October 2014 to January 2015. Extraction DNA was performed from dormant oak buds freed from bud scales using a Sorb-A-GMO reagent kit (Sintol, Russia).

The intersimple sequence repeat- (ISSR-) method was performed for the analysis of the isolated DNA using six

Table 2: Phenological cycle of *Q. robur* varieties in 2014.

Phenological phases	Calendar date		
	Q. r. var. *praecox* N 1	*Q. r.* var. *tardiflora* N 1	*Q. r.* var. *tardiflora* N 2
Bud	10.IV	24.IV	21.IV
Beginning of shoot growth	14.IV	26.IV	25.IV
Leafing	14.IV	26.IV	25.IV
Efflorescence	20.IV	26.IV	25.IV
Full leafy	7.V	12.V	12.V
End of flowering	30.IV	3.V	2.V
End of shoot growth	15.V	18.V	18.V
Secondary shoot growth	26.V	—	28.V
Full shoot maturation	10.VII	—	10.VII
Mass staining	15.X	15.X	15.X
Massive maturation of seeds	4.IX	7.IX	10.IX
Massive fruit abscission	10.IX	14.IX	14.IX
Massive defoliation	27.X	30.X	Do not fall down

Table 3: The total number of samples of *Quercus* L.

Species	Variety	Number of samples
Quercus robur	*Quercus robur* var. *praecox*	5
Quercus robur	*Quercus robur* var. *tardiflora*	2
Quercus robur	*Q. robur* var. *"fastigiata"*	1
Quercus libani	—	1
Quercus macrocarpa	—	1
Quercus rubra	—	1
Quercus petraea	—	1

Table 4: ISSR primer sequences.

Primer name	Primer sequence	Annealing temperature (°C)
UBC 811	$(GA)_8C$	53
UBC 835	$(AG)_8YC$	52
UBC 841	$(GA)_8YC$	52
UBC 857	$(AC)_8YG$	52
UBC 878	$(GGAT)_4$	53
UBC 880	$(GGAG)_4$	53

Y is any pyrimidine.

oligonucleotide primers (two lines of three primers) with different annealing temperatures (Table 4) from "100/9 University British Columbia (UBC)" kit (University of Colombia, USA) [25, 27].

2.3. ISSR Amplification and Electrophoresis. The PCR mix was prepared calculating the following quantities of reagents per sample: H_2O (DD), 15.8 μL; 10xdNTP, 2.5 μL (25 mM); 10x PCR buffer, 2.5 μL; Mg, 2.5 μL (25 mM); mutant Taq-polymerase, 0.2 μL (5 unit/μL); DNA sample, 1 μL and 0.5 μL of primer (10 OU/μL). The total volume of the mixture was 25 μL.

Amplification was performed with a Thermal Cycler T100 (BioRad). Amplification protocol is as follows: 1, 94°C, 1.00 min; 2, 94°C, 0.30 s; 3, 52°C/53°C, 0.45 s; 4, 72°C, 2.00 min; 5, 35 cycles from the second point; 6, 72°C, 5.00 min; 7, storage at 12°C.

Separation of DNA fragments was performed by electrophoresis on a 2% agarose gel using TBE-buffer (Tris, boric acid, EDTA), at voltage of 100 V and duration 1.5 hours. DNA was stained with SYBR Green I.

DNA staining dye produced SYBR Green I of ratio 1 μL of dye 5 μL of DNA. Shoot made in GelDoc XR+ system (BioRad) software version 4.1 ImageLab.

Length marker of 100–1500 bp DNA fragments was added to 7 μL per well.

2.4. Statistical Analysis. Computer processing of the derived electropherograms was performed using the program PyElph 1.4, followed by its presentation in the form of a matrix of binary data. Being dominant markers, the ISSRs were scored as presence (1) or absence (0) in order to construct binary matrices for statistical analysis. Only reproducible fragments (in repeated experiments) were considered, whereas the variation in intensity was ignored. Computer analysis of matrices was conducted using MS Excel. Hierarchical cluster analysis of the data was performed by Neighbor-Joining (NJ) and Unweighted Pair Group Average (UPGMA) methods [30], using programs TREECON (version 1.3b) and PyElph 1.4 [31].

3. Results and Discussion

In our work, we used six ISSR primers to test genetic affinity of phenological forms *Q. robur*. The obtained data showed that marker UBC 878 has a lower specificity as compared with all varieties of *Q. robur*.

There was a slight amplification of PCR product in this case that, however, did not affect the analysis of the obtained amplicon spectra (Figure 1).

