BIOLOGIJA. 2006. Nr. 1. P. 53–59

Assessment of genetic polymorphism in natural populations of ostrich fern (*Matteuccia struthiopteris* L., Dryopteridaceae) in Lithuania by using RAPD markers

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Randomly amplified polymorphic DNA analysis was carried out to examine genetic diversity, population structure and polymorphism level among and within seven natural populations of ostrich fern (*Matteuccia struthiopteris* L., Dryopteridaceae) in Lithuania. In total, 43 plants were analysed using 10 polymorphic decanucleotide primers of random sequence. 73 polymorphic bands ranging in size from 470 to 2600 bp were derived using selected primers. The average value for Shannon's phenotypic diversity index for all seven populations was $I = 0.0498$ ($SD = 0.0534$, $P = 0.02$), mean heterozigosity was 0.033 ($SD = 0.038$, $P = 0.03$), total gene diversity $H_t = 0.3385$ ($SD = 0.0164$), gene diversity among populations $G_{st} = 0.9009$ and gene flow $N_m = 0.0550$. PCA and UPGMA analysis revealed a high degree of interpopulation differentiation. Genetic differences among populations were due to the absence or presence of the same alleles in different populations, but not to the frequency of alleles in each population. Analysis of molecular variance (AMOVA) revealed that 9% of the total molecular variance was due to an individual variation within the populations and 91% to the differences among populations ($\Phi_{PT} = 0.909$, $P = 0.001$, $df = 6$). A weak but statistically significant correlation ($r = 0.354$, $P = 0.047$) between Nei's genetic distances and the geographic location of populations was established using the Mantel test. The very low intrapopulation differentiation shows that clonal propagation is prevailing in populations of ostrich fern in Lithuania. The parameters of genetic diversity and a correlation of genetic distance with the geographic location revealed the existence of population isolation by distance forasmuch as migration is very low.

Key words: clonality, genetic diversity, *Matteuccia struthiopteris*, RAPD, population genetic structure

INTRODUCTION

Matteuccia struthiopteris (L.) Todaro (Dryopteridaceae) is a hemicryptophytic fern, which has circumpolar distribution in the north temperate and sub-boreal zones of the northern hemisphere [1, 2]. Its area spreads from North America, through Europe to East Asia. In Europe it is mostly confined to northeastern parts and usually along floodplains, springy sites, and its occurrence may be considered as an indicator of moist eutrophic soils [2]. *Matteuccia* is the only fern species in Lithuania with the dimorphic fronds: sterile trophophylls and fertile sporophylls [2–4]. Ostrich fern often forms extensive mono-dominant stands as the light at the ground level in the most vigorous and dense stands of *Matteuccia* is reduced to a level insufficient for the growth of competitors [2]. *M. struthiopteris* populations are long-lived and theoretically they are immortal due to continuous clonal growth [5]. Stands of *Matteuccia* spread asexually by the colonization of surrounding soil by stolons developed on the rhizomes. Ramets (erect rootstocks with a projecting crown of fronds) connected to the mother plant by rhizome may genetically represent the same individual (genet) in a population. Plants of sexual origin have not been recorded in dense fern stands [4, 6]. The lack of sexually reproduced new *Matteuccia* plants have also

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been reported from other areas [7], and this feature may be related to the location of population according to the species areal [8]. Plants of *Matteuccia* disperse by air and water dispersal of spores, and water dispersal of crowns and/or stolons from populations along riverbanks. Establishment of young plants from gametophytes has not been studied, for in spite of the prodigious production of spores per fertile frond, very few spores germinate [5].

