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Genetic diversity of warty cabbage (*Bunias orientalis* L.) revealed by RAPD and ISSR markers

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Abstract

Agricultural weed warty cabbage (*Bunias orientalis* L., *Brassicaceae*) is an alien species in Lithuania scattered through the whole country. We used RAPD and ISSR techniques to study genetic variation within and between six populations of this species in Lithuania. High level of DNA polymorphism was established in the study populations by RAPD technique. Five selected RAPD primers produced 71 reliable bands, of which 71.8% were polymorphic at the species level. Moderate level of DNA polymorphism was revealed using ISSRs. Six ISSR primers produced 94 scorable bands. The level of DNA polymorphism using ISSR markers was 39%. Among six studied populations of *B. orientalis* the highest values of genetic diversity parameters using both techniques were determined in Belmontas population, the least in Vingis population. The pattern of clustering of genotypes in these RAPD and ISSR-based UPGMA dendrograms is different. No correlation between genetic and geographic distances of populations and individuals was found. The correlation coefficient for the elements of the RAPD and ISSR genetic distance matrices was calculated. There was no significant correlation between these two matrices, which indicate that RAPD and ISSR markers revealed the unrelated features of DNA polymorphism in *B. orientalis*.

Key words: *Bunias orientalis*, exotic plant species, RAPD, ISSR, genetic diversity.

Introduction

Agricultural weed warty cabbage (*Bunias orientalis* L., *Brassicaceae*) is very common in agricultural landscape of some Baltic countries (Birnbaum, 2006). This alien species recently entered a phase of rapid spread (Steinlein et al., 1996; Dietz et al., 1999). It is common from south-eastern to the west and north Europe, except the Iberian peninsula and Greece (Dietz et al., 1999; Kobyłko et al., 2009). *B. orientalis* occupies rather different habitats, but especially prefers those subject to anthropogenic soil perturbations (Dietz et al., 1999). Other important biological features include either sexual or vegetative (by root fragments) reproduction, usually perennial life cycle, ability to synthesize secondary plant metabolites glucosinolates (Harvey et al., 2010). Known as a weed of cultivated and waste ground, on the other hand it is considered a valuable fodder plant (Karpenko et al., 1990). *B. orientalis* is an alien species in Lithuania, distributed diffusely through the whole country. It was first recorded in 1898 in Klaipėda (Gudžinskas, 1997).

Many of the studies concerning this species invasion success have concentrated on the species ecology or on the community vegetation structure characteristics (Steinlein et al., 1996; Dietz et al., 1999; Woitke, Dietz, 2002; Harvey et al., 2010). However, the colonization of new ranges and habitats depends also on genetic diversity potential of the species and causes changes in genetic diversity. Besides genetic diversity, the organization of genetic variation among populations is likely to change during invasions (Henry et al., 2009).

Molecular markers are widely used to study genetic diversity and population genetic structure of invasive plants. These include allozyme, RAPD (random amplified polymorphic DNA), ISSR (inter-simple sequence repeat), AFLP (amplified fragments

length polymorphism) and SSR (simple sequence repeat) (Bossdorf et al., 2005). There is a single study where DNA (RAPD) markers were used in analysis of genetic variation dynamics in cohorts *B. orientalis* during the 9 years of population development (Dietz et al., 1999). In this study, the authors showed that molecular variance within cohorts did not change with cohort age and molecular variance of the whole population did not change with the age of the population. However, this work was carried out on the single population covering an area of about 350 m^2. In this context, the study of population genetic structure of *B. orientalis* based on the analysis of several populations using different molecular methods can be interesting from the ecological and agronomical point of view. It is recognized in numerous cases that population genetic structure changes during the invasion process and this knowledge can assist in the management of invasive species (Ren et al., 2005; Edwards et al., 2006; Schachner et al., 2008).

Multiple ecological, evolutionary and demographic factors, including population bottlenecks, founder effect, influence the genetic diversity of exotic plant species in its new area (Barrett, Kohn, 1991; Novak, Mack, 2005; Schachner et al., 2008). The aims of our study were to explore the amount and distribution of genetic variation in *B. orientalis*. For more precise and complete assessment of genetic diversity in invasive populations of this species we used RAPD and ISSR techniques.

Materials and methods

This study was carried out during the period of 2010–2011 at the Department of Botany and Genetics, Faculty of Natural Sciences of Vilnius University.

