Random Amplified Polymorphic DNA Analysis of Genetic Diversity of *Taxus baccata* L. in Provenances Baltic Sea Countries

TOMAS KUISYS[1, 2], DONATAS NAUGŽEMYS[2], AUDRIUS SKRIDAILA[1], SILVA ŽILINSKAITĖ[1], DONATAS ŽVINGILA[2]

[1]*Botanical Garden of Vilnius University, Kairėnų 43, LT-10239, Vilnius, Lithuania.*

[2]*Department Botany and Genetics of Vilnius University, M. K. Čiurlionio 21, LT-03101, Vilnius, Lithuania.*

Kuisys, T., Naugžemys, D., Skridaila, A., Žilinskaitė, S. and Žvingila, D. 2007. Random amplified polymorphic DNA analysis of genetic diversity of *Taxus baccata* L. in provenances of Baltic Sea countries. *Baltic Forestry*, 13 (2): 184–189.

Abstract

Taxus baccata L. is extinct species in natural stands of Lithuania and rare in neighbouring states. The paper presents a study of genetic diversity of *Taxus baccata* growing in natural populations close allocated to Lithuanian border in neighbouring Baltic Sea countries. The main aim of this investigation was to assess genetic based possibilities of regeneration of *Taxus baccata* population in Lithuanian forests. In this study we used RAPD markers to assess genetic diversity of 58 *T. baccata* samples of provenances from Latvia, Lithuania, Poland and Russian Kaliningrad region. We identified 58 RAPD profiles on the basis of 64 amplified DNA fragments. 73.4% of RAPD bands were polymorphic. Four provenance specific bands were identified. Results demonstrate rather high level of DNA polymorphism and genetic differentiation of studied *T. baccata* provenances.

Key words: *Taxus baccata* L., provenances, RAPDs, genetic diversity

Introduction

English yew (*Taxus baccata* L., Taxaceae) is a species notable for specific ecological demands. Although the area of the species extends from South Scandinavia to Greece and from North-West Africa to the Baltic States, this species is entered in the Red Data Books of several countries (Navasaitis 2004). This is strictly outcrossing dioecious plant, which pollen is transported by wind and seeds are mainly spread by birds (Hilfiker *et al*. 2004a). The biological peculiarities of this species allow to call it "an old rare species" (Holderegger 1997) that is thought to be adapted to the isolated type of habitats and grows as individual trees or in groups under the shade of other trees.

In Lithuania the English yew, according to the literature, disappeared in the 19th century (Natkevičaitė 1932, Lietuvos TSR flora 1959, Navys 2000). *Taxus baccata* specimens were described in Švėkšna and collected in the herbarium of J. Pabrėža. In 1823 Prof. J. Jundzill took part in an expedition in Žemaitija (West Lithuania) to study forests in the environs of Švėkšna town and indicated this species too (Natkevičaitė 1932, Regelis 1936). The last date when it was mentioned growing as natural habitants - 1863 in Trakai district (Navys 2000). Since ancient times, because of the valuable wood of *Taxus baccata*, it was used in production of furniture and weapons, daily needs and folk medicine. Alkaloid taxol that is reckoned by some authors as very efficient anti-tumour agent is synthesized in the plants of *Taxus* (Wani *et al*. 1971, Croteau *et al*. 2006).

Remains of natural populations of this species are observed in the Baltic Sea countries – Lithuania's neighbours (Latvia, Poland, and Russian Kaliningrad Region). Ecological conditions in certain areas (forests) of Lithuania are suitable for regeneration of population of this species flourishing in maritime climate (Navys 2000). Two oldest examples of English yew growing in Lithuanian parks (Švėkšna and Šateikiai) are nearly 200-years old and could be the remnants of population that existed formerly in Lithuania. Moreover, the English yew is grown as a decorative plant in some parks of Lithuania, as well as botanical gardens and numerous private gardens in all regions of this Baltic state.

