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BIOLOGICAL CHARACTERIZATION, PHENOTYPIC AND GENOTYPIC STRUCTURE OF PREDATORY STINKBUG *Perillus bioculatus* Fabr. (*Heteroptera*, *Pentatomidae*) POPULATION IN KRASNODAR REGION

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Abstract

Perillus bioculatus Fabr. (1775) is a predatory bug in the family *Pentatomidae*, a prospective North American entomophage of the Colorado potato beetle. Earlier, Colorado potato beetle eggs were deemed the most preferable for the predator feeding and the main obstacle for perillus acclimatization on new territories. However, in monitoring perillus populations that have acclimatized in several areas of Krasnodar region for the last decade, we revealed the predators' feeding on ragweed leaf beetle (*Zygogramma suturalis* Fabr.) larvae and ragweed cutworm *Tarachidia candefacta* Hübn. (*Noctuidae*: *Lepidoptera*) caterpillars and also on all growth stages of Colorado potato beetle and other insects. In Krasnodar Krai perillus can give annually three generations and is present in three phenoforms, with red-black, orange-black and white-black scutellum. The abundance of *P. bioculatus* is influenced by feeding conditions and by other entomophages, e.g. egg-eaters of genus *Trissolcus* (*Hymenoptera*: *Scelionidae*) and tachina flies (*Diptera*: *Tachinidae*). Analysis of scutellum color inheritance in seven different crosses, including reciprocal ones, between red, orange (or yellow) and white parents showed that the F_1 and F_2 progeny from all combinations, including white individuals crossed with each other, have only red and orange shield (1:1). PCR analysis of *P. bioculatus* bugs in F_2 from crossing insects of different phenoforms revealed that the bugs with the same phenoform in F_2 are genetically different from each other for the frequency of certain RAPD markers. These data indicate non-Mendelian inheritance of scutellum coloration trait in perillus that additionally attract attention to the point. RAPD (random amplification of polymorphic DNA)- and ISSR(inter simple sequence repeat)-PCR analysis detected intra- and inter-population variation of the predatory bugs molecular genetic structure from different zones of the Krasnodar Krai. PCR analysis with almost all used specific primers revealed statistically significant differences ($p = 0.05$) in molecular genetic structure and DNA polymorphism for the populations studied. Geographic populations from the village of Staro-Nizhesteblievskaya and the city of Krasnodar (Krasnodar Krai) were genetically close while there genetic identities with the Moldavian village (Crimean region, Krasnodar Krai) population were low. This comports with their geographical location and indicates geographic variability of *P. bioculatus* populations' genetic structure. The researches executed by us confirm plasticity of this species and prospects for its further acclimatization within Colorado potato beetle area.

Keywords: predatory bugs, *Perillus bioculatus* Fabr., adventive species, acclimatization, PCR analysis, molecular genetic similarity, Colorado beetle, entomophages, biocontrol

Nowadays, Colorado beetle (Leptinotarsa decemlineata Say) has acclimatized in Europe and Asia from the southern regions of Denmark to Spain and Portugal, from the North-West of the European territory of Russia to Siberia and the Far East [1-4]. The growth of the Colorado beetle population in the absence of permanent pesticide treatments can be limited by adverse weather conditions, lack of feed, or by entomophages and entomopathogens. However, until recently within the European areal of this pest, specialized entomophages that could reduce its number were absent. Despite the successful use of natural enemies against many species of ticks and harmful insects, such techniques against the Colorado beetle are either absent or limited to small areas, although its effective natural enemies of Colorado beetle are well known [5].

Systematically applied insecticide treatments ensure the preservation of crop yields [6], but lead to very large and in many cases unnecessary costs due to the lack of accounting for the useful activities of natural enemies of pests [7-10].

The opportunity to use the biological agents against the Colorado beetle attracted the attention of scientists after the pest spread widely in the United States, Canada, and then in Europe [11]. The subject of these studies was pathogenic microorganisms as the basis of microbiological preparations, local entomofauna (to identify effective natural enemies of *L. decemlineata*); the works on the introduction of entomophages of the Colorado beetle from North America were carried out [5, 12, 13].