Plant material. A general description of the six Lithuanian *B. orientalis* populations studied is given in Table 1. A total of 90 plants from these populations were sampled. From 8 to 20 plants were collected from each population.

Table 1. Characteristics of *Bunias orientalis* populations

Population	District	Collecting coordinates (long. E, lat. N)	Habitat
Belmontas	Vilnius	25°20′45″, 54°40′51″	Mown meadow, roadside
Seredžius	Kaunas	23°25′31″, 55°04′47″	Mown meadow
Darsūniškis	Kaišiadorys	24°07′06″, 54°44′14″	Polder of Nemunas river
Viduklė	Raseiniai	22°50′52″, 55°24′56″	Meadow near railway
Vingis	Vilnius	25°13′55″, 54°40′56″	Vingis park, Botanical Garden of Vilnius
Vilkpėdė	Vilnius	25°12′40″, 54°38′09″	Railway embankment

Genomic DNA extraction, RAPD and ISSR analyses. Total genomic DNA was isolated from ground material of fresh leaves using CTAB method (Doyle, Doyle, 1990). DNA quantity and quality were measured with a spectrophotometer and electrophoretically.

Each 20 µL RAPD and ISSR polymerase chain reaction contained 2 µL 10 × PCR buffer (“Fermentas”, Lithuania), 200 µM dNTPs; 1 unit Taq polymerase (“Fermentas”, Lithuania), 300 µM $MgCl_2$, 0.4 µM of the primer and approximately 20 ng of DNA. The RAPD-PCR were carried out for 4 min at 94°C, followed by 45 cycles of 1 min at 94°C, 1 min at 35°C, and 1 min at 72°C, followed by a final extension step of 5 min at 72°C. The ISSR-PCR conditions were: 1 cycle of 7 min at 94°C; 32 cycles of 30 s at 94°C, 45 s at primer specific temperature, and 2 min at 72°C; 1 cycle of 7 min at 72°C. All reactions were run at least twice. Amplifications were resolved on a 1.5% agarose gel (4 h, 4 V/cm) and stained with ethidium bromide.

Data analysis. Amplified bands were scored in a size range from 0.3 to 2 kb. Differences in RAPD phenotypes of study individuals were used to calculate genetic distance, which was used here as a measure of genetic variation. Binary data matrixes of RAPD and ISSR phenotypes were made. Each locus was considered as bi-allelic. The presence of the DNA fragment (allele) was represented with ‘‘1’’ and the absence was represented with ‘‘0’’.

Commonly used indicators of molecular diversity within populations were calculated: Nei’s gene diversity (Nei, 1978), the percentage of polymorphic bands (P) and Shannon’s index of diversity (I).

To avoid strict Hardy-Weinberg equilibrium postulation, the phenetic analysis was adopted. In this case each PCR product is assumed to represent a profile (phenotype). Analysis of molecular variance (AMOVA) (Excoffier et al., 1992) was used to partition variance to hierarchical levels (among individuals within populations and between populations) from the matrix of squared Euclidian distances between all pairs of study individuals (Tansley, Brown, 2000).

An unweighted pair group method with arithmetic mean (UPGMA) cluster analysis based on pairwise Nei’s unbiased genetic distances (Nei, 1978) was used to assess genetic relationships among populations and to present the results as dendrogram.

Results and discussion

Polymorphisms detected using RAPD and ISSR analysis. It has been shown that different markers might reveal different classes of variation (Russell et al., 1997; Hou et al., 2005). In the study of invasive species *B. orientalis* we analysed polymorphism of RAPD and ISSR loci. A pilot experiment was carried out to select primers producing clear and reproducible RAPD and ISSR phenotypes. From an initial screening five RAPD primers and six ISSR primers (Table 2) were selected.

These five selected RAPD primers produced 71 reliable bands, of which 52 (71.8 ± 20.15%) were polymorphic at the species level. The number of bands per primer ranged from 13 to16 with an average of 14.2 ± 1.3% bands/primer. Size of RAPD bands was from 380 to 1800 bp. Six ISSR primers produced 94 scorable bands corresponding to 15.67 ± 6.28% bands per primer. The level of DNA polymorphism established using this method was 39 ± 15.62%. The size of ISSR bands ranged from 390 to 1800 bp. All primers used in this study produced polymorphic DNA bands. In both cases rare alleles at the species level were identified, but they were not included in the analysis. In total 22 such RAPD and 27 ISSR alleles were established. In contrast to RAPD, no identical ISSR phenotypes were established among individual sampled plants.