Main purpose of this work was to conduct genetic diversity studies of remnants of natural English yew

populations in the neighbouring Baltic Sea countries in areas allocated nearest to the Lithuanian border in order the results of this investigation could be used for elaboration and implementation of the programme for regeneration of natural population of this species in Lithuania. Especially important for elaboration of such a programme is selection of parental plant material. Collecting genetic material from low numbers or improperly chosen individuals can cause low effective population size and inbreeding depression, as well as reduce the adaptive evolutional potential of such population (Barret and Kohn 1991). Indeed the same problems occur in the natural English yew populations in Baltic Sea countries, which are critically small and rather isolated geographically.

Methods of molecular markers are among the most efficient modern ways to assess genetic diversity and determine the genetic structure of a population. Nowadays molecular methods are rather widely used in forest regeneration works (Zhang *et al*. 2006, Blakesley *et al*. 2004, Romero-Severson *et al*. 2003). One of these methods - the RAPD [random amplified polymorphic DNA] technique is at the moment very popular. It is based on PCR [polymerase chain reaction] - one of PCR modifications (Williams *et al*. 1990). Last year RAPD markers have been used for definition of variation found between *Taxus* species, identification of interspecific hybrids (Collins *et al*. 2003), determination of *T. baccata* population genetic structure and dynamics (Hilfiker *et al*. 2004 a, Hilfinker *et al*. 2004 b).

Due to even random distribution of the RAPD markers within a genome and higher polymorphism detected this assay method is superior to allozyme analysis; moreover, its simplicity and lower expenditures for investigations overcome the microsatellite markers. Despite some criticism, mainly for lack of reproducibility, it is suggested that after proper optimization, the RAPD is a reliable sensitive and reproducible assay (Atienzar and Iha 2006). Therefore, the RAPD is widely applied in plant populations investigation (Nybom 2004, Nybom and Bartish 2000). Another limitation of RAPD markers is their dominant nature (Wiliams *et al*. 1990). This peculiarity of the RAPDs impedes direct estimations of allele frequency and can bias calculations of population differentiation (Lynch and Miligan 1994). This problem can be overcome by using analysis of molecular variance (AMOVA), which is not influenced by the dominance problem (Huff *et al*. 1993, Diaz *et al*. 2001).

The objective of our study was to evaluate, quantify and compare genetic diversity in provenances of *T. baccata* growing in different countries (Latvia, Lithuania, Poland, Russian Kaliningrad region).

Matterial and methods

Plant material. Four provenances of *T. baccata* from Poland, Latvia, Kaliningrad and Lithuania region were analyzed in this study (Fig. 1; Table 1). Number of individuals analyzed per provenance ranged from 2 to 28. Fresh leaf material of individual's (plants) was collected and kept on the ice. Then this plant material was stored at -40°C up to extraction of DNA. In total, 58 samples were applied for DNA analysis.

Figure 1. Locations of four studied provenances of *Taxus baccata* L. Provenances designations are following: Latvia (Sl - Slitere, Em - Embrekši, Ze - Zentene, Da - Daiķi), Lithuania (Šv - Švėkšna, Ša - Šateikiai), Kaliningrad (Za - Zaicev, Ce – Celau), Poland (Cz – Czeski, CJ – Cisowy Jar, W – Węzewo)

Table 1. Number of investigated *Taxus baccata* L. samples in different provenances

Provenance	Site	Numbers of sampled plants	
Latvia	Slitere	11	28
	Embrekši	1	
	Zentene	3	
	Daiķi	13	
Kaliningrad	Zaicev	7	11
	Celau	4	
Poland	Czeszki	1	17
	Cisowy Jar	15	
	Węzewo	1	
Lithuania	Šateikiai	1	2
	Švėkšna	1	

DNA extraction. Genomic DNA was extracted using a CTAB protocol (Doyle and Doyle 1990). DNA

concentration and quality were measured using Eppendorf BioPhotometer.

RAPD-PCR. Decamer primers purchased from Carl Roth GmbH were used. Six primers were selected for further analysis (Table 2). Reactions were performed in 25 µl volumes and consisted of 2.5 µl 10x *Taq* reaction buffer, 3.0 mM $MgCl_2$, 0.2 mM dNTP, 1 µM primer, 1 U *Taq* DNA polymerase (MBI Fermentas) and 50 ng/µl genomic DNA. PCRs were conducted using Biometra TPersonal thermocycler according to the following program: 4 min at 94°C, followed by 35 cycles of 60 s at 94°C, 60 s annealing at 36°C, 2 min extension at 72°C and last extension cycle for 5 min at 72°C.