The investigations on the acclimatization of the North American predatory bug *Perillus bioculatus* Fabr. have begun in France in the 1930s and resumed in the 1950s and 1960s actively [14]. In Europe, the representatives of the subfamily *Asopinae*, the *Perillus bioculatus*, *Podisus maculiventris* Say, *Oplomus nigripennis* var. pulcher Dull., as well as parasitic flies of the genus *Doryphorophaga*, were introduced [15-17]. Since 1966, *Perillus bioculatus* was brought to Belgium, France, Germany, Italy, the USSR, Slovakia, Yugoslavia, and Ukraine for the purpose of natural regulation of the *L. decemlineata* population [18, 19] which became insensitive to the used insecticides quickly [20-23] and showed cross-resistance [24-27]. An international working group of entomologists from Germany, Belgium, Czechoslovakia, Hungary, Bulgaria, and Poland was created for the acclimatization of the entomophage to carry out breeding, production and introduction of *Perillus bioculatus* in agrocenoses [14].

In the USSR, works on the introduction of this predatory bug (as the supposed most promising bioagent for the control of the Colorado beetle) were carried out in 1960-1970, but the attempts of acclimatization and/or seasonal colonization failed. In the last 25-30 years, the research of *Perillus bioculatus* in Russia has not been reported. In 2008, a naturally acclimatized population of *Perillus bioculatus* was found in Krasnodar [28, 29]. To identify the causes of its naturalization and adaptation, determine the areal and assess the prospects for practical application against Colorado beetle, monitoring of the biological characteristics of the predator and its morphogenetic structure in the Krasnodar population were carried out [30, 31]. In recent years, geographical populations of *P. bioculatus* Fabr. have also been identified in the Ust-Labinsky and Abinsky Districts of the Krasnodar Territory. Earlier, *Perillus bioculatus* was found in several areas of Kuban, Adygea, Rostov Region, Ukraine, Moldova, Turkey, and other countries, which gives the reason to assume its large-scale acclimatization in the Old World countries [32].

This paper summarizes the data on the biology and phylogeny of predatory double-eyed soldier bug *Perillus bioculatus* naturally acclimatized in the Krasnodar Territory. The geographical variability of the genetic structure and diversity of the Krasnodar population of this species new for Europe have been revealed for the first time with a description of the biotic factors affecting the dynamics of its population.

The work objective was to study the biological characteristics and phenetic structure of *Perillus bioculatus* populations by the color of the scutellum, including the analysis of the inheritance of this trait with the assessment of the genetic similarity of *P. bioculatus* populations.

Techniques. *P. bioculatus* from different geographical populations (Krasnodar Territory, Rostov Region, and the Republic of Adygea) collected in 2014-2015 were studied. Observations were carried out under stationary crop rotation (All-Russian Research Institute of Plant Biological Protection — ARRIPBP) and in the farms of the Krasnodar Territory which use organic agriculture systems (2014-2016). Eggs, larvae, and adults of Colorado beetle per 1bush (potato varieties of Gollandka, Udacha, Lugovskoy), the number of ragweed leaf beetle *Zygogramma saturalis* Fabr. per 1 m^2 area (plants, soil surface and its layer of 0-12 cm) were counted, as well as larvae and imago of *Perillus bioculatus* [33].

In lab tests, *Perillus bioculatus* was grown on eggs and larvae of *L. decemlineata*, as well as on caterpillars of wax moth (*Galleria mellonella* L.) of the 2nd and 3rd instars. Optimal conditions for the development of *Perillus bioculatus* were 24-25 °C, 70-80% relative humidity, and 16 h photoperiod. The previously developed technique for storage of adult bugs (imagoes) was used [29]. The methods to overcome diapause were applied to continuously maintain the laboratory populations (V.Ya. Ismailov et al., RF patent No. 2 14122878/10, 2015).

During hybridological analysis (reciprocal crossing), one pair of mature bugs of different phenological forms in different combinations, i.e. orange♂ × red♀, red♂ × orange♀, red♂ × white♀, white♂ × red♀, red♂ × red♀, orange♂ × orange♀, white♂ × white♀, were placed in Petri dishes in 5 repetitions. Insects were fed with eggs and larvae of Colorado beetle; after feeding of the 2nd and 3rd generations of the predator, the ratio of phenological forms was counted.