Table 2. Sequences and annealing temperature of the RAPD and ISSR primers used in the study and the polymorphism of scored DNA bands

Primer	Sequence 5'→3'	Annealing t °C	Size of DNA bands (bp)	Number of DNA bands		P[1] %
				polymorphic	monomorphic	
			RAPD			
Roth A_01	CAGGCCCTTC	35	500–1800	16	0	100
Roth A_02	TGCCGAGCTG	35	380–1450	8	5	62
Roth A_03	AGTCAGCCAC	35	520–1150	12	3	80
Roth A_04	AATCGGGCTG	35	510–1600	10	4	71
Roth A_05	AGGGGTCTTG	35	610–1650	6	7	46
			Sum	52	19	
			Average	10.4 ± 3.85	3.8 ± 2.59	71.80 ± 20.15
			ISSR			
ISSR I-32	$(AGC)_4C$	39	470–1450	4	12	19
ISSR I-34	$(AGC)_4GG$	46	390–1800	7	16	30
ISSR I-18	$GTG(CT)_7C$	51	500–1300	13	9	59
ISSR I-50a	$CCA(GCT)_4$	46	490–1500	5	11	32
ISSR_A	$CTC(GT)_8$	51	600–1200	3	5	38
ISSR_C	$A(GA)_7TG$	51	490–990	5	4	56
			Sum	37	57	
			Average	6.17 ± 3.60	9.5 ± 4.51	39 ± 15.62

P[1] – polymorphism

Within and between population variability. The percentage of polymorphic RAPD loci per population showed similar trends for both types of DNA markers. It ranged from 25.35 (Vingis) to 52.11 (Belmontas) for RAPD loci, while for ISSR loci this parameter varied from 14.89 (Vingis) to 27.66 (Belmontas) (Table 3).

The mean value of polymorphism within populations was 34.27 ± 9.33% for RAPD and 20.57 ± 4.4% for ISSR markers. The summary value of genetic variation of Shannon index for all loci determined on the basis of RAPD analysis was 0.280 ± 0.241 and from ISSR – 0.170 ± 0.250. The highest level of variability also exhibited Belmontas population; the lowest variability was detected in Vingis population. The extreme values of Nei's gene diversity in RAPD analysis were established also in Belmontas (0.176) and Vingis population (0.104). This same trend was observed in the ISSR analysis – the highest value of Nei's gene diversity − in Belmontas population (0.105), the least – in Vingis population (0.059).

The level of population differentiation was similar in both analyses − 24% of total RAPD and 25% of total ISSR genetic diversity AMOVA allocated among populations. This is moderate level of population differentiation. These values are quite congruent with the level of genetic differentiation established for species with mixed mating systems using allozyme analysis (21.2–24%) (Hamrick, Godt, 1996) and using RAPD (26−27%) (Nybom, Bartish, 2000; Patamsytė et al., 2010).

Table 3. Estimates of genetic diversity at RAPD and ISSR loci of *Bunias orientalis* populations

Population	Plants per population	P[1] %	h[2]	I[3]
		RAPD		
Belmontas	20	52.11	0.176 ± 0.196	0.265 ± 0.284
Seredžius	20	35.21	0.117 ± 0.182	0.176 ± 0.264
Darsūniškis	14	29.58	0.105 ± 0.178	0.156 ± 0.259
Viduklė	14	32.39	0.112 ± 0.185	0.167 ± 0.266
Vingis	8	25.35	0.104 ± 0.192	0.150 ± 0.272
Vilkpėdė	14	30.99	0.114 ± 0.189	0.168 ± 0.271
	Average	34.27 ± 9.33		
	Summary of genetic variation statistics for all loci		0.175 ± 0.170	0.280 ± 0.241
		ISSR		
Belmontas	20	27.66	0.105 ± 0.184	0.153 ± 0.265
Seredžius	20	23.40	0.091 ± 0.178	0.133 ±0.255
Darsūniškis	14	19.15	0.071 ± 0.155	0.105 ± 0.226
Viduklė	14	19.15	0.080 ± 0.167	0.116 ± 0.241
Vingis	8	14.89	0.059 ± 0.147	0.087 ± 0.213
Vilkpėdė	14	19.15	0.072 ± 0.158	0.105 ± 0.229
	Average	20.57 ± 4.40		
	Summary of genetic variation statistics for all loci		0.110 ± 0.172	0.170 ± 0.250

