BIOLOGIJA. 2006. Nr. 3. P. 16–20

The use of ISSR method for the assessment of bee genetic diversity

V. Paplauskienė,

V. Čeksterytė,

I. Pašakinskienė,

D. Tamašauskienė,

J. Račys

Lithuanian Institute of Agriculture, LT-58344 Akademija, Kėdainiai distr., Lithuania
E-mail: violeta@lzi.lt

Genomic DNA assays of bee races *Apis mellifera caucasica* and *Apis mellifera carnica* and three lines of the latter were performed with eleven simple sequence repeat primers. Using these primers in PCR there were amplified 60 DNA fragments of which 66.7% were found to be polymorphic. The number of fragments produced in the DNA profiles of different bee races and lines varied from 2 to 10, with their sizes varying within 350–3000 bp.

The genetic diversity of the individuals of *A. m. caucasica* and *A. m. carnica* races was revealed by using $AC(GACA)_4$, $(GACA)_4GT$, $(ATG)_5GA$, $(TCC)_5GT$, $(AC)_8YT$ and $(CT)_8A$ primers in PCR. Race and line-specific DNA profiles were obtained. Fragments specific to *A. m. caucasica* race were produced using $(TCC)_5GT$, $(AC)_8YT$ and $(AC)_8T$ primers in PCR, and those specific to *A. m. carnica* lines were amplified by $(AC)_8YT$ primer. Having used eleven simple sequence repeat primers for DNA fingerprinting of bees, on average in 6.8% of the cases there were generated 5–7 types of DNA profiles, in 70.4% of cases 2–4 types of profiles, in the rest of 22.8% of cases any no polymorphism was revealed between the bee races and within the lines tested.

Key words: honeybee, DNA, polymorphism, ISSR fingerprinting, primers

INTRODUCTION

Most studies on honeybee phylogeny concerned external morphological features [1–4]. On the basis of morphometric studies, honey bee *Apis mellifera* L. subspecies were grouped into four lineages: east (and south-east) European (C), west (and north) European – Saharan African (M), sub-Saharan – African (A), and near-eastern (O) [5]. The bee queens of *A. m. carnica* are mostly used by Lithuanian beekeepers. The characteristic features of *A. m. carnica* bees are docility and high honey production [6]. The population of the oldest Lithuanian bees of *Apis mellifera mellifera* L. has become extinct in most apiaries in Lithuania. The bees of *A. m. caucasica*, *A. m. carnica* and *A. m. carpatica, A. m. ligustica* and Bukfast (hybrid bees) introduced into Lithuania have superseded the indigenous bees. *A. m. caucasica* bees in Lithuania exhibit a satisfactory overwinter survival, however, in the course of time they have become aggressive [2, 7]. The new bee hybrids were developed by crossing the introduced species [8]. The assessment of bee populations by morphological features alone can be imprecise. New genetic methods of mitochondrial DNA haplotypes and microsatellite loci have been employed to study the genetic variations of honey bees [9–12]. Analyses of mtDNA of bees collected from queen-producing apiaries of the United States showed that only 4% of bees from 142 commercial queen-breeding apiaries had *Apis m. mellifera* L. traits [13]. Bees from the other 136 apiaries had 97% of mtDNA haplotypes of *A. m. carnica* and *A. m. ligustica* bees. Analysis of polymorphism of the locus COI-COII of mtDNA bees *Apis m. mellifera* L. Bashkir population from south Ural showed that only in one reserve (Burzyanskii) local bees were purebred. The other part of former *Apis m. mellifera* L. of Bashkir population in Ural is currently represented by hybrids of local bees with southern bee population [14]. The electrophoretical investigation of eighteen enzymes of *Apis m. mellifera* L. and *A. m. carnica* bees from Czechia showed the polymorphism for five enzymes [15].

The aim of the present study was to investigate the polymorphism of *A. m. carnica* and *A. m. caucasica* bees by the inter-simple sequence repeats (ISSR) method.