Ostrich fern is a quite rare and endangered species in Lithuania. Populations of this species are clearly restricted and sparse in the country. This species is widely known for its ornamental value in the whole world and as an economic fern in America where edible fiddleheads are a spring vegetable [2, 5, 9]. There is no threat for ostrich fern to be exterminated in Lithuania because of its nutritive quality as a vegetable, but the distribution of this species is too narrow to be indifferent and this is a critical case of interest. *M. struthiopteris* is protected by law in the majority of Central European countries [1]. There have been carried out different studies on the physiology [10] and propagation [11], morphology [4] and anatomy [12], structural variations [13], demography [4] and composition of ostrich fern populations [1, 14]. Although some systematic analyses and phytogeographic studies have been published [2], the genetic composition of *Matteuccia* populations remains unclear. Only a few articles can be found to deal with the ostrich fern population genetic structure or genetic diversity using allozyme systems [9]. The present study is a new insight to the attributes of *M. strutiopteris* populations that have been studied genetically very modestly up to now. As there is no available specific information about the genetics of this species, the RAPD method was chosen as the most appropriate.

MATERIALS AND METHODS

Plant material. Seven natural populations of ostrich fern (*M. struthiopteris*) were analysed in Lithuania using RAPD markers: three populations in western Lithuania (Naujoji Akmenė, Šilutė and Telšiai districts), one population in the eastern part (Anykščiai distr.), and two populations in the central part of Lithuania (Kaišiadorys and Panevėžys districts). The last – Pilis island – population is different from the rest because it is located on the island in Lake Plateliai (Þemaitija regional park). The tops of fronds (10 cm) and croziers (coiled young fronds) from the top of the rootstocks of 10–12 ferns growing at a distance no less than 3 m were collected for analysis from each population. Plants were picked up from the whole area occupied by this species going lengthways the population. Plant material was collected in August–September of 2004 and July–August of 2005. In total, 43 samples collected from seven populations were analysed using RAPD (Table 1).

DNA extraction and amplification. Genomic DNA was extracted from 135 mg of frond or 100 mg of crozier tissue using liquid nitrogen and the Genomic DNA Purification Kit (Fermentas, Lithuania) in accordance with recommendations of manufacturer. DNA concentration and purity were measured spectrophotometrically (BioPhotometer, Eppendorf, Germany). Ten decanucleotide primers (Roth, Germany) of random sequence (GC 70–80%) were chosen from three primer kits (Kit 270, Kit 380, Kit 470). Amplification was performed in a thermocycler (Mastercycler personal 5332, Eppendorf, Germany) and the amplified DNA bands were separated electrophoretically under the conditions described earlier [14]. Amplification was performed at least in two independent experiments for each sample. Data from clear, unambiguous and reproducible bands were used for analysis.

Data analysis. Amplified DNA bands were estimated as distinct DNA loci with two alleles. Monomorphic loci had only one allele (present = 1) while polymorphic loci had both (present = 1 and absent = 0) alleles.

The number of polymorphic loci, measures of heterozygosity and the Shannon's index of phenotypic diversity (I), genetic distances (GD) among populations, parameters of genetic diversity (H_t, G_{ST}, h) and

Table 1. **Sampling locations (districts in brackets), coordinates and sample sizes of individuals analyzed in populations of ostrich fern (*Matteuccia struthiopteris* L.) in Lithuania**

No	Population	n	Latitude N	Longitude E
1	Anykščiai (Anykščiai distr.)^{NE}	7	55°34'	25°05'
2	Krekenava (Panevėžys distr.)^{NE}	8	55°35'	24°11'
3	Rambynas (Šilutė distr.)^{W}	4	55°02'	22°06'
4	Pilis island (Telðiai distr.)^{W}	5	56°02'32,1"	21°50'50,0"
5	Plokštynė (Telšiai distr.)^{W}	7	56°02'35,3"	21°50'49,5"
6	Venta (N. Akmenė distr.)^{NW}	7	56°09'	22°35'
7	Lomena (Kaiðiadorys distr.)^{C}	5	54°57'	24°26'
	total	**43**		

^{NE} – northeastern, ^{C} – central, ^{W} – western, ^{NW} – northwestern part of Lithuania.