Twenty individuals were selected from each population to study genetic polymorphism. DNA was isolated by a modified CTAB method (V.I. Kil. Method of assessing DNA polymorphism of insect populations by RAPD and ISSR-PCR — recommendations. Krasnodar, 2009) with corresponding reagents (DiaEm, Russia). Purity (according to OD_{260}/OD_{280}) and concentration of DNA preparation were determined spectrophotometrically (Hitachi Perkin Elmer 124, Hitachi, Ltd., Japan).

DNA polymorphism was evaluated by RAPD- (random amplification of polymorphic DNA) and ISSR (inter-simple sequence repeat)-PCR methods (V.I. Kil. Method of assessing DNA polymorphism of insect populations by RAPD and ISSR-PCR — recommendations. Krasnodar, 2009) (iCycler, Bio-Rad Laboratories, Inc., USA) with electrophoretic separation of amplicons (Sub Cell-GT, a power source Power Pac-Basic, Bio-Rad Laboratories, Inc., USA; reagents of OOO DiaEm, Russia; Dialat Ltd., Russia.) DNA-polymerase (5 IU/μl, Dialat Ltd., Russia), a molecular weight marker (M 100bp, OOO SibEnzim, Russia) and standard primers designed in the Operon Technology, Inc. (the USA) (OP) and the University of British Columbia (Canada) (UBC), i.e. UBC880 (ISSR) and OPA02, OPA07, OPA20, OPB01, OPE07, UBC450, UBC531 (RAPD) (synthesized at OOO SibEnzim, Russia), were used. Amplified fragments were separated in 1.8% agarose (AppliChem GmbH, Germany). Photo documentation was performed on the transilluminator ESC-20-M (Vilber Lourmat, France). The compared DNA samples were amplified in one PCR in 2 analytical repeats.

The frequency of DNA markers and its variability were estimated by χ^2-criterion, the reliability of differences between the sample averages by Student's *t*-criterion. Statistical differences were estimated at 5% significance level. Polymorphism indicators were calculated as a percentage of polymorphic fragments to the total number of DNA markers. The genetic diversity of populations and genetic distances were estimated according to M. Nei and C. Schennon with POPGENE v1.31 software (https://sites.ualberta.ca/~fyeh/index.html) [34].

Results. The populations of *Perillus bioculatus* were studied both in the field and in the laboratory. The modes of preservation of adult bugs (up to 10-15 days at 4 °C) were optimized for this purpose [29]. The laboratory population of *P. bioculatus* was maintained continuously due to the developed method of overcoming diapause by means of 10-15-day preservation of insects at low temperatures (3-4 °C) followed by laboratory breeding under optimal conditions (V.Ya. Ismailov et al., RF patent No. 2 14122878/10, 2015).

Biological features and phenetic structure of *Perillus bioculatus* populations. Observations of the populations of *P. bioculatus* and its victims were carried out during regular annual and seasonal observations. In 2015, the overwintered beetles *L. decemlineata* appeared in the 3rd ten-day period of April, the first egg-laying was on May 1 to May 3. In the first ten-day period of May, the first individuals of *P. bioculatus* were noted on the experimental site on potato plants; in the second ten-day period of May, the number of the bug began to increase gradually. At the beginning of the growing season, *P. bioculatus* was represented by a red phenological form; in the third ten-day period of May, individuals of a white phenological form appeared, and only in the third ten-day period of August a small number of bugs with an orange colored scutellum appeared. In this population, 80.0% (603 insects) were individuals with red-black scutellum, 17.0% (128 insects) were orange-black and 3.0% (23 insects) were black-white (total sample size of 754 insects). In 2012, the shares of these phenological forms were 71.3% (445 insects), 17.9% (111 insects) and 10.8% (69 insects), respectively; in 2013 these were 74.9% (540 insects), 20.1% (144 insects) and 5.0% (37 insects), in 2014 — 70.0% (480 insects), 15% (103 insects) and 15% (103 insects). The proportion of *P. bioculatus* individuals with different colors of the scutellum was compared over the years by χ^2-criterion in a form applicable to estimate the similarity of two or more empirical distributions [35]. The result was a value of $\chi^2 = 11.7$, which is less than the standard for the 5% statistical significance ($x^2_{st.[df = 11]} = 19.7$). In other words, the ratio of individuals with different coloring does not depend on the year of the study (differences by year are statistically unreliable).