MATERIALS AND METHODS

Samples of Caucasian (*A. m. caucasica*) and Carniolan (*A. m. carnica*) bee races for genetic tests were collected from the breeding colonies of the Lithuanian Institute of Agriculture apiary. Multiplication of *A. m. caucasica* bee queens was started at the Lithuanian Institute of Agriculture from the stocks imported from the

Russian Institute of Apiculture's bee queen breeding apiary situated in Krasnodar region, Krasnaya Poliana. *A. m. caucasica* bees perform well collecting nectar from red clover [16]. The line C_{262} of *A. m. carnica* bee race has been developed at the Lithuanian Institute of Agriculture and is characterised by a high honey production and pollen collection and a low swarming rate. Bee queens of the Vigor line of the same race were brought to the Lithuanian Institute of Agriculture breeding apiary from the Czech Institute of Apiculture based in Dole. This line was developed using instrumental bee insemination. The bees of this line are resistant to diseases, especially to chalk brood, are not prone to excess swarming and are noted for high honey production. Carniolan *A. m. carnica* bee queens from Slovenia, further referred to as (C_{SLOV}), were introduced three years ago, i. e. they have not fully acclimatized yet. They are distinguished by a very high honey production, good nest cleaning skills and a high royal jelly production; however, they demonstrate a high swarming rate.

A. m. caucasica and *A. m. carnica* bee races and lines were represented by 10–12 individuals. The bees were placed in 2 ml Eppendorf test-tubes and frozen at –20 °C. DNA was extracted following the CTAB-based extraction protocol [17]; 750 µl of extracting buffer was used per bee. Polymerase chain reactions (PCR) were carried out in 25 µl in an Eppendorf Master Cycler Gradient thermocycler using the following primers: $(AGAC)_4GC$, $AC(GACA)_4$, $(GACA)_4GT$, $(GACA)_4CT$, $(ATG)_5GA$, $(CTC)_5GT$, $(TCC)_5GT$, $(AC)_8G$, $(AC)_8T$, $(AC)_8YT$ and $(CT)_8A$. Twenty five microlitre aliquots or PCR mixtures contained 10 × Mg^{2+}-free buffer; 2.0 mM dNTP mix; 50 mM $MgCl_2$, 2.5 µM primer; 50 ng DNR; 2.0 U DyNAzyme II polymerase (Finnzyme OY). Amplification products were analysed in 1.5% agarose gel, and electrophoresis was carried out in 1×TAE buffer. GeneRuler™ DNA Ladder Mix (Fermentas) was used as the DNA fragment size marker. The gels were analysed in UV light by staining with ethidium bromide.

RESULTS AND DISCUSSION

Tetranucleotide motif primers used for bee DNA assays generated formation of 2–7 fragments (Table 1). While using $(AGAC)_4GC$ primer in PCR, DNA profiles of bees were found to possess seven fragments, their size ranging from 750 to 3000 bp. The DNA profiles of *A. m. caucasica* bees were identical. Most of the individuals of *A. m. carnica* C_{262} and Vigor lines had identical profiles with *A. m. caucasica*. The profiles of the rest of $C_{262,}$ Vigor and C_{SLOV} individuals had lost some of the fragments or had been supplemented with the novel ones. The number of polymorphic fragments in the individuals of *A. m. carnica* lines tested varied from 3 to 6. The primer $AC(GACA)_4$ produced two fragments in the DNA profile. Two profile types were identified according to the deployment of the latter fragments: possessing one fragment and possessing two fragments. The type II DNA profile was more specific to the bees of Vigor and C_{SLOV} lines of *A. m. carnica* and *A. m. caucasica* race, 70.0–81.8% of the individuals tested possessed this profile type. The type I DNA profile was more frequent among the individuals of C_{262} line of *A. m. carnica* race.

