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Analysis of Gene Flow between Generations of Various Years in Population of Stag Beetle (*Lucanus cervus L.*) Based on *RAPD* and *ISSR* DNA Markers.

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ABSTRACT

Polymerase chain reaction method (*RAPD*- and *ISSR*-markers) was used to show high level of gene flow between generations of various years in population of stag beetle (*Lucanus cervus L.,* 1752), that lives in oak wood near Belgorod town. The data obtained prove experimental studies, according to which some animal units breed in other generation due to uneven maturation of larvae. This contributes to gene exchange between groups of various years and provides genetic integrity of population and species.
Keywords: stag beetle, reproductive groups, gene flow.

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INTRODUCTION

It is known that an average developmental cycle of stag beetle (*Lucanus cervus L.*, 1752) lasts for 5 years. Within the first four years larvae are developed in earth-ball part of trunk as well as in thick roots of old trees, feeding on wood substances. Pupation occurs on the fourth year in September. Imagoes overwinter below ground [1]. An outgo for reproduction in investigational area occurs in May-July (rarely in August). Males die after copulation, and females die after oviposition. Thus, generations of various years are isolated from each other as well as form separate mendelian subpopulations. At the same time, there is information stating that in laboratory experiments some animal units of this species at larva stage may develop faster (for three of four years instead of five or six years), and, thus, they could develop in other generation [2].

According to our observations, a small part of animal units in imago stage go out to the surface in August, i.e., when reproductive period is already over. One cannot rule out that these could be quadrennial beetles, which went through accelerated developmental variation as a result of favourable combination of circumstances (optimal temperature, plenteous feed etc.). Their descendents, having gone through full five-year ontogenesis, may cause timeframe shift, breeding in other reproductive group yet.

Studying genetic processes that are flowing in such generations of this specially protected red-listed species may clarify whether it is naturally possible [3].

The main objective of this work was to asses the level of gene flow between generations of various years in population of stag beetle near Belgorod, basing on biochemical markers.

METHODS

Collection of bugs was being performed from 2006 to 2010 in broadleaved woodlands, located near Belgorod town not far from "Sokol" factory (50°38′31.41″n.l, 36°32′56.80″E). Considering the fact that study species' population reduces and study species is protected, we selected limited samples form populations, the number of which didn't exceed 10% of their total number.

Genetic analysis was performed with the usage of polymerase chain reaction, particularly, using *RAPD* (*Random amplified polymorphic DNA*) [4] and *ISSR* (*Inter simple sequence repeats*) methods [5]. Amplification was performed in thermal cyclers MJ Mini and MyCycler (*Bio-Rad*). Reaction was perfomed in 25 mcl of the mixture, containing 20 ng of genomic DNA, PCR buffer (mM of tris- HCl (pH 8,8), 16 мМ $(NH_4)_2SO_4$, 5 мМ of β-mercaptoethanol, 7 мМ of EDTA, 3 мМ of MgCl), 0,25 мМ of dNTP, 0,5 µm of primer, 1 unit of Taq DNA polymerase (inhibited for hot start). Reaction proceeded in the following conditions: hot start – 2 min/94°C, 40 cycles (denaturation – 30s/94°C, primer's annealing – 30s/ (36 °C for *RAPD* and 55°C for *ISSR*), synthesis – 2 min/72°C), additional synthesis – 10 min/72°C), cooling to 4°C. PCR products were divided via electrophoresis in 2% agarose gel with the usage of TAE buffer (cooled to +4° C), 100 V – 45 min. Blocks were coloured with ethidium bromide.

According to images of amplified fragments obtained in the course of electrophoresis, binary matrixes were composed, in which the presence of stripe would be noted as "1" (allelic gene *p*), and the absence was noted as "0" (allelic gene *q)*. Considering that while using *RAPD* method non-specific amplified products may occur, we used clearly identified and reproductive amplicons for the analysis.

We extracted 16 locuses (48 in total) in the range from 3K to 200 of base pairs from this species with the usage of two *RAPD* primers: *OPF* 5 (5′-CCGAATTCCC-3′), *OPF 12* (5′-ACGGTACCAG-3′) and one *ISSR* primer *UBC 809* (5'-$(AG)_8$G-3').

Data processing was performed with the help of GenAlEx 6.5 [6] and POPGENE 32 [7] programs.

MAIN PART

Obtained data show that reproductive groups of various years have similarly high level of genetic diversity (Table 1).

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Table 1: Indices of genetic variability by DNA-locuses in study groups of *L. cervus* of various years (*N* stands for sample size, *P* is a percentage of polymorphic locuses, A_e stands for effective number of allelic genes, *I* is Shannon index; H_0 stands for medium observed heterozygosity)

Year	*N*	*P%*	*A*	A_e	I_{sh}	H_e
2006	35	100	2,00±0,00	1,67±0,02	0,587±0,008	0,399±0,007
2007	17	100	2,00±0,00	1,71±0,03	0,592±0,013	0,405±0,011
2008	44	100	2,00±0,00	1,72±0,02	0,604±0,007	0,415±0,007
2009	45	100	2,00±0,00	1,68±0,02	0,591±0,007	0,402±0,006
2010	19	100	2,00±0,00	1,69±0,03	0,585±0,013	0,399±0,012

Table 2: Genetic differentiation indices of studied groups of *L. cervus* from various years by DNA locuses (abbreviations are expanded in the text).