gene flow (N_m) were computed with PopGene1.31 [16] assuming all loci to be dominant and in Hardy–Weinberg equilibrium. Genetic distances among individual ferns were estimated according to the Nei and Li formula [17] using TREECON for Windows V 1.3b [18]. UPGMA (Unweighted Pair-Group Method for Arithmetic Averages) cluster analysis based on these genetic distances generated a dendrogram representing the relationships among the populations [18]. GenAlEx_6b4 [19] software package was used for the performance of the principal coordinates analysis (PCA) – to plot the relationship between genetic distances matrix elements based on their principal coordinates. Analysis of molecular variance (AMOVA) was performed using the same software. AMOVA estimated the partitioning of the total genetic variation into intrapopulational and interpopulational (Φ_{PT}) values. Variance components were tested statistically by nonparametric permutation procedures using 999 permutations. The Mantel test has been performed to find out if there is a relationship between geographic location (latitude, longitude) of populations and their genetic distances.

RESULTS

One hundred and four DNA bands were generated in the analysis in total, but 31 of them (29.8%) were not included in the analysis because of their monomorphic pattern. RAPD was carried out using 73 polymorphic loci. The percentage of polymorphic loci for each primer varied from 57.1 to 85.7% with an average of 70.2% (Table 2). Amplified band sizes ranged from 470 to 2600 bp and an average number of band per primer was 7.3. Analysis of band patterns (Fig. 1) showed that all bands had a frequency of ≥5% and no bands with a frequency of ≤5% were amplified. Moreover there were no any bands with a low frequency (5% ≤ X ≤ 25%) either. From 5 to 9 bands had a frequency of 25% ≤ X ≤ 50%, the rest of bands were more than 50% frequent in all seven populations. Five private alleles each present in only one population were detected. Two private alleles were specific for Anykščiai population, and the other three were specific for Krekenava, Plokštynė and Venta populations respectively (Fig. 1). There were 14 zero alleles (data not shown) which were absent only in one population, but they were not considered as private given that fewer mutations contribute to the occurrence of 0 allele than of the allele that is present. Data in Table 3 show a very low number of polymorphic loci for each population – the mean is only seven. The lowest and the highest percentage of amplified polymorphic loci differ 18 times: 1.4% in the Plokštynė population and 24.7% in the Venta population (mean 9.4%, SD 8.9). This parameter of polymorphism is totally opposite when compared to the polymorphism level (70.2%) for all seven populations together in Table 2. These results reveal that genetic differences among the populations are due to the absence or presence of the same alleles in different populations, but not to the frequency of alleles in each population (Table 3).

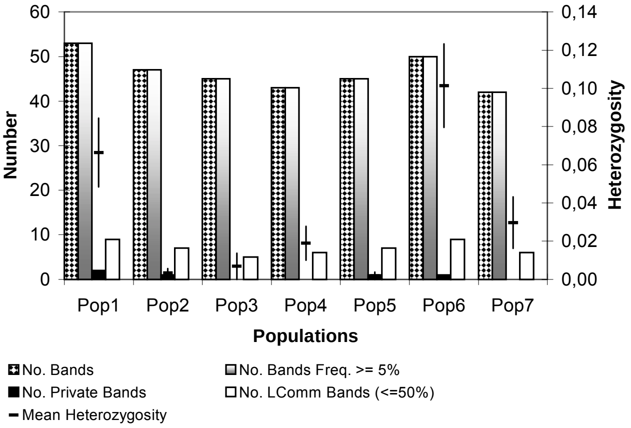

Fig. 1. RAPD band patterns and mean heterozygosity across *Matteuccia struthiopteris* L. populations in Lithuania

Table 2. **Sequences of ten primers used in the research, polymorphic band size range, total number of amplified bands, monomorphic and polymorphic bands and percentage of polymorphic bands for each primer**