Using the other tetranucleotide primers, fewer fragments were obtained in the DNA profiles; moreover, their size varied within a narrower range. In total, 19 fragments were amplified by tetranucleotide motif primers, of which 68.4% were polymorphic. The largest number (9) of polymorphic DNA fragments was identified among the individuals of *A. m. carnica* Vigor line, while among the individuals of *A. m. caucasica* only three polymorphic fragments were found. On replacing the two-base GT 'anchor' by CT, fewer frag-

Table 1. **ISSR products generated by 11 primers in honeybee *A.m. caucasica* and *A. m. carnica* bees**

Primer	Number of reproducible bands	MW range, bp	Number of polymorphic bands	Number of polymorphic bands			
				A. m. caucasica	Lines of *A. m. carnica*		
					C_{262}	Vigor	C_{SLOV}
$(AGAC)_4GC$	7	750–3000	7	0	4	6	3
$AC(GACA)_4$	2	1500–1700	1	1	1	1	1
$(GACA)_4GT$	6	700–1500	4	1	1	2	3
$(GACA)_4CT$	4	480–900	1	1	0	0	0
$(ATG)_5GA$	5	350–1400	3	2	2	2	2
$(TCC)_5GT$	10	500–1300	9	6	4	1	2
$(CTC)_5GT$	3	400–1200	1	1	0	0	0
$(AC)_8G$	4	400–750	2	0	1	2	1
$(CT)_8A$	5	550–1400	3	2	3	2	2
$(AC)_8YT$	10	600–1550	6	3	5	3	2
$(AC)_8T$	4	450–1200	3	0	2	1	0
Total	60	350–3000	40	17	23	20	16

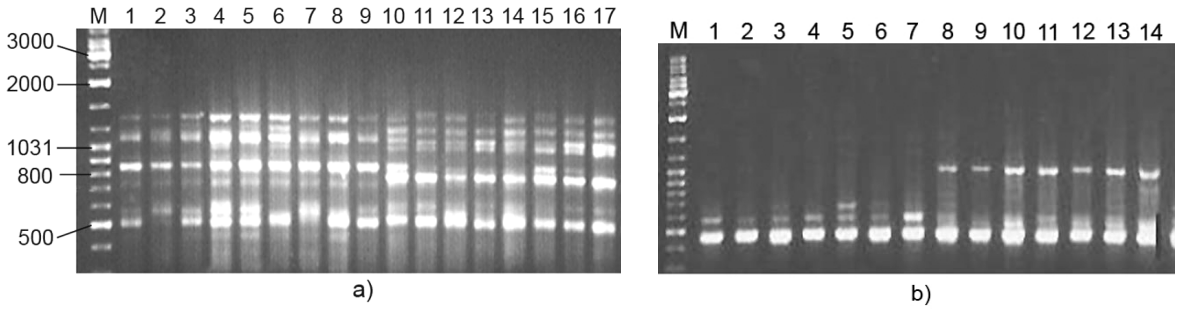

Figure. *A. m. caucasica* and *A. m. carnica* C_{262} bee DNA profiles amplified with: a) – primer $(TCC)_5GT$: 1–9 – *A. m. carnica* C_{262}; 10–17 – *A. m. caucasica*; b) – primer $(AC)_8T$: 1–7 – *A. m. carnica* C_{262}; 8–14 – *A. m. caucasica*. M – molecular size marker

ments were produced with $(GACA)_4CT$ primer as compared with $(GACA)_4GT$ primer. The profiles of *A. m. carnica* race lines and of most of *A. m. caucasica* race bees produced by $(GACA)_4CT$ primer were found to be identical. A small part of *A. m. caucasica* individuals did not contain 620 bp fragment in their profiles.

Of all trinucleotide motif primers used in PCR, the largest number of DNA fragments was amplified by $(TCC)_5GT$ primer. Using this primer, in bee DNA profiles 10 fragments were produced, of which only one (1100 bp) was common to all profile types, the others being polymorphic (Fig. 1a). The bee individuals assayed by this primer were identified to possess 15 DNA profile types, of which seven were specific only to *A. m. caucasica* individuals. Their profiles contained a specific 800 bp fragment which was not found in *A. m. carnica* individuals. In the profiles of the latter individuals we identified a fainter 750 bp fragment, which was not present in the profiles of *A. m. caucasica* individuals. In the range of fragments 1000–1300 bp, *A. m. caucasica* bee profile is represented by four while *A. m. carnica* by 2–3 fragments. Part of DNA profiles of *A. m. carnica* lines were identical, but part of the individuals of C_{262} line had distinctive fingerprints. Most of the profiles (63.6–80.0%) of line C_{262} of *A. m. carnica* and *A. m. caucasica* bees, generated by $(ATG)_5GA$ primer, are represented by five fragments, while in the rest of the profiles as well as in those of *A. m. carnica* Vigor and C_{SLOV} individuals, 2 or 3 fragments are lost. A low diversity of DNA profiles was recorded on using other trinucleotide motif primers in PCR. The fingerprints of *A. m. carnica* lines produced by $(CTC)_5GT$ primer were identical and contained two fragments. The same profiles were found for part of *A. m. caucasica* individuals, the rest being supplemented with a larger 1200 bp fragment; they accounted for 63.6%. Other tri- and tetranucleotide motif primers are used for the genetic DNA studies of honey-bee, bumble-bee, and many other insect species [18].

