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CLONAL DIVERSITY OF *OTIORHYNCHUS LIGUSTICI* AND *O. RAUCUS* (COLEOPTERA, CURCULIONIDAE) IN CENTRAL UKRAINE

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Clonal Diversity of *Otiorhynchus ligustici* and *O. raucus* (Coleoptera, Curculionidae) in Central Ukraine. Morozov-Leonov, S. Yu., Nazarenko, V. Yu. — The clonal diversity in four weevil populations of two species, *Otiorhynchus ligustici* (Linnaeus, 1758) and *O. raucus* (Fabricius, 1777), from vicinity of Kyiv is analyzed. Polyclonality and inter-population differentiation in both species is demonstrated. The obtained data about two weevil species clonal diversity are compared with those known for European species *O. scaber* (Linnaeus, 1758).
Key words: weevil, *Otiorhynchus,* clonal structure, genetic variation.

Introduction

Alternative forms of sexual reproduction in animals (such as parthenogenesis, gynogenesis, hybridogenesis) have traditionally attracted interest of researchers. At the same time they are known mainly for invertebrates. In particular, among the insects about 900 species are reproduced by a similar mode (Normark, 2003). The parthenogenetic reproduction is widely known among species belonging to the family Curculionidae (Insecta, Coleoptera). Most of the scientific investigations devoted to the species of this family are realized on the species of the genus *Otiorhynchus* (Stenberg et al., 2003). The genus *Otiorhynchus* Germar, 1822 includes 1288 species belonging in 113 subgenera in the Palaearctic Region (Magnano, Alonso-Zarazaga, 2013). The population genetic structure has been studied by far only in the Western European species *O. scaber* (Suomalainen, Saura, 1973; Stenberg et al., 2000; Stenberg et al., 2003). The genetic structures of other *Otiorhynchus* species populations inhabiting Eastern Europe have not been analyzed yet. The aim of this study was to analyze the clonal variability of the other *Otiorhynchus* species of genus in Eastern Europe. The two model species, *Otiorhynchus* (*Cryphiphorus*) *ligustici* (Linnaeus, 1758) and *O.* (*Choilisanus*) *raucus* (Fabricius, 1777),have been chosen.

Subspecies structure of both species is not allocated (Magnano, Alonso-Zarazaga, 2013), though Mazur (1993) described a subspecies *Otiorhynchus* (*Choilisanus*) *raucus* (Fabricius, 1777), based on the ecological and morphological differences.Both species reproduce parthenogenetically over much of its range, though a single *O. raucus* male has been described by Mazur (2003).

Material and methods

We sampled in three localities in the vicinity of Kyiv, Ukraine. The samples were taken in the spring and summer of 2016 of the following localities (table 1). Three of the four populations we studied are on the right bank of the Dnieper. Only the population from the vicinity of Panfily is located on the left Dnipo bank.

Samples of *Otiorhynchus ligustici* — "*O. ligustici* 1" (the vicinity of the Village Hutir Vilny (50.318452, 30.518464), 20 specimens) and "*O. ligustici* 2" (near Bila Tserkva City (49.808380, 30.059087), 5 specimens).

Samples of *Otiorhynchus raucus* — "*O. raucus* 1" (the vicinity of the Village Hutir Vilny (50.318767, 30.533248), 14 specimens) and "*O. raucus* 2" (near Panfily Village (50.225285, 31.740811), 6 specimens). Weevils were hand-collected and transported to the laboratory for probes preparation.

Electrophoretic variability of esterase (Es-1, 2, 3), malate dehydrogenase (Mdh) (for both species); muscle proteins (Pt) (for *Otiorhynchus ligustici*), aspartate aminotransferase (Aat) and acid phosphatase (Acph) (for *Otiorhynchus raucus*) was studied. The thoracic segments of every weevil were frozen during 12 hours, then used in the acrylamide electrophoresis. Sample preparation, electrophoretic analysis of enzymes and non-enzyme proteins, the data interpretation were performed by standard methods (Mezhzherin, Peskov, 1992).

The following parameters were calculated for samples of both parental species.

The observed number of clones (Nclon) was determined by direct count of electrophoretically distinct multilocus phenotypes.

The effective number of clones (Neff) is calculated by using formula Neff = 1 / Σ (Pi^2), where Pi — the frequency of the i-phenotype in a sample (Parker, 1979).

The clonal heterozygosity (Het) is calculated by the formula similar to the expected heterozygosity one for one gene H = (1–Σ (Pi^2))* n / (n–1), where Pi — the frequency of the i-phenotype in a sample, n is a sample volume (Nei, Roychoudhury, 1974).

The clonal diversity index (Geff) is calculated according to the formula Geff = (Neff-1) / (n–1) (Dorken, Eckert, 2001).

Results

Electrophoretic spectra of the studied proteins. Most of the examined heterozygous electrophoretic spectra are asymmetric (figs 1, 2). However, in some specimens electrophoretic spectra are symmetrical, especially in case of esterase-3 (fig. 1). Furthermore, one *O. ligustici* specimen has a spectrum Es-$3^{88/100/112}$, which is in agreement with data published earlier (according to them, both studied species are triploid) (Lokki, Saura, 1980; Stenberg et al., 1997). Heterozygous spectra of Mdh in all specimens of both species show clear asymmetry (figs 2, 3), which also confirms the polyploid nature of their genome.