Primer	Sequence (5'→3')	Polymorphic band size range (bp)	Total number of bands	Monomorphic bands	Polymorphic bands	Polymorphism (%)
Kit 270-1	GTCTCGTCGG	540–1800	10	2	8	80.0 ± 12.6
Kit 270-3	GTGTAGGGCG	500–1900	12	3	9	75.0 ± 12.5
Kit 270-4	CGGGTCGATC	590–1500	11	4	7	63.6 ± 14.5
Kit 380-2	ACTCGGCCCC	540–2200	18	7	11	61.1 ± 11.5
Kit 380-3	GGCCCCATCG	770–2100	15	3	12	80.0 ± 10.3
Kit 380-9	ACGGCGGCTC	1050–1600	7	3	4	57.1 ± 18.7
Kit 470-4	GGACCGCTAG	470–2100	10	4	6	60.0 ± 15.5
Kit 470-5	CATGTCCGCC	1000–2500	8	2	6	75.0 ± 15.3
Kit 470-7	CTATCGCCGC	960–2600	7	1	6	85.7 ± 13.2
Kit 470-10	CGCAGACCTC	790–2600	6	2	4	66.7 ± 19.2
		total	**104**	**31**	**73**	**70.2 ± 4.5**

Table 3. **Number and percentage of polymorphic loci, pattern of genetic diversity for each population of *Matteuccia struthiopteris* L. in Lithuania**

Population	Number of polymorphic loci	Percentage of polymorphic loci (%)	Observed number of alleles, N_a	Mean heterozigosity	Shannon index	h
Anykščiai	14	19.2	1.1918 (0.3964)	0.066 (0.018)	0.0988 (0.2205)	0.0664 (0.1539)
Krekenava	2	2.7	1.0274 (0.1644)	0.003 (0.002)	0.0066 (0.0394)	0.0033 (0.0199)
Lomena	5	6.8	1.0685 (0.2543)	0.030 (0.013)	0.0423 (0.1613)	0.0297 (0.1148)
Pilis island	5	6.8	1.0685 (0.2543)	0.019 (0.009)	0.0302 (0.1174)	0.0189 (0.0763)
Plokštynė	1	1.4	1.0137 (0.1170)	0.002 (0.002)	0.0036 (0.0309)	0.0019 (0.0161)
Rambynas	3	4.1	1.0411 (0.1999)	0.007 (0.007)	0.0203 (0.1028)	0.0132 (0.0692)
Venta	18	24.7	1.2466 (0.4340)	0.101 (0.022)	0.1467 (0.2665)	0.1015 (0.1871)
Mean (SD)	**7 (7)**	**9.4 (8.9)**	**1.0939** **(0.0893)**	**0.033** **(0.038)**	**0.0498** **(0.0534)**	**0.0602** **(0.0713)**

h – Nei's [27] gene diversity; standard deviations are given in brackets.

Table 4. **Nei's unbiased measures of genetic identity (above *** diagonal) and genetic distance (below *** diagonal) [19]**

Population	Anykščiai	Krekenava	Rambynas	Pilis island	Plokštynė	Venta	Lomena
Anykščiai	***	0.6638	0.6677	0.6498	0.6387	0.6791	0.6444
Krekenava	0.4097	***	0.6423	0.6134	0.5779	0.6702	0.5967
Rambynas	0.4040	0.4427	***	0.6551	0.6567	0.5632	0.6672
Pilis island	0.4311	0.4888	0.4229	***	0.6769	0.6458	0.5769
Plokštynė	0.4483	0.5484	0.4206	0.3902	***	0.6557	0.5007
Venta	0.3870	0.4001	0.5741	0.4372	0.4221	***	0.6372
Lomena	0.4394	0.5163	0.4046	0.5501	0.6918	0.4507	***

The average value of Shannon's information index of phenotypic diversity (I) for all seven populations was 0.0498 (SD = 0.0534, P = 0.02) ranging from 0.0036 in the Plokštynė population to 0.1467 in the Venta population. The values of Nei's [17] gene diversity based on the frequency of alleles varied from 0.0019 (SD = 0.0161) in the Plokštynė population to 0.1015 (SD = 0.1871) in the Venta population. Mean heterozigosity was 0.033 (SD = 0.038, P = 0.03) ranging from 0.002 (Plokštynė pop.) to 0.101 (Venta pop.). The same tendency was observed for the number of alleles (N_a): the lowest value of N_a was observed in the Plokštynė population (1.0137) and the highest in the Venta population (1.2466).