Among the dinucleotide motif primers, $(AC)_8YT$ was generated the largest number of DNA fragments. In the fingerprints of *A. m. caucasica* bees there was found a prominent 1550 bp fragment which we did not identify in *A. m. carnica* individuals. Some differences were noted between *A. m. carnica* lines. When this primer was used in PCR, *A. m. carnica* Vigor and C_{SLOV} lines had 750 bp fragment, however, C_{262} individuals did not possess it. The 680 bp fragment, which was rather frequent in C_{262} and C_{SLOV} individuals, was not found in the Vigor line of this race. While assaying various bee colonies of *Apis m. mellifera* and *Apis m. caucasica*, there were identified differences in DNA profiles among the colonies belonging to different races [19].

$(AC)_8G$ generated formation of fragments within a narrow (400–750 bp) range. DNA profiles possessing 400 and 600 bp fragments were specific to *A. m. caucasica* bees and 27.3% of individuals of *A. m. carnica* Vigor and C_{SLOV} lines. The profiles of the other line of this race had one or two supplementary fragments. DNA profiles of *A. m. caucasica* bees amplified by this primer were identical. Using $(AC)_8G$ primer but a different DNA extraction method and PCR in different conditions, the fragments produced in the DNA profiles of *Apis mellifera* L. from Russia and Italy varied within a much wider range [20]. On replacing the ‘anchor’ bases G by A, i.e. using $(AC)_8T$ primer, the same number of fragments was produced, but they varied within a wider (450–1200 bp) range (Fig. 1b). The DNA profiles of *A. m. caucasica* bees amplified by this primer were also identical. While comparing the profiles of *A. m. caucasica* and *A. m. carnica* it was noted that the fingerprints of the latter did not have 1200 bp fragment, but some profiles were supplemented with a novel 700 bp fragment. Individuals of the C_{SLOV} line of *A. m. carnica* subspecies also had identical DNA fingerprints. Using dinucleotide motif primers in PCR, 23 fragments were produced, the largest number of polymorphic ones (eleven) being identified among the individuals of *A. m. carnica* C_{262}. Reliable results of genotype identification of *Apis mellifera* genotypes were obtained on isolating DNA from a bee wing tip sample (1.3 mm^2) [21].

Using tetra-, tri- and dinucleotide motif primers, in total 60 fragments were generated in the DNA profiles of *A. m. caucasica* and *A. m. carnica* bees. The fragments varied within 350–3000 bp. Other authors have reported narrower DNA fragment ranges (400–2000 or 50–500 bp) generated while assaying *A. mellifera* L. bees [22].

Table 2. **Inter-SSR profile type frequency (%) in *A. m. caucasica* and *A. m. carnica* bees**

Primer motif	DNA profiles	*A. m. caucasica*	Lines of *A. m. carnica*		
			C_{262}	Vigor	C_{SLOV}
Tetra-repeats	Monomorphic	25.0	25.0	25.0	25.0
	Polymorphic	75.0	75.0	75.0	75.0
Tri-repeats	Monomorphic	0	33.3	33.3	33.3
	Polymorphic	100	66.7	66.7	66.7
Di-repeats	Monomorphic	50.0	0	0	25.0
	Polymorphic	50.0	100	100	75.0