Method	Primer	Locus	*Ht*	*Hs*	*Gst*	*Nm*
RAPD	*OPF 5*	1	0,345	0,337	0,023	21,61
		2	0,425	0,424	0,005	107,79
		3	0,371	0,368	0,010	50,72
		4	0,441	0,434	0,015	32,58
		5	0,418	0,403	0,035	14,00
		6	0,435	0,386	0,112	3,95
		7	0,438	0,411	0,062	7,58
		8	0,425	0,423	0,004	115,88
		9	0,447	0,446	0,001	503,41
		10	0,439	0,436	0,007	73,91
		11	0,436	0,419	0,038	12,53
		12	0,383	0,377	0,016	31,67
		13	0,441	0,437	0,008	61,20
		14	0,413	0,409	0,009	53,88
		15	0,423	0,399	0,055	8,58
		16	0,432	0,419	0,029	16,60
	OPF 12	1	0,379	0,376	0,007	67,27
		2	0,390	0,364	0,067	6,93
		3	0,445	0,432	0,029	16,76
		4	0,448	0,431	0,038	12,81
		5	0,441	0,431	0,023	21,21
		6	0,413	0,408	0,014	35,86
		7	0,400	0,383	0,042	11,55
		8	0,417	0,411	0,014	35,22
		9	0,405	0,402	0,007	72,14
		10	0,389	0,378	0,027	18,20
		11	0,433	0,423	0,024	20,65
		12	0,395	0,392	0,006	87,76
		13	0,410	0,398	0,030	16,43
		14	0,362	0,357	0,014	36,43
		15	0,455	0,443	0,026	18,76
		16	0,427	0,421	0,014	35,25
ISSR	*UBC 809*	1	0,412	0,402	0,024	20,58
		2	0,321	0,319	0,006	77,10
		3	0,436	0,419	0,040	11,91
		4	0,416	0,409	0,016	31,67
		5	0,421	0,414	0,016	30,67
		6	0,459	0,454	0,010	47,80
		7	0,368	0,366	0,006	83,65
		8	0,395	0,394	0,004	142,54
		9	0,439	0,434	0,010	48,76
		10	0,486	0,469	0,036	13,50
		11	0,385	0,379	0,016	30,65
		12	0,418	0,408	0,024	20,51
		13	0,378	0,373	0,013	37,10
		14	0,399	0,387	0,030	16,03
		15	0,403	0,386	0,043	11,24
		16	0,406	0,393	0,031	15,54
Medium			0,414±0,004	0,404±0,004	0,024	20,41

Evaluation of degree of generations' differentiation of *L. cervus* based on the model suggested by M.Ney [8] by DNA locuses (Table 2) showed obviously low disintegration of studied generations by all detected locuses. This is witnessed by small percentages of interpopulation gene diversity in general diversity (G_{st}).

At this, medium gene flow (*Nm*) was equal to 20,41 of animal units in population and was comparable to equivalent value that had been obtained by us earlier based on Write's *F*-statistics on isoenzyme markers F_{st}=0,012, *Nm*=21,0) [9].

Besides, all noted locuses in various generations of *L. cervus* are marked by high level of expected percentage of heterozygous genotypes, which was calculated for the whole populations (H_T) and large mean for all subpopulations values of interpopulation gene diversity ($\overline{Hs}$).

Even more disintegration of various generations was shown by analysis of molecular dispersion (AMOVA) [10]. According to the data received, the measure of discrepancy between reproductive groups doesn't exceed 1% (Table 3). That said, genetic differentiation index Φ_{st} appeared to be very low (0,006), and gene flow level equaled 24,6 animal units of all the populations.

Table 3: Results of molecular dispersion analysis (AMOVA) by DNA locuses in studied groups of *L. cervus* of various years.

Source of variation	*df*	*SS*	*MS*	*V*	*%*	Φ_{st}	*P*	*Nm*
Between populations	4	56,713	14,178	0,073	1%	0,006	0,050	24,6
In populations	155	1847,506	11,919	11,919	99%			
Total	159	1904,219	26,098	11,993				

It should be noted that similar analysis that had performed by us earlier for assessment of differentiation level between various populations of *L. cervus* in investigational area showed their larger disintegration and lesser level of gene exchange (Φ_{st}=0,047, *Nm*=3,97) [11].

Table 4: Values of genetic similarity index between study groups of *L. cervus* of various years

Years	2006	2007	2008	2009	2010
2006	1,000				
2007	0,957	1,000			
2008	0,979	0,957	1,000		
2009	0,975	0,963	0,975	1,000	
2010	0,936	0,922	0,953	0,946	1,000

SUMMARY

Therefore, conducted molecular genetic analysis allowed clarifying that reproductively isolated generations of various years in stag beetle's populations are marked with high genetic similarity.

CONCLUSION

Minor genetic distance between reproductively isolated groups of *L. cervus* of various years, noted by DNA spectra, which to a greater extent reflect genetic automatic processes, demonstrates that there is a limited genetic information transfer between these groups.

The data obtained verify laboratory tests' results [2] and results of our observations, according to which some animal units breed in other generation due to uneven maturation of larvae.

Gene exchange between groups of various years provides genetic integrity of population and *L. cervus* species, and given identified high genetic variability, it favours its viability increase.

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