[1] – the percentage of polymorphic loci, [2] – Nei's (1973) gene diversity, [3] – Shannon's information index

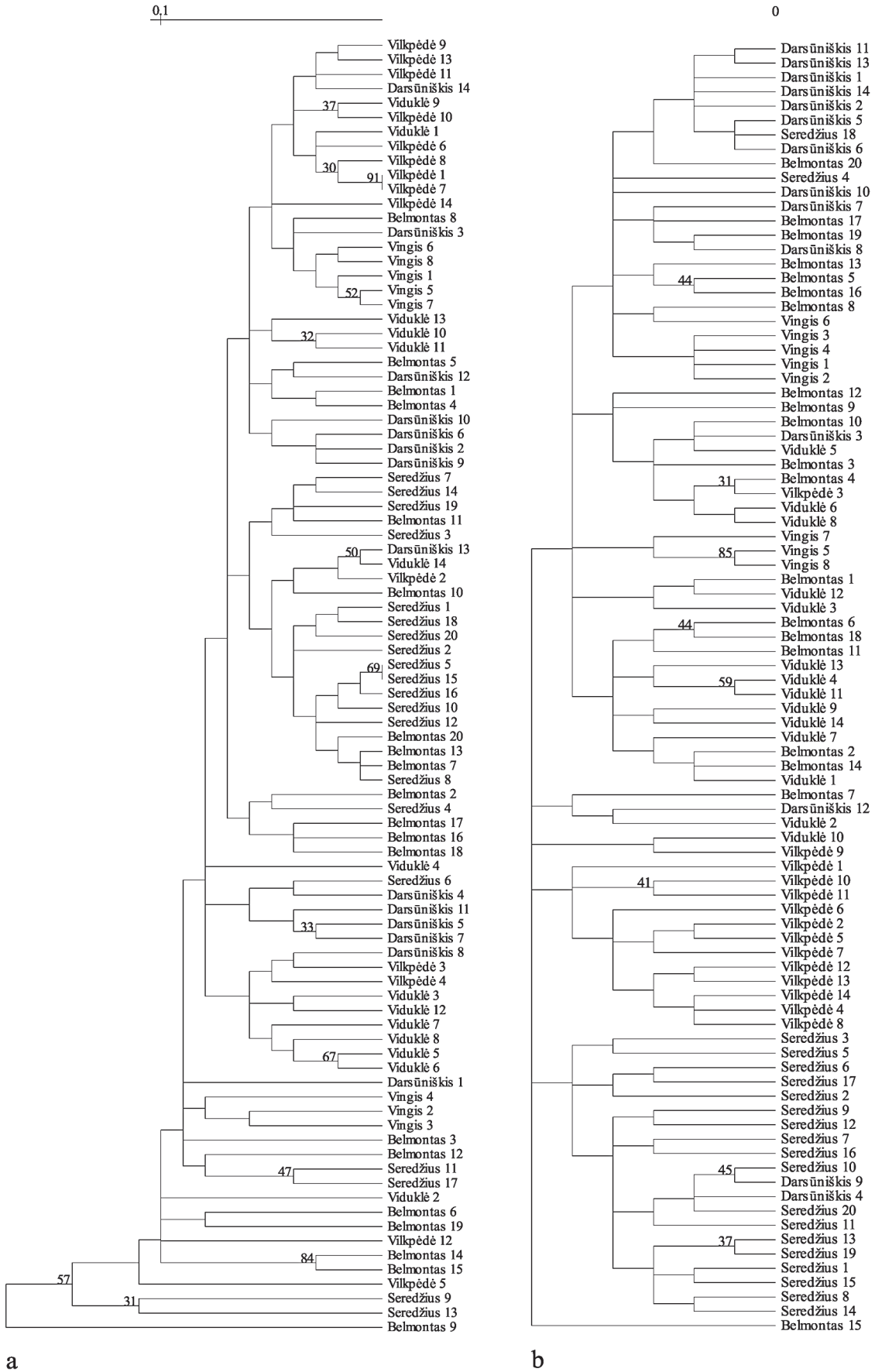
Cluster analysis. Pairwise genetic distances between individual plants in populations of *B. orientalis* estimated using RAPD and ISSR markers were 0.0–0.204 and 0.0–0.760, respectively. These genetic distances obtained using RAPD and ISSR markers were compared. The low correlation coefficient was established ($r = 0.245$, $p < 0.05$), which indicates that RAPD and ISSR markers revealed the unrelated features of DNA polymorphism.