Now, in the natural conditions of the Krasnodar Territory and the Republic of Moldova [36, 37], red, white, and also orange individuals can be met. In the studies conducted by E.M. Shagov (the city of Mukachevo, Transcarpathian Region, 1968), insects with yellow (orange) and white color of the scutellum were described [38]. E.M. Shagov noted that the higher the temperature, the lighter the color of the scutellum in *P. bioculatus* [38]. The observations of *P. bioculatus* in the natural conditions of the Krasnodar Territory confirm this dependence. In early May 2014, *P. bioculatus* was represented by the red morph (mean ten-period temperature in this period was about 20 °C), by the middle of May, orange individuals were noted (about 23 °C) and only by the middle of July (about 24.5 °C) a white morph of insects was found. The data we obtained are consistent with the report on the cleavage of the enzyme that affects the formation of the *P. bioculatus* scutellum color at elevated temperatures [39]. However, such dependence was not observed during lab culture. In the climatic chamber under the same conditions (the chamber supports the “day-night” mode) insects also had different colors of the scutellum (mainly red, rarely orange), while the white color was extremely rare.

It was found that *P. bioculatus* develops in three generations in the Central zone of Krasnodar Territory and its number during the whole vegetation period is from 2 up to 15 insects per 1 m^2 of a potato field, which is enough to suppress the population of Colorado beetle and to abolish chemical treatments.

The observations have shown that the *P. bioculatus* population acclimatized in the Krasnodar Territory, when compared to that introduced in 1960-1970, has expanded trophic connections with the Colorado beetle: initially, *P. bioculatus* ate only eggs of this phytophage [14, 40], while the naturalized population had all the openly living stages of *L. decemlineata* (eggs, larvae of all ages, and adults) as a fodder base. Such feeding behavior of the predator allowed overcoming the asynchronous development of *P. bioculatus* and *L. decemlineata* in the second and third ten-day periods of April and the first ten-day period of May, which increases the survival rate of the predator and its bioregulatory activity.

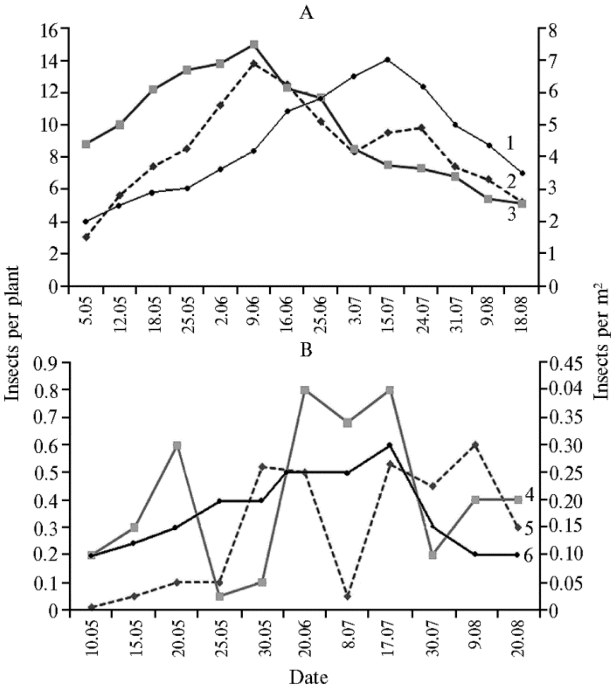

Fig. 1. Population dynamics of adults of ***Leptinotarsa decemlineata*** **Say,** ***Zygogramma saturalis*** **Fabr. and** ***Perillus bioculatus*** **Fabr. during the growing season in 2014** (A) **and 2015** (B): 1 — *Z. suturalis*, 2 — *L. decemlineata*, 3 — *P. bioculatus,* 4 — *L. decemlineata*, 5 — *P. bioculatus*, 6 — *Z. suturalis* (the stationary crop rotation of ARRIPBP, Krasnodar).