Table 2. Primers used in RAPD analyses, numbers and size of generated bands

Primer (Carl Roth)	Primers sequence 5'• 3'	No. of DNA bands	Numbers of monomorphic loci	Numbers of polymorphic loci	Percentage of polymorphic loci (%)	DNA bands size range (bp))
170-04	CGC ATT CCG C	12	2	10	83.3	500-1680
170-05	GAG ATC CGC G	9	4	5	55.6	450-1300
170-08	CTG TAC CCC C	9	2	7	77.8	400-1250
170-10	CAG ACA CGG C	12	3	9	75	640-2000
380-01	ACG CGC CAG G	13	2	11	84.6	500-2300
380-03	GGC CCC ATC G	9	4	5	55.6	450-2000
	Total:	64	17	47	73.4	400-2300

Amplification products were separated on 1.5% agarose gel in 1.0x TBE buffer and visualized by staining with ethydium bromide and photographed over UV light using BioDocAnalyze system (Biometra). Each reaction was performed at least twice in independent experiments. Only clear and reproducible bands were taken for data analysis.

Data analysis. Data were organized in a binary matrix with 1 indicating band presence and 0 indicating band absence. This matrix was analyzed using TREECON for Windows (Van De Peer and De Wachter 1994). Genetic distance matrix was generated according to Nei and Li (Nei and Li 1979). Clustering analysis was performed using Unweighted Pair - Group Method of arithmetic Averages (UPGMA) method algorithm. The significance level of dendrogram branches was determined using 1000 bootstrap replicates. The Shannon index of diversity (I), Nei (1973) gene diversity (h), coefficient of gene differentiation (G_{ST}) and effective number of alleles (n_e) were calculated using POPGENE v.1.32 (Yeh *et al*. 1999).

Principal coordinate analysis (PCA) and distribution of DNA fragments in *T. baccata* provenances was performed using software package GenAlEx v.6. Mean heterozygosity was calculated assuming Hardy – Wainberg equilibrium for each polymorphic locus (Nei, 1978) using GenAlEx v.6 (Peakall and Smouse 2006).

Results and discussions

The 6 chosen primers amplified a total of 58 scorable RAPD bands that ranged in size from 400 to 2300 bp (Table 2). Every one of primers generated between 9 and 13 DNA fragments with a mean of 10.7. 73.4 % of the RAPD bands were polymorphic, which is similar to the value obtained by Collins *et al*. (2003). These authors considered a species separation of *T. baccata*, *T. canadensis* and *T. cuspidata* and origins of their hybrids on the basis of the RAPD and cpDNA data. The highest number of polymorphic bands was established in the *T. baccata* group (86.5% polymorphism). The average number of species polymorphic loci determined in our study was 47 (Table 2). Fifty eight unique RAPD banding patterns were observed, i.e. any of the samples have not signified an identical banding pattern and UPGMA cluster analysis based on GDxy values indicated patterns of genetic relationship among individual plants from different provenances (Fig. 2). This demonstrates the existence of genetic variation in the studied plant groups.

The Nei's gene diversity (h) within studied plant group provenances of *T. baccata* ranged from 0.1901 Kaliningrad provenances to 0.3751 Latvian provenances (Table 3).

Table 3. Calculated values of genetic diversity indices in provenances of *Taxus baccata*

Provenance	Polymorphism (%)	Number of effective alleles, n_e	Nei's gene diversity, h	Shannon's information index, I
Poland	78.72	1.5162	0.2913	0.4288
Kaliningrad	53.19	1.3173	0.1901	0.2849
Latvia	87.23	1.6840	0.3751	0.5392
Mean ± SD	73.05 ± 17.71	1.5058 ± 0.184	0.2855 ± 0.093	0.4176 ± 0.127

The coefficient of gene differentiation among studied plant groups was 0.218. This indicated that 21.8% of the total genetic diversity existed among groups, while 78.2% existed within groups. This result indicates the genetic specificity of *T. baccata* germplasm from different countries. The level of genetic differentiation established in our study was higher than one detected by Hilfiker *et al*. (2004 b). The mean value of molecular variance established in that study ranged from 4.34 in the small populations to 5.02 in the large populations. This discrepancy could be explained by less isolation of *T. baccata* populations in Switzerland than in the Baltic Region.