AMOVA analysis revealed that only 9% of the overall molecular variance was due to the differences within populations and 91% was due to the interpopulational differences (Φ_{PT} = 0.908841, p = 0.001, df = 6). The estimated average number of migrants per generation (N_m) is only 0.055. This means that 18 generations are required for one migrant to arise.

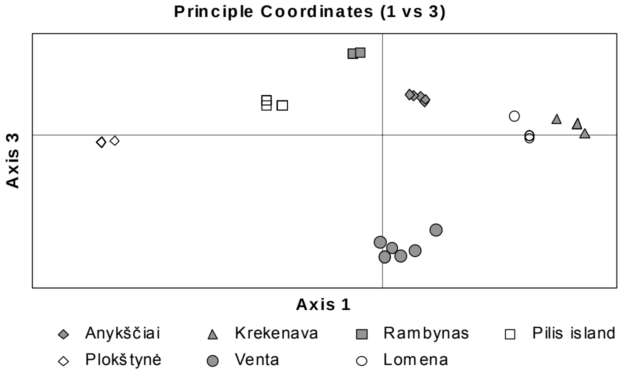

Fig. 2. Principal coordinates analysis representing relationship among individuals of seven populations of *Matteuccia struthiopteris* L.

The Genetic Nei's distance and identity [20] among pairs of populations are given in Table 4. The lowest genetic distance was detected between Anykščiai and Venta populations (GD = 0.3870) and the highest between Lomena and Plokðtynė populations (GD = 0.6918). Principal coordinate analysis has shown that populations don't overlap in space

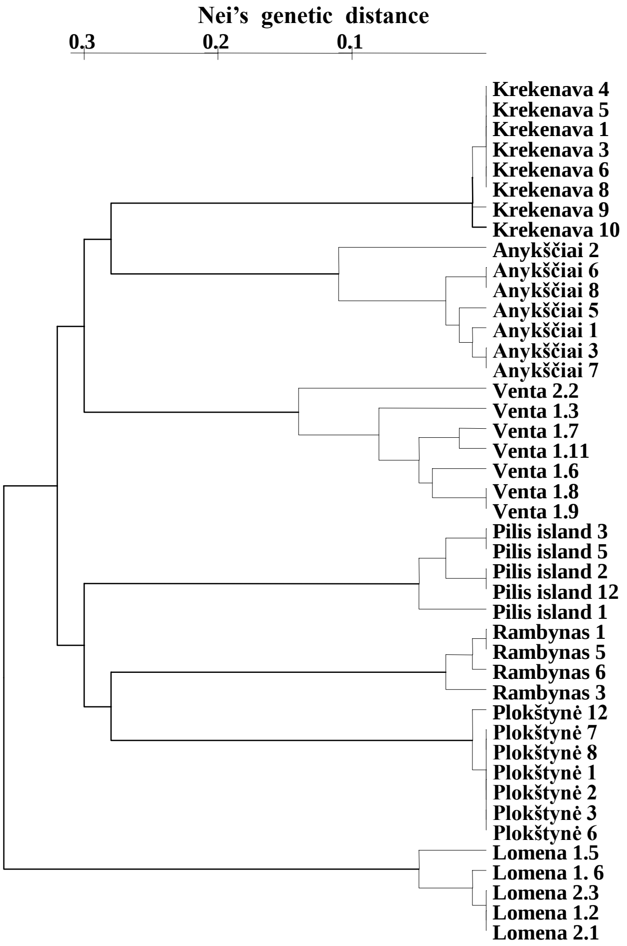

Fig. 3. Clustering of seven *Matteuccia struthiopteris* L. populations based on Nei's genetic distance using the UPGMA method

and individuals belonging to the same population are close to each other, forming separate groups located differently when compared to their actual geographic localization. Two populations (Pilis island and Plokðtynė) are the closest geographically, but in PCA analysis they are very distant (Fig. 2).