In the village of Moldavanskoye (the Krymsky District, Krasnodar Territory, OOO Chistaya eda), the population of *P. bioculatus* was represented by the red and orange phenological forms in the ratio of 4:1. Annual planting of potatoes and other solanaceous crops (tomatoes and eggplants) in the farm, certified according to the organic standard, contributed to the development of the fodder base of the entomophage and created conditions for the development of its two summer generations. Chemical treatments in the fields of this enterprise were not carried out, and *P. bioculatus* during the entire vegetation period restrained the number of Colorado beetle on the nightshade crops at a level below the economic threshold of harmfulness.

To assess the impact of biotic factors on the dynamics of natural populations of *P. bioculatus*, we observed the predator at different stages of its development in natural stations. First of all, we studied the fodder base of *P. bioculatus* was studied, including estimates of both the predatory bug and its victims, the Colorado beetle *L. decemlineata* and ragweed leaf beetle *Z. suturalis* (Fig. 1). These observations revealed the determining role of a fodder base in dynamics of the predatory bug *P. bioculatus* population. For example, the decline of ragweed leaf beetle and the Colorado beetle populations in accounting stations (experimental fields of ARRIPBP) was the determining factor during a significant decrease in the predator population which reached in 2010-2012, 20-30 insets per 1 m^2 but was only 2-3 insects per 1 m^2 in 2014-2016. At the same time (in 2014-2016), in the South-Eastern regions of the Republic of Adygea, where the density of ragweed leaf beetles' and potato fleas' populations was high, the number of entomophages also remained high (up to 10-15 insects per 1 m^2).

Another equally important biotic factor is the activity of other entomophages. In some years, the significant infection of the eggs of *P. bioculatus* with parasitic egg-eater *Trissolcus vassillievi* (Mayr) (*Hymenoptera*: *Scelionidae*) occurred, from 5 to 28%. *Phasiinae* flies (subfamily *Phasiinae*, family *Tachinidae*) infected 3-15%, and ectoparasitic mites up to 10% of the predatory imagoes.

P. bioculatus preferred the eggs of the Colorado potato beetle as food, but no less actively ate larvae of all instars and adult beetles. In natural conditions, in addition to the Colorado beetle, the predator fed larvae and imagoes of ragweed leaf beetle and caterpillars of olive-shaded bird-dropping moth *Tarachidia candefacta* Hübn.

Inheritance of scutellum color in *Perillus bioculatus*. In seven different combinations of crosses (including reciprocal) between red, orange (or yellow) and white parental forms in the offspring (both in F_1 and F_2), including the crossing of white individuals with white, only individuals with the red and orange scutellum were produced in a ratio of 1:1. Unlike natural, laboratory population did not contain white phenological forms. The lack of uniformity in F_1, the absence of white phenological forms and the equal ratio of red and orange morphotypes in F_2 indicate the non-Mendelian nature of the trait inheritance and its conditionality (along with the genetic component) with insect conditions (temperature, photoperiod duration, etc.).

Comparative analysis of geographical populations of *Perillus bioculatus* by DNA markers. In 2015, new geographical populations of *P. bioculatus* were discovered in the village of Varenikovskaya (Krasnodar Territory, the Krymsky District), in the village of Shuntuk (the Maikop station of the Vavilov All-Russian Research Institute of Plant Genetic Resources, the Republic of Adygea) and the Aksaysky District of the Rostov Province. By comparison of their survival depending on a type of feed, the population most suitable for lab culture was selected. The biological indicators of the population of *P. bioculatus* collected on potato plants in the village of Moldavanskoye were significantly different from that of Krasnodar.