Genetic analysis of material collected from two oldest in Lithuania *Taxus baccata* specimens (growing in Šateikiai and Švėkšna parks, West Lithuania) has been also performed. Comparison of studied material demonstrated that analyzed Lithuanian samples resembled with some of the examples from the Kaliningrad provenances, as indicated in the dendrogram (Fig. 2). However, we cannot make statistically reliable conclusions about the genetic relationship of the oldest Lithuanian individuals to those in Latvian and Kaliningrad Region provenances, since the material we have tested is not sufficient. That is why other genetic parameters of *T. baccata* from Lithuanian provenance are not given in the paper, as is known the genetic diversity correlates to the size of population (Frankham 1996). Nevertheless, the investigations carried out do not dispose the possibility that the English yew individuals in the parks of Šateikiai and Švėkšna can be

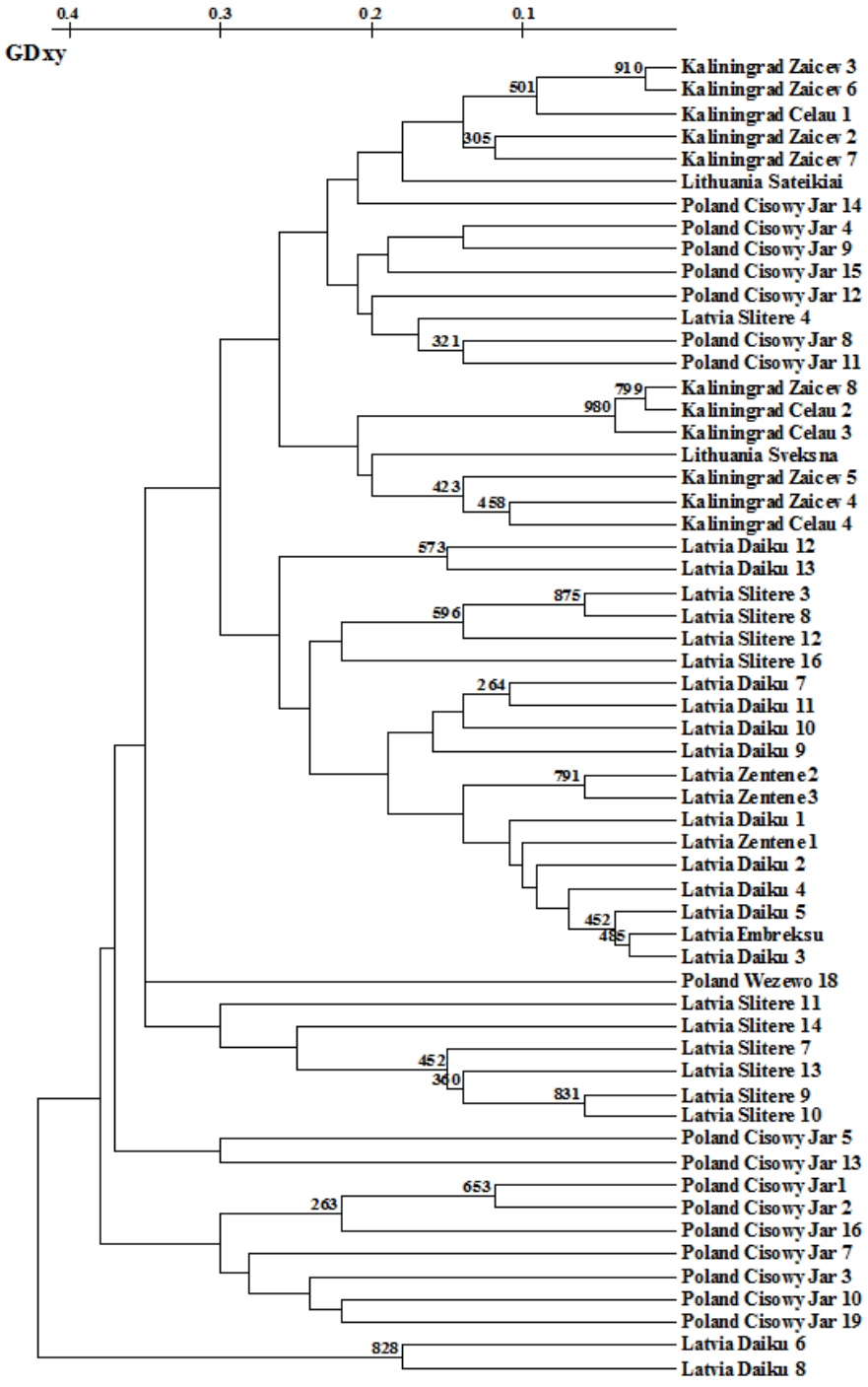

Figure 2. Bootstrapped (1000 iterations) cluster analysis (UPGMA) of 3 provenances based on the genetic distance among 58 *Taxus baccata* plants (Nei and Li's 1979)

remnants of the former natural Lithuanian population. Especially that some literature sources (Natkevičaitė 1932, Regelis 1936) have mentioned facts of still naturally growing English yew in forest adjacent to Švėkšna in the first half of the 19th century.