A dendrogram based on genetic distance values shows that individuals of each population form a separate subcluster (Fig. 3). Genetic distances inside a populations are about 3 times lower when compared to genetic distances among populations. Clustering seems to be in concordance with the geographical location of the populations: subclusters of Anykščiai, Krekenava and Venta populations are on the same branch forming a cluster of north eastern and north western Lithuanian populations. The second cluster consists of Pilis island, Plokðtynė and Rambynas subclusters, which form a separate group of western Lithuanian populations. The only population from the central part of Lithuania (Lomena subcluster) is the most distinct from the others (Fig. 3). The most homogeneous subclusters are Krekenava and Plokðtynė, the most heterogeneous being Anykščiai and Venta. But this conclusion cannot be assumed as very reliable because of the small sample size in all populations. The Mantel test revealed a weak but statistically significant correlation (r = 0.354, P = 0.047) between Nei's genetic distances and geographic locations of the populations (Fig. 4).

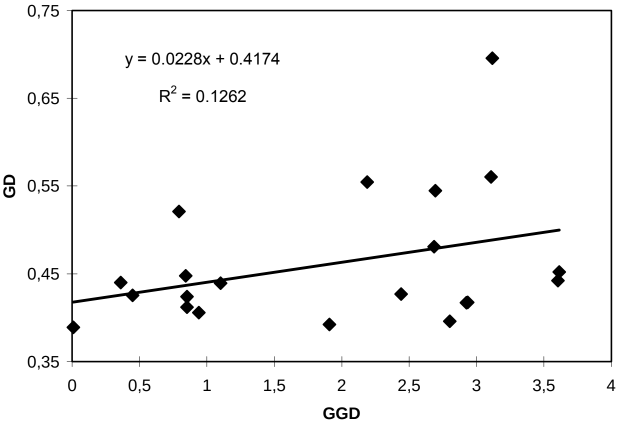

Fig. 4. Linear relationship between Nei's genetic distance (GD) and geographic distance (GGD) of *Matteuccia struthiopteris* L. populations (Mantel test). Geographic distance was generated from latitudinal and longitudinal data using GenAlEx_6b4 software [19]

DISCUSSION

The genetic structure of a plant population is influenced by many factors: the pattern of species distribution [14, 21], plant mating mode [22] and breeding system, diaspore dispersal mode and life form [21]. It is known that in northern, cold areas most species reproduce by clonal growth, and the low probability of success in sexual reproduction favors asexual reproduction in cold climates [8]. *Matteuccia struthiopteris* is a clonal fern and there was reported an increase in production of ramets (vegetative reproduction) northward and an increase of production of sporophytes (sexual reproduction) southward [4, 8]. Results of our research on genetic diversity within populations correspond to the above-mentioned ideas about different fern ecotypes due to the environmental conditions (especially temperature) [4].

Our research revealed a low intrapopulational variation (Table 4, Fig. 3), and the proportion of polymorphic loci among ostrich fern populations was similar to the polymorphism level observed in other clonally propagating plants [22]. Nevertheless, the level of ostrich fern intrapopulational polymorphism was the lowest among compared clonal plant species *Paris quadrifolia* [22], *Turnera intermedia* [23], *Aster cantoensis* [24]. This exceptionally low level of intrapopulational fern polymorphism might be explained by the effect of allelopathy [4, 5]. The lower percentage (7.7%) of polymorphic loci compared to our data (9.4%) was reported only by Zhong-ren [9] in a comparative allozyme study in N. America populations of *M. struthiopteris*. These populations were thought to arise from spores from the Old World (Asia), because founder populations are often more structured than source populations, have fewer alleles and are less heterozygous. Although ostrich fern is able to propagate clonally using subterranean rhizomes, the frequency of heterozygotes in natural populations and in the sporophytes raised from gametophytes studied by Zhong-ren revealed that *Matteuccia* is probably a sexual random-mating species [9].