As a result, the starting population of the village of Varenikovskaya and the city of Krasnodar was the most adapted to lab culture. When feeding with wax moth (*Galleria mellonella* L.), the number of eggs laid per female was to 70.4-81.6 pcs, whereas the population of the village Moldavanovskoye was unsuitable for the lab breeding of the predator. Winged bugs in this population when feeding its larvae with Colorado beetle were only 21.3%, and when converted to food with an additional victim, the wax moth, the survival of the Moldavanovskoye population of *P. bioculatus* fell sharply, which led to the loss of stock laboratory population, while the Krasnodar one continued to successfully develop and multiply.

The revealed features (along with differences in the phenotypic structure) lead to a conclusion about genotypic differences of *P. bioculatus* populations from the vicinity of Krasnodar and the Krymsky District.

1. DNA polymorphism and genetic structure of *Perillus bioculatus* Fabr. geographical populations (Krasnodar Territory, 2014))

Primer	DNA polymorphism, %		DNA fragment per individual, M±SEM		Number of detected DNA markers, n	Differences between samples in DNA marker frequency, χ^2
	SN	K	SN	K		
OPA02	100	100	7,9±1,4	8,2±0,7	26	25,6
OPA20	100	100	4,0±1,3	5,0±0,8	22	18,5
UBC880	100	100	4,0±0,7	2,0±0,2*	11	14,9

Note. SN means the village of Staro-Nizhesteblievskaya, K means the city of Krasnodar.
* Differences between samples are statistically significant ($t_{act.} \geq t_{05}$).

This was confirmed by the PCR-DNA analysis of *P. bioculatus* bugs, performed with two (OPA02, OPA20) RAPD and one (UBC880) ISSR primers. As a result, no differences in DNA polymorphism between the Krasnodar and Staro-Nizhesteblievskaya (the Krymsky District, Krasnodar Territory) populations of insects (sample of 2014) were found (Table 1).

The high genetic similarity (0.99) was observed between the studied samples (Table 2). Intrapopulation genetic diversity in the studied geographical samples (Krasnodar and Staro-Nizhesteblievskaya) did not differ (see Table 2). The obtained data indicate a high migratory capacity of the species and that the samples under investigation probably belong to the same geographical population.

2. Genetic diversity of *Perillus bioculatus* Fabr. geographical populations in different years (Krasnodar Territory)

Indicator	2014		2015	
	SN	K	SN	M
Genetic diversity according to Shennon, I±SD	0.38±0.18	0.36±0.18	0,28±0,25	0,43±0,21*
Genetic identity according to Nei	0.99		0.86	

N o t e. SN means the village of Staro-Nizhesteblievskaya, K means the city of Krasnodar, M means the village of Moldavanskoye.
* Differences between samples are statistically significant ($t_{act.} \geq t_{05}$).

In 2015, the additional primers (OPA07, OPB01, OPE07, and UBC450) were used to obtain more detailed information on *P. bioculatus* polymorphism. The PCR analysis of the samples (from the village of Staro-Nizhesteblievskaya and the village of Moldavanskoye, 2015) revealed statistically significant differences in the genetic structure and DNA polymorphism with almost all used primers (Fig. 2, Table 3). The genetic diversity of these samples varied and their genetic similarity was relatively low (0.86) (see Table 2).