The UPGMA cluster analysis was carried out on the basis of estimated genetic distances and dendrograms were drawn which show genetic relationships among individual plants studied (Fig. 1 a, b).

The pattern of clustering of genotypes in these RAPD and ISSR-based UPGMA dendrograms is different. The RAPD-based dendrogram shows that clusters are heterogeneous, they are composed of small groups of genetically related, usually 3–9, individuals. Some plants show identical RAPD phenotypes, which indicate, that they might have arisen as a result of vegetative reproduction. According to published information, *B. orientalis* has high capacity of regeneration from root fragments (Steinlein et al., 1996; Birnbaum, 2006). The ISSR-based UPGMA dendrogram of all individuals showed some population structure. Dendrogram joins some accessions of the same populations into compact groups. For example, this is intrinsic for Seredžius and Vilkpėdė populations. But in most cases one to several individuals at any given population clustered with individuals from another population. In contrast to RAPD markers, ISSR-based dendrogram indicated that all 90 plants could be distinguished using the ISSR markers in spite of the moderate level of DNA polymorphism established using this type of DNA markers. Higher potential of ISSR markers to detect DNA polymorphism was reported by many authors (Parsons et al., 1997; Fernández et al., 2002; Hou et al., 2005; Bhuyan et al., 2007).

The resulting RAPD and ISSR-based UPGMA dendrograms are different (Fig. 2 a, b). No shared clustering was detected. The differences found among the dendrograms generated by RAPD and ISSR analyses could be partially explained by the different number of PCR products analysed, different character and properties of RAPD and ISSR loci (Fernández et al., 2002; Hou et al., 2005).

This result was supported by the absence of correlation between genetic and geographical distances of study populations detected in our study ($p > 0.05$). Similar results were obtained by Dietz et al. (1999) who showed that genetic variation at the level of both the whole population and of cohort groups of *B. orientalis* was independent of spatial variation. The results of UPGMA cluster analysis and the lack of correlation between genetic and geographic distances suggests that populations have received migrant genotypes from many sources (Ge et al., 2003). According to Bossdorf et al. (2005), multiple introductions of invasive plants appear to be the rule rather than the exception. So, our study of genetic diversity of populations of *B. orientalis* possibly indicates the multiple and random introduction of this invasive species in Lithuania.



Note. The numbers on the branches indicate bootstrap values above 30%.

Figure 1. UPGMA dendrograms, constructed using RAPD (a) and ISSR (b)-based genetic distance matrixes, estimated according to Nei and Li (1979)

Genetic distances among populations (Nei, 1978) based on RAPD and ISSR data were also estimated using *PopGene* computer program (Table 4).

Molecular markers are important tools in the study of plant invasions, because they provide information about invasion pathways and the amount of genetic variation introduced (Bossdorf et al., 2005). According to our data, this study is the first attempt to assess DNA polymorphism among different populations of *B. orientalis*. Our study of six populations of invasive *B. orientalis* species monitors the current state of genetic diversity of this species in Lithuania.

Table 4. Genetic distances (Nei, 1978) among *Bunias orientalis* populations for RAPD (below diagonal) and ISSR (above diagonal) data

	Belmontas	Seredžius	Darsūniškis	Viduklė	Vingis	Vilkpėdė
Belmontas		0.0261	0.0183	0.0148	0.0323	0.0400
Seredžius	0.0394		0.0174	0.0370	0.0517	0.0580
Darsūniškis	0.0614	0.0588		0.0291	0.0367	0.0480
Viduklė	0.0827	0.0782	0.0415		0.0397	0.0361
Vingis	0.0971	0.1081	0.0819	0.1047		0.0423
Vilkpėdė	0.0766	0.0706	0.0290	0.0368	0.0660	