Table 5: Analysis of information content of the primers.

	Name of primers				
	UBC 811	UBC 835	UBC 841	UBC 857	UBC 880
Maximum number of amplicons	10	12	11	8	11
Minimum number of amplicons	6	3	3	2	5
Monomorphic fragments (unique), total number	7	8	9	5	10
Average percentage of polymorphism of ISSR	93.6	88.8	85.9	54.0	91.6

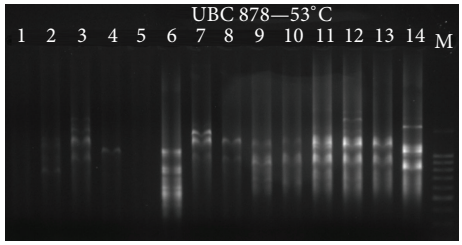

Figure 1: PCR detection of oak trees using the primer UBC 878. 1, *Q. r.* var. *praecox* 1; 2, *Q. r.* var. *praecox* 2; 3, *Q. r.* var. *praecox* 3; 4, *Q. r.* var. *praecox* 4; 5, *Q. r.* var. *praecox* 5; 6, *Q. r.* var. *tardiflora* 1; 7, *Q. r.* var. *tardiflora* 2; 8, *Q. r.* f. *fastigiata*; 9, *Q. macrocarpa*; 10, *Q. libani*; 11, *Q. rubra*; 12, *Q. petraea* 1; 13, *Q. petraea* 2; 14, *Q. petraea* 3; M, the number of the base pair.

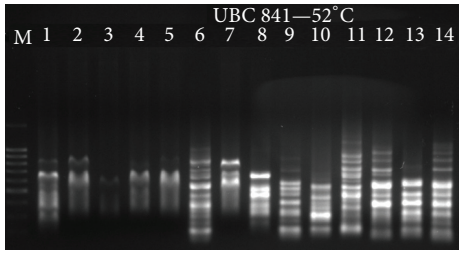

Figure 2: PCR detection of oak trees using the primer UBC 841. 1, *Q. r.* var. *praecox* 1; 2, *Q. r.* var. *praecox* 2; 3, *Q. r.* var. *praecox* 3; 4, *Q. r.* var. *praecox* 4; 5, *Q. r.* var. *praecox* 5; 6, *Q. r.* var. *tardiflora* 1; 7, *Q. r.* var. *tardiflora* 2; 8, *Q. r.* f. *fastigiata*; 9, *Q. macrocarpa*; 10, *Q. libani*; 11, *Q. rubra*; 12, *Q. petraea* 1; 13, *Q. petraea* 2; 14, *Q. petraea* 3; M, the number of the base pair.

Markers UBC 841 and UBC 880 showed the highest sampling specificity (Figures 2 and 3).

A similar result was obtained previously by López-Aljorna and coauthors in the study of *Quercus suber* L. [25].

When analyzing the obtained matrix it was determined that the total number of the amplicons did not exceed 12 (UBC 835) and was not less than 2 (UBC 857). The average number of amplified fragments was 7 of five primers. The quantity of monomorphic (unique) fragments ranged from 5 (UBC 857) to 10 (UBC 880). The average percentage of polymorphism for primer UBC 811 was 93.6%; for the UBC 835 88.8%; for UBC 841 85.9%; for UBC 857 54.0%; for UBC 880 91.6% (Table 5).

The obtained results allowed constructing the dendrogram of a genetic similarity of the material (Figures 4 and 5).

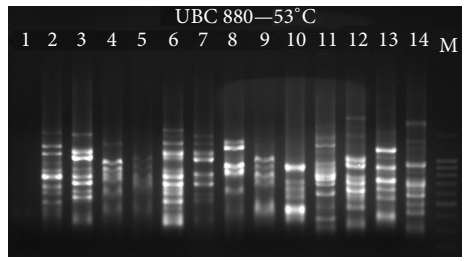

Figure 3: PCR detection of oak trees using the primer UBC 880. 1, *Q. r.* var. *praecox* 1; 2, *Q. r.* var. *praecox* 2; 3, *Q. r.* var. *praecox* 3; 4, *Q. r.* var. *praecox* 4; 5, *Q. r.* var. *praecox* 5; 6, *Q. r.* var. *tardiflora* 1; 7, *Q. r.* var. *tardiflora* 2; 8, *Q. r.* f. *fastigiata*; 9, *Q. macrocarpa*; 10, *Q. libani*; 11, *Q. rubra*; 12, *Q. petraea* 1; 13, *Q. petraea* 2; 14, *Q. petraea* 3; M, the number of the base pair.