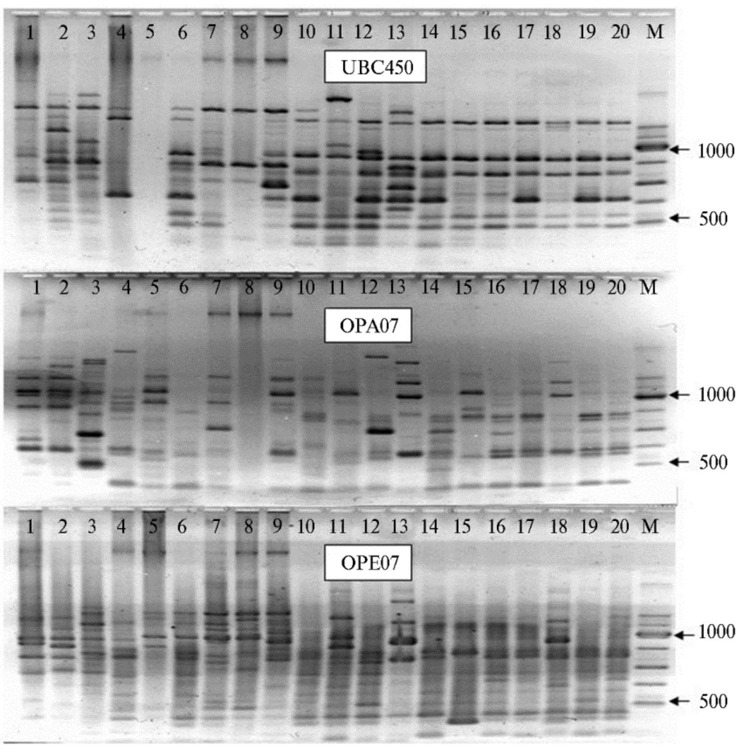

Fig. 2. RAPD-PCR analysis of *Perillus bioculatus* Fabr. from different geographical populations (Krasnodar Territory, 2015): 1-10 — the village Staro-Nizhesteblievskaya; 11-20 — the village Moldavanovskoye; UBC450, OPA07, OPE07 — the used primers; M — molecular weight marker (M 100bp, SibEnzim, Russia), 1.8% agarose.

3. DNA polymorphism and genetic structure of *Perillus bioculatus* Fabr. geographical populations (Krasnodar Territory, 2015 год)

Primer	DNA polymorphism, %		DNA fragment per individual, $M \pm SEM$		Number of detected DNA markers, n	Differences between samples in DNA marker frequency, χ^2
	SN	M	SN	M		
OPA02	100	100	6.2±1.4	5.8±1.0	25	25.1
OPA07	100	100	7.1±0.4	7.0±1.0	19	32.1*
OPA20	76.9	100	7.6±0.5	2.9±0.6*	10	30.5*
OPB01	75.0	100	5.5±0.5	5.0±0.7	12	23.4*
OPE07	93.3	100	10.1±0.7	9.8±1.0	21	50.8*
UBC450	100	100	5.1±1.2	6.3±0.7	11	27.8*

N o t e. SN means the village of Staro-Nizhesteblievskaya, M means the village of Moldavanskoye.
* Differences between samples are statistically significant ($t_{act.} \geq t_{05}$, $\chi^2_{act.} \geq \chi^2_{05}$).

The PCR analysis showed that the low viability of the starting populations of *P. bioculatus* from the village of Moldavanovskoye and its food preferences, which distinguish this population from the others, are due to genetic differences.

In general, it can be concluded that since 2008, when the first reports of acclimatization of the predatory bug *P. bioculatus* in the Krasnodar Territory [28, 32] had appeared, entomophage, due to the high migration capacity and the acquisition of additional trophic connections, managed to naturalize well in these new living conditions. Similar data were obtained on the rapid acclimatization of predatory bug throughout the entire territory of Moldova [36, 37], as well as in Turkey [39, 41, 42], Bulgaria [43], and Serbia [44]. The revealed significant influence of the fodder base and entomophages on the natural number of this bug allows us to suggest that native species of entomophages have adapted to the useful adventive species and are able to reduce its number. In some years, this can neutralize the useful activity of natural populations of the predator, thence, it is necessary to improve methods of artificial cultivation of *P. bioculatus* and create optimal conditions for its activation and reproduction in field conditions (the lack of chemical treatments and the presence of the fodder base).

Thus, in the Krasnodar Territory, acclimatized North American predatory bug *Perillus bioculatus* Fabr. has become an effective natural regulator of the Colorado beetle population. The study of the biological characteristics of *P. bioculatus* revealed a significant impact of a fodder base on the number of natural populations of the predator and indigenous species of parasitoids. The data obtained by crossing insects of different phenological forms of *P. bioculatus* indicate the non-Mendelian nature of the inheritance of the scutellum color and the different manifestations of this trait in the studied populations of the predator. The detailed genetic analysis of this trait and its variability depending on the environmental conditions is the fact of scientific interest and is also of practical importance for assessing the effectiveness of natural populations of entomophage. The results of the evaluation of predatory activity, the viability of the entomophage and the PCR analysis of differences in the genetic structure in the studied geographical populations are consistent.

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