The spatial distribution of populations in most cases of this study was in agreement with the geographic localization, except Pilis island and Plokðtynė populations in PCA analysis (Fig. 2). Lithuania is too small in territory to make any conclusions about its colonization events in the past based on the genetic data. However it is clear that the colonization of the territory of Lithuania must have been aided by spores. Ferns produce large quantities of small diaspores which are mainly dispersed by wind [24]. Persistent spore banks play an important role in the establishment and regeneration of populations and in the colonization of new sites [25]. The spores of ferns contain a reduced number of chromosomes, and this could be one of the reasons of such a big interpopulational diversity due to random fertilization events. A support of this idea is provided by a high value of genetic diversity among the populations (G_{st} = 0.9009, Φ_{PT} = 0.909) and the high genetic Nei distances even among geographically very close populations (Pilis island and Plokštynė populations).

Estimation of genetic distances has indicated that in all seven populations plants from the same population are more related to each other than plants from the different populations. The population dendrograms reflect the same tendency of strongly genetically related individuals inside each population. These data and a low gene flow itself indicate that there is almost no exchange of genetic material among populations on the temporal and spatial scale in Lithuania.

Our results imply that in case of establishment of each population only one genet was employed, which has expanded by vegetative propagation creating an extensive stand (population) of many ramets, but still only one genet. In each population a 2 × 2 m sample plot was studied for morphological measurements, demographic data. Results of the plots also show the genetic identity of the populations studied and that their asexual reproduction [26].

Thus, the parameters of genetic diversity and a correlation of genetic distance and geographical localization of *Matteuccia* populations revealed the existence of population isolation by distance and the prevalence of clonal propagation in the fern species *Matteuccia struthiopteris* in Lithuania.

ACKNOWLEDGEMENTS

This work was partially supported by the Norwegian Centre for International Cooperation in Higher Education (grant CCP 03/02: ENLINO Master program network).

Received 1 December 2005
Accepted 23 February 2006

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LIETUVOS PAUPINIO JONPAPARČIO (*MATTEUCCIA STRUTHIOPTERIS* L., DRYOPTERIDACEAE) POPULIACIJŲ GENETINIO POLIMORFIZMO TYRIMAI PANAUDOJANT RAPD ŽYMENIS

Santrauka

Atsitiktinai pagausintos polimorfinės DNR (APPD) metodu ištirta septynių Lietuvos paupinio jonpaparčio populiacijų struktūra, genetinė vidupopuliacinė ir tarppopuliacinė įvairovė. Ištirti 43 augalai panaudojant 10 atsitiktinės sekos pradmenų, kurių dėka buvo amplifikuoti 73 polimorfiniai lokusai, tačiau vidutinis vidupopuliacinis lokusų polimorfizmas tesiekė 9,4%. Genetine populiacijų analize nustatyta, kad ir kiti vidupopuliacinės genetinės įvairovės rodikliai yra žemi: vidutinis Shannon fenotipinės įvairovės indeksas I = 0,0498 (SD = 0,0534, P = 0,02), vidutinis heterozigotiškumas H = 0.033 (SD = 0,038, P = 0,03). Nustatytas žemas genų srautas tarp populiacijų (N_m = 0,0550). Molekulinės įvairovės analizė rodo, kad net 91% (Φ_{PT} = 0,909, P = 0,001, df = 6) įvairovės nulemia tarppopuliaciniai skirtumai, tuo tarpu vidupopuliaciniai skirtumai ne tokie reikšmingi (nulemia tik 9% molekulinės įvairovės). Klasterinės analizės duomenimis, populiacijos sudaro atskirus kompaktiškus klasterius, kurių grupavimasis atspindi geografinę populiacijų padėtį. Mantel testas patvirtino linijinę priklausomybę tarp populiacijų geografinės padėties ir genetinių atstumų (r = 0,354, P = 0,047). Tai rodo, kad dėl geografinio nuotolio tarp šių populiacijų egzistuoja izoliacija. Genetinė Lietuvos paupinio jonpaparčio populiacijų analizė atskleidė, kad populiacijos tarpusavyje labai skiriasi, tačiau kiekviena atskira populiacija yra sudaryta tik iš vieno genetinio individo, kuris kloninę populiaciją suformavo besidaugindamas vegetatyviai.