The obtained dendrogram was compared to the taxonomic position of the studied species within the genus. Intrageneric taxonomy of these species is shown in Figure 6 [32, 33].

Dendrograms obtained by different methods (Figures 4 and 5) were matched with an intrageneric taxonomy of the studied species (Figure 6). In result, the preference was given to the Neighbor-Joining (NJ) method.

It should be noted here that both methods, NJ and UPGMA, allow clearly identifying clade of «*Q. r. var. praecox*» and «*Q. petraea*». Evidently, both these methods will be effective in analyzing the polymorphism in collection of samples from *Q. robur* and *Q. petraea*, two closely related species.

The dendrogram of Figure 5 showed three principal groups:

(i) Group 1 with *Q. libani* Oliv. and *Q. rubra* L. samples. Both species are most taxonomically distant from *Q. robur*.

(ii) Group 2 «*Q. r. var. praecox*», which includes all samples of the variety, as well as sample N 2 (*Q. r.* var. *tardiflora*) that is logical as far as this sample belongs to the same seed generation as that of *Q. r.* var. *praecox* N 4 and N 5 (all samples of these oaks have a common origin).

(iii) Group 3 «*Q. petraea*», which also includes samples of *Q. robur* "fastigiata" and *Q. macrocarpa* that does not contradict to the genera phylogeny.

(iv) *Q. r.* var. *tardiflora* N 1 out of clades.

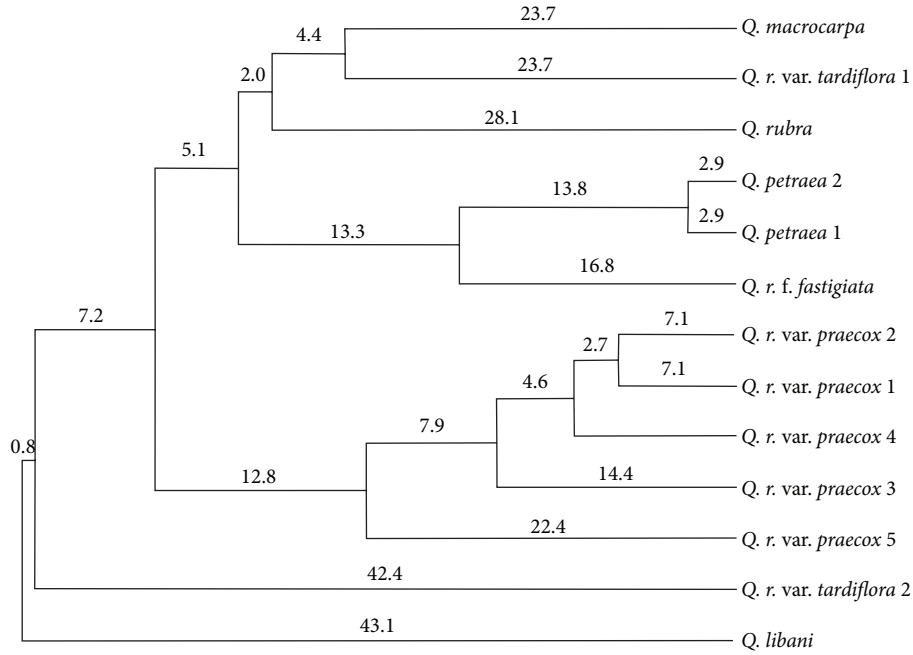

Figure 4: Dendrogram representing the genetic affinity of *Quercus* built by UPGMA on the basis of the electropherogram of primer 841.