The use of tetranucleotide motif primers did not always reveal bee diversity; 25% of these primers generated formation of monomorphic DNA profiles between *A. m. caucasica* and *A. m. carnica* races and within the individuals of the latter lines (Table 2). Identical DNA profiles of *A. m. caucasica* bees were produced by $(AGAC)_4GC$ and of all lines of *A. m. carnica* by $(GACA)_4CT$ primer. The highest DNA polymorphism in *A. m. caucasica* and *A. m. carnica* C_{262} bees, when using trinucleotide motif primers, was identified with $(TCC)_5GT$, which generated formation of 6–7 types of profiles. The profiles of *A. m. caucasica* race bees amplified by the other trinucleotide motif primers were also polymorphic, whereas those of part of *A. m. carnica* race lines were found to be identical. ISSR fingerprints generated with all dinucleotide motif primers revealed genetic diversity between the individuals of *A. m. carnica* lines C_{262} and Vigor. Polymorphism of the individuals of *A. m. caucasica* race and *A. m. carnica* C_{SLOV} was indicated by 50 and 25% of dinucleotide sequence primers, respectively. According to the results of molecular analysis, mtDNA of the *A. m. carnica* from Slovenia and Croatia honeybee populations were homogeneous [23].

Using eleven simple sequence repeat primers for DNA fingerprinting of bees, on average in 6.8% of the cases 5–7 types of DNA profiles and in 70.4% 2–4 types of profiles were generated; in the rest 22.8% of cases no polymorphism was revealed between the bee races and within the lines tested. Hunt & Page tried 68 primers for *Apis melifera* L., of which 13 proved to be suitable for the analysis of bee genetic diversity [24].

A. m. caucasica race-specific fragments were produced using $(TCC)_5GT$, $(AC)_8YT$ and $(AC)_8T$ primers. Some differences were also noted between the DNA profiles of the individuals of *A. m. carnica* lines tested amplified by $(AC)_8YT$ primer.

CONCLUSIONS

1. The genetic diversity of individuals of *A. m. caucasica* and *A. m. carnica* races was revealed using $AC(GACA)_4$, $(GACA)_4GT$, $(ATG)_5GA$, $(TCC)_5GT$, $(AC)_8YT$ and $(CT)_8A$ primers.

2. *A. m. caucasica* race-specific (discriminating) fragments were obtained by $(TCC)_5GT$, $(AC)_8YT$ and $(AC)_8T$ primers, and those specific to *A. m. carnica* lines by $(AC)_8YT$ primers.

Received 19 February 2006
Accepted 27 May 2006

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V. Paplauskienė, V. Čeksterytė, I. Pašakinskienė, D. Tamašauskienė, J. Račys

ISSR METODO PANAUDOJIMAS ĮVERTINANT BIČIŲ GENETINĘ ĮVAIROVĘ

S a n t r a u k a

Dviejų bičių rasių *Apis mellifera caucasica* ir *Apis mellifera carnica* skirtingų linijų genominės DNR tyrimams panaudota vienuolika paprastųjų pasikartojančių sekų pradmenų. PGR naudojant šiuos pradmenis buvo amplifikuota 60 DNR fragmentų,

kurių 66,7% polimorfiški. Skirtingose bičių rasėse bei linijose gautuose DNR profiliuose fragmentų skaičius kito nuo 2 iki 10, jų dydžiai – 350–3000bp ribose. *A. m.caucasica* ir *A. m.carnica* rasių individų genetinė įvairovė buvo atskeista PGR naudojant $AC(GACA)_4$, $(GACA)_4GT$, $(ATG)_5GA$, $(TCC)_5GT$, $(AC)_8YT$ ir $(CT)_8A$ pradmenis. Gauti specifiniai bičių rasių bei linijų DNR profiliai. *A. m. caucasica* rasę ženklinantys fragmentai gauti PGR naudojant $(TCC)_5GT$, $(AC)_8YT$ ir $(AC)_8T$, o *A. m. carnica* rasės linijas – $(AC)_8YT$ pradmenis. Panaudojus 11 pradmenų, vidutiniškai 6,8% atvejų gauti 5–7 tipų DNR profiliai, 70,4% atvejų gauti 2–4 tipų profiliai, likusieji 22,8% atvejų neatskleidė polimorfiškumo tirtų bičių rasių bei linijų viduje.