The genetic structure of investigated *T. baccata* provenances is illustrated by PCA (Fig. 3). It shows that the most plants from Poland and Kaliningrad provenances tend to group together and are more genetically similar than individuals from Latvian provenances.

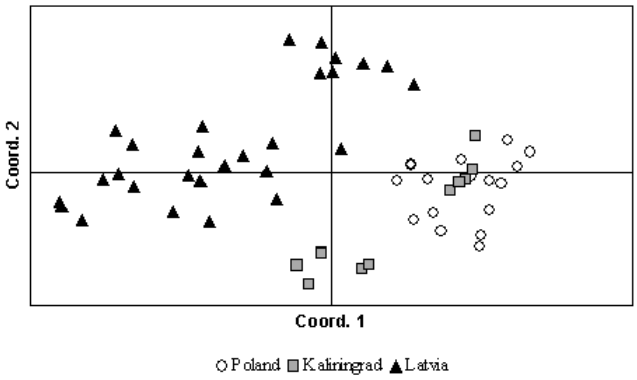

Figure 3. Two-dimensional representation of principal coordinate analysis of *Taxus baccata* provenances. Coordinate 1 extracted 31.78% of the variance and Coordinate 2 extracted 19.67% of the variance

The study of mean heterozygosity level within *T. baccata* provenances demonstrates that the highest one – was in Latvia provenance (0.37) and the lowest one was in Kaliningrad provenances (0.19). A few private bands only were detected: one in Kaliningrad provenance and three in Latvian provenance (Fig. 4). This rather low frequency of individual bands indicates that most of the divergence between populations derives from band frequency differences. Nowadays is taken a view that the higher heterozygosity causes the better adaptation (David 1998). Our observations indirectly consist with this point of view. In Latvia provenance, with the highest level of mean heterozygosity, were observed numerous individuals of English yew in-fruit, more healthy and by far larger. Whereas those studied in Kaliningrad Region were notable for worse morphology, scrubby and less productive (data not shown). In order to regenerate the English yew population in Lithuanian forests, it would be more expedient to take the parental propagation material from yews growing in Latvia.