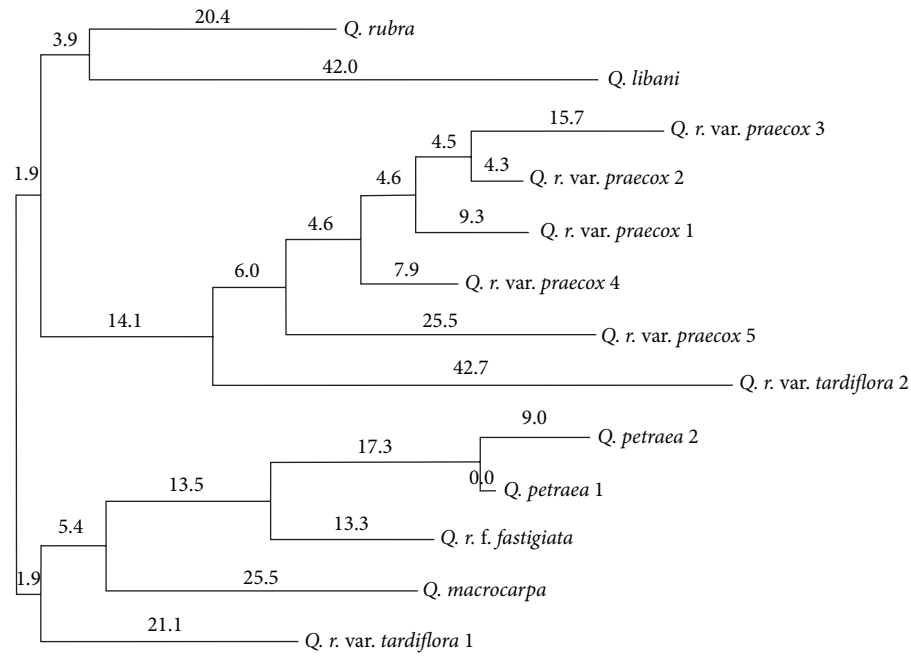

Figure 5: Dendrogram representing the genetic affinity of *Quercus* built by NJ on the basis of the electropherogram of primer 841.

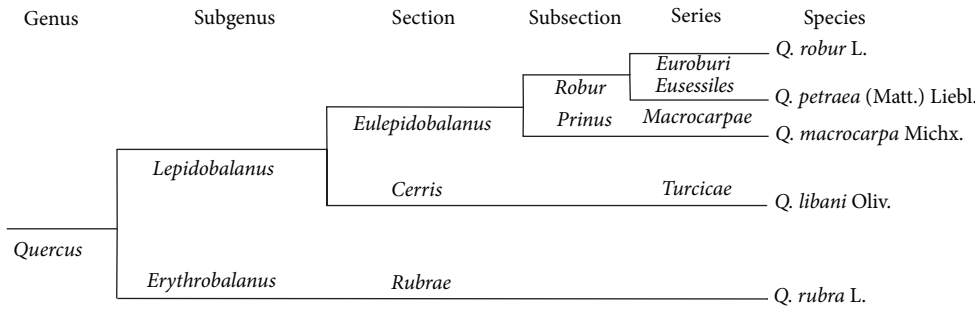

Figure 6: Dendrogram of intrageneric taxonomy of the genus oak (*Quercus*).

It is significant that in result of dendrogram construction by UPGMA method (Figure 4) this sample appeared to be in a more kinship with North American species, *Q. rubra* and *Q. macrocarpa* (one clade), than with *Q. r.* var. *praecox*.

4. Conclusions

Analyzing the obtained matrix it was determined that the maximum number of amplicons is 12 (UBC 835) and minimum number of amplicons is 2 (UBC 857). The average percentage of polymorphism for primer UBC 811 was 93.6%; for UBC 835 88.8%; for UBC 841 85.9%; for UBC 857 54.0%; for UBC 880 91.6%.

It was showed by analyzing the obtained dendrogram (Figure 5) that *Q. libani* and *Q. rubra* samples form Group 1. It is not contrary to the intrageneric taxonomy of the genus oak. Group 2 includes all samples of the variety *Q. robur* var. *praecox* and sample of the variety *Q. robur* var. *tardiflora* N 2. All samples of these oaks have a common origin. The sample *Q. robur* var. *tardiflora* N 2 belongs to the same seed generation as that of *Q. robur* var. *praecox* N 4 and *Q. robur* var. *praecox* N 5. Group 3 includes all samples of *Q. petraea* and also includes the samples of *Q. robur* "fastigiata" and *Q. macrocarpa* that does not contradict to the genera phylogeny (Figure 6). Sample of *Q. robur* "fastigiata" belongs to the seed generation of *Q. robur* var. *praecox*. Sample *Q. r.* var. *tardiflora* N 1 is out of clades.

Analysis of polymorphism of intersimple sequence repeats can be used successfully for DNA fingerprinting of varieties of *Q. robur*. The revealed polymorphism of two samples of *Q. robur*. var. *tardiflora* indicated that similar phenological forms can be based on genetically far-distinct populations, lines, generations, and forms within the *Q. robur* species. Therefore the phenological features of *Q. robur* should not always be considered to be related with its valuable biological and economical characteristics, as it has been done in a number of studies [17–23]. It was shown also that the sample N 1 of *Q. r.* var. *tardiflora* from the artificial population of the Botanical Garden of SFU has unique characteristics of its phenology, living longevity, and characteristics obtained by ISSR-analysis.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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