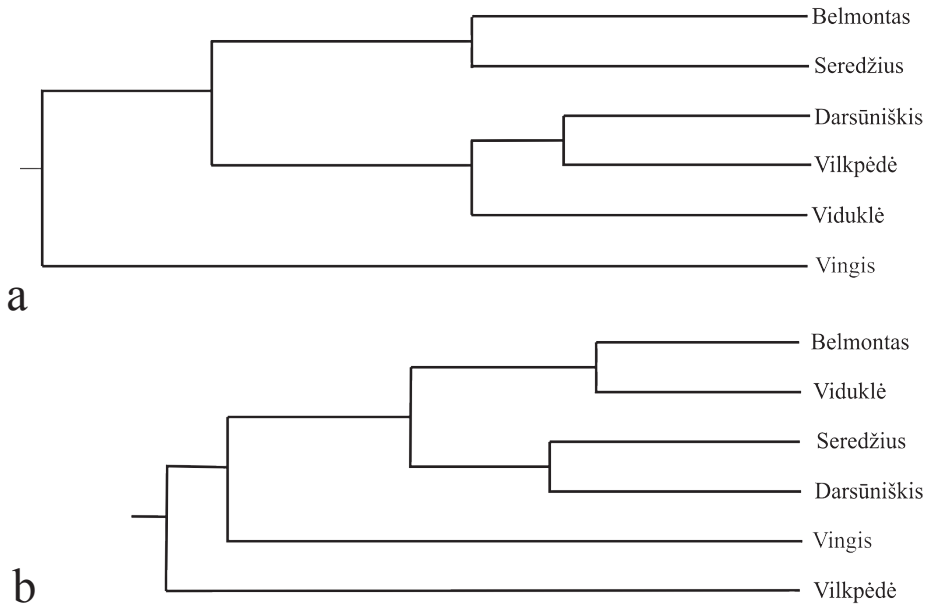


Figure 2. Dendrograms of *Bunias orientalis* populations constructed by UPGMA cluster analysis of the matrixes of Nei's (1978) genetic distances estimated on the basis of RAPD (a) and ISSR (b) data

Conclusion

In this study we selected RAPD and ISSR primers producing scorable and reproducible PCR products suitable for analysis of genomic DNA of invasive *B. orientalis* species. High level of DNA polymorphism was established in the study populations using RAPD technique. Five selected RAPD primers produced 71 reliable bands, of which 71.8% were polymorphic at the species level. Six ISSR primers produced 94 scorable bands. The level of DNA polymorphism using ISSR markers was 39 ± 15.62%. Among the six *B. orientalis* populations studied, the highest values of genetic diversity parameters using both techniques were determined in Belmontas population, the least in Vingis population. No correlation between genetic and geographic distances of populations and individuals was found. Our study of genetic diversity of *B. orientalis* populations indicates the multiple and random introduction of this invasive species in Lithuania.

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Rytinės engros (*Bunias orientalis* L.) genetinės įvairovės tyrimas, naudojant RAPD ir ISSR žymenis

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Santrauka

Rytinė engra (*Bunias orientalis* L., *Brassicaceae*) – svetimkraštė augalų rūšis, nevienodai gausiai aptinkama Lietuvos teritorijoje. Taikant RAPD (angl. *random amplified polymorphic* DNA) ir ISSR (angl. *inter-simple sequence repeats*) žymenų metodus, tirtas šios rūšies šešių populiacijų genetinės įvairovės pasiskirstymas. RAPD metodu nustatytas aukštas DNR polimorfizmo lygis (71,8 %), ISSR metodu nustatytas vidutinis DNR polimorfizmas (39,0 %). Didžiausi populiacijų genetinės įvairovės rodikliai būdingi Belmonto, mažiausi – Vingio parko engros populiacijai. Ištirtų 90-ies augalų giminingumą atspindinčios dendrogramos, gautos naudojant RAPD ir ISSR žymenis, skyrėsi tarpusavyje. Nenustatyta priklausomybės tarp populiacijų geografinių ir genetinių atstumų. Reikšmingos koreliacijos tarp genetinių atstumų matricų, sudarytų naudojant RAPD ir ISSR duomenis, nebuvimas rodo, kad skirtingų tipų DNR žymenys atspindi nevienodus DNR polimorfizmo aspektus.

Reikšminiai žodžiai: *Bunias orientalis*, svetimkraščiai augalai, RAPD, ISSR, genetinė įvairovė.