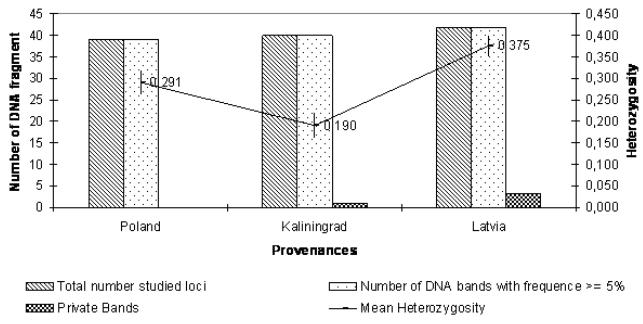

Figure 4. The level of average expected heterozygosity in the study populations of *Taxus baccata*

Conclusions

1. The study of *Taxus baccata* L. provenances reported in this paper provides a more detailed insight into genetic constitution of autochthonous specimens that survived until nowadays in areas adjacent to Lithuania.

2. The results of our investigation demonstrate a rather high level of DNA polymorphism and genetic differentiation of *T. baccata* provenances in Latvia, Poland and Russian Kaliningrad region.

3. Examples of Polish and Kaliningrad regions *T. baccata* provenances are closer genetically related. The level of mean heterozygosity of examples from Latvian provenance is higher.

4. For potential regeneration of *T. baccata* population in Lithuania, the parental material for propagation seems to be better fitting from Latvia. Its higher heterozygosity is related to higher flexibility of plants and their wider potential for adaptation under more diverse ecological conditions.

References

Atienzar, F.A. and Iha, A.N. 2006. The random amplified polymorphic DNA (RAPD) assay and related techniques applied to genotoxicity and carcinogenesis studies: a critical review. *Mutation Research,* 613: 76-102.

Barrett, S.C.H. and Kohn, J.R. 1991. Genetic and evolutionary consequences of small population size in plants: implications for conservation. In: Falk D.A. and Holsinger K.E. (Eds), Genetics and Conservation of Rare Plants. *Oxford University Press*, p. 3–30.

Blakesley, D., Pakkad, G., James, C., Torre, F. and Elliott, S. 2004. Genetic diversity of *Castanopsis acuminatissima* (Bl.) A. DC. in northern Thailand and are selection of seed trees for forest restoration. *New Forests*, 27: 89-100.

Collins, D., Mill, R.R. and Möller, M. 2003. Species separation of *Taxus baccata*, *T. canadensis* and *T. cuspidate* (Taxaceae) and origins of their reputed hybrids inferred from RAPD and cpDNA data. American *Journal of Botany,* 90(2): 175-182.

Croteau, R., Ketchum, R.E.B., Long, R.M., Kaspera, R. and Wildung, M.R. 2006. Taxal biosynthesis and molecular genetics. *Phytochemistry Reviews,* 5: 75-97.

David, P. 1998. Heterozygosity – fitness correlations: new perspective on old problems. *Heredity,* 80: 521-537.

Doyle, J.J. and Doyle, J.L. 1990. Isolation of plant DNA from fresh tissue. *Focus,* 12: 13–15.

Frankham, R. 1996. Relationship of genetic variation to population size in wildlife. *Conservation Biology,* 10: 1500-1508.

Hilfiker, K., Gugerli, F., Schütz, J-P., Rotach, P. and Holdergger, R. 2004 a. Low RAPD variation and female – biased sex ratio indicate genetic drift in small populations at the dioecious conifer *Taxus baccata* in Switzerland. *Conservation Genetics,* 5: 357-365.

Hilfiker, K., Holdergger, R., Rotach, P. and Gugerli, F. 2004 b. Dynamics of genetic variation in *Taxus baccata*: local versus regional perspectives. *Can. J. Bot.*, 82: 219-227.

Holderegger, R. 1997. Recent perspectives in conservation biology at rare plats. *Bull Geobot Just ETH*, 63: 109-116.

Huff, D.R., Peakall, R. and Smouse, P.E. 1993. RAPD variation within and among natural populations of outcrossing buffalograss [*Buchloe dactyloides* (Nutt.) Engelm.]. *Theoretical and Applied Genetics*, 86: 927-934.

Lietuvos TSR flora [Flora of Lithuanian SSR]. 1959. Vilnius, I t., 87-89, (in Lithuanian).

Lynch, M. and Milligan B.G. 1994. Analysis of population genetic structure with RAPD markers. *Molecular Ecology,* 3: 91-99.

Natkevičaitė, M. 1932. Kukmedis (*Taxus baccata* L.) [English yew (*Taxus baccata* L.]. *Kosmos*: 3-4, (in Lithuanian).

Navasaitis, M. 2004. Dendrologija [Dendrology]. Vilnius, *Margi raštai*, 111-115 p., (in Lithuanian).

Navys, E. 2000. English Yew (*Taxus baccata* L.) in forests of Baltic States and the main reasons for its distinction from Lithuania. *Baltic Forestry,* 6 (2): 41-46 (*Brief Reports*).

Nei, M. 1978. Estimation of average heterozygosity and genetic distance from a small number of individuals. *Genetics*, 89: 583-590.

Nei, M. and Li, W.H. 1979. Mathematical model for studying genetic variation in terms of restriction endonucleases. Proceedings of National Academy of Science USA, 76: 5269-5273.

Nybom, H. 2004. Comparison of different nuclear DNA markers for estimating intraspecific genetic diversity in plants. *Molecular Ecology,* 13: 1143-1155.

Nybom, H. and Bartish, J.V. 2000. Effects of life history traits and sampling strategies on genetic diversity estimates obtained with RAPD markers in plants. *Perspectives in Plant Ecology, Evolution and Systematics*, 3/2: 93-114.

Peakall, R. and Smouse, P. 2006. GenAlEx v.6, Genetic Analysis in Excel. Population genetic software for teaching and research. *Mol Ecol Not*; 6: 288-295.

Regelis, K. 1936. J. Jundzilos kelionė per Žemaitiją. *Gamta*, 3: 190-195.

Romero-Severson, J., Aldrich, P., Feng, J., Sun, W. and Michler, Ch. 2003. Chloroplast DNA variation of northern red oak (*Quercus rubra* L.) in Indiana. *New Forests,* 26: 43-49.

Van de Peer, Y. and De Wachter, R. 1994. TREECON for Windows: a software package for the construction and drawing of evolutionary trees for the Microsoft Windows environment. *Computer applications in the biosciences*, 10: 569-570.

Wani, M.C., Taylor, H.C., Wall, M.E., Coggan, P. and McPhast, A.T. 1971. The isolation and structure of taxal, a novell antileukemic and antitumor agent from *Taxus brevifolia. J. Am.Chem. Soc*., 93: 2325-2327.

Williams, J.G.K., Kubelik, A.R., Livak, K.J., Rafalski, J.A. and Tingey, S.V. 1990. DNA polymorphisms amplified by arbitrary primers is useful are genetic markers. Nucleic Acids Research. 18: 6531–6535.

Yeh, F.C., Yang, R. and Boyle, T. 1999. POPGENE v.1.31, Microsoft Window-based Freeware for Population Genetic Analysis.

Zhang, X., Chen, X-Y. and Zhang, D. 2006. Effect of regeneration method on RAPD-based genetic variation of *Cyclobalanopsis glauca* (Fagaceae). *New Forests*, 32: 347-356.

Received 20 March 2007
Accepted 30 October 2007

RAPD АНАЛИЗ ГЕНЕТИЧЕСКОГО ПОЛИМОРФИЗМА В ПРОВЕНАНЦИЯХ *TAXUS BACCATA* L.

Т. Куйсис, D. Наугжемис, А. Скридайла, С. Жилинскайте и Д. Жвингила

Резюме

Тис европейский (*Taxus baccata* L.) является редким исчезающим видом в соседних с Литвой странах. В Литве этот вид считается исчезнувшим в XIX в. Для оценки генетического полиморфизма в 58 образах *T. baccata* из Литвы, Латвии, Польши и Калининградской области РФ мы использовали метод RAPD – анализа с шестью праймерами. На основе 64 RAPD локусов были определены 58 RAPD спектра, что показывает генетические различие между всеми исследованными образцами. Уровень полиморфизма ДНК составил 73,4%. RAPD – анализ показал, что существует довольно высокая генетическая дифференциация между провененциями тиса разного происхождения.

Ключевые слова: *Taxus baccata* L., генетический полиморфизм, RAPD анализ