Each studied specimen of both species had asymmetric electrophoretic spectra of some or all of the studied proteins (figs 1–3). The proportion of weevils, whose heterozygous electrophoretic spectra all are asymmetrical is equal to 0.16 for *O. ligustici* (table 1) and 0.60 for *O. raucus* (table 2).

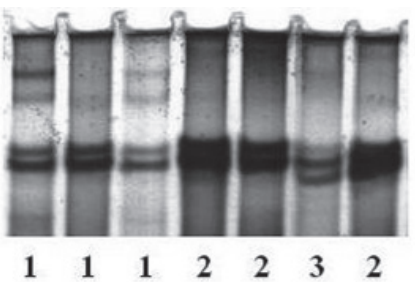

Fig. 1. The electrophoretic spectra of the escterase-3 in the muscle of *Otiorhynchus ligustici* specimens: 1 — phenotype Es-$3^{88/100/100}$; 2 — phenotype Es-$3^{88/100}$; 3 — phenotype Es-$3^{88/100/112}$.

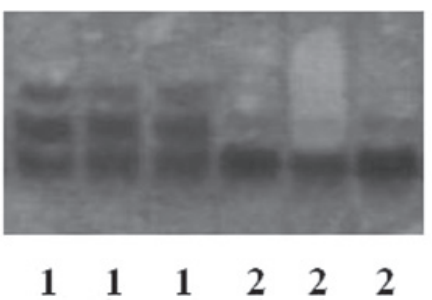

Fig. 2. The electrophoretic spectra of the malate dehydrogenase in the muscle of *Otiorhynchus ligustici* specimens: 1 — phenotype Mdh$^{79/100/100}$; 2 — phenotype Mdh^{100}.

The clonal structure of the studied species. Parallel analysis of the studied proteins electrophoretic spectra for each specimen has enabled us to identify a lot of clones in each sample studied (tables 1, 2). None of analyzed populations was monoclonal. The number of clones in a sample varies from 2 to 7. The inequality of clones frequencies causes the fact that effective number is lower than observed one and not exceed 3.64 (*Otiorhynchus ligustici*) and 4.90 (*Otiorhynchus raucus*).

In the *Otiorhynchus ligustici* population No. 1 the L4 clone predominates quantitatively with frequency 0.45, in the population No. 2 the two identified L8 and L9 clones have the frequencies that are approximately equal (0.60 and 0.40, respectively). In the *Otiorhynchus raucus* population No. 1, the maximum frequency is that of R4 clone (0.36). In population No. 2 of this species, the quantitative predominance of clone R7 (0.83) is sure. This high clone frequency is the cause for the relatively low level of this population genetic diversity (see below).

Genetic variation level. The level of genetic variation in *O. ligustici* populations is relatively high and shows no significant inter-population differences (Het = 0.60–0.76, Geff = 0.14–0.23, $P < 0.05$, table 1). These data are in contrast with the clones spatial distribution among populations.

The level of genetic variation in *O. raucus* populations is more variable, but the inter-population differences are also unreliable (Het = 0.33–0.86, Geff = 0.08–0.30, $P < 0.05$, table. 2).

Table 1. The electrophoretic phenotypes numbers in two populations of *Otiorhynchus ligustici* from vicinities of Kyiv

Clone	Gene			Population	
	Es-3	Mdh	Pt	*O. ligustici* 1	*O. ligustici* 2
				Hutir Vilny	Bila Tserkva
L1			100 / 108	4	
L2	88/100	79/100/100	100 / 100 / 108	3	
L3			100 / 108 / 108	1	
L4			100 / 108	9	
L5	88/100/100	79/100/100	100 / 100 / 108	1	
L6			100 / 108 / 108	1	
L7	88/100/112	79/100/100	100	1	
L8	112	100	103 / 110		3
L9			103 / 103 / 110		2
Sample volume				20	5
Nclon				7	2
Neff				3,64	1,92
Het				0.76	0.60
Geff				0.14	0.23

Note. Abbreviations see "Material and methods".

The geographic distribution and inter-population genetic differentiation. A comparison of the identified clones frequencies demonstrates significant differentiation between populations within a species *Otiorhynchus ligustici* (fig. 4). Moreover, the genetic differences were observed in two of the three genes analyzed. The sample No. 2 (Bila Tserkva) has fixed on Es-3^{112} allele that is present in the sample No. 1 (Hutir Vilny) with very low frequency — no more than 2 %. The gene encoding the muscle protein in the sample No. 2 has two alleles — Pt^{103} and Pt^{110} that are absent in sample No. 1. Finally, all the specimens in the sample No. 1 have heterozygous genotype $Mdh^{79/100/100}$, while the specimens in the sample No. 2 have the homozygous one (Mdh^{100}). Respectively, for *O. ligustici* no clones common to both populations are identified. Of the 9 clones identified, the clones L1–L7 are found in population No. 1 exclusively, while clones L8–L9 — in the population No. 2. It should be noted that these populations are separated by relatively small distance — not more than 70 km.

Table 2. The electrophoretic phenotypes numbers in two populations of *Otiorhynchus raucus* from vicinities of Kyiv

Clone	Gene				Population	
	Es-3	Mdh	Aat	Acph	*O. raucus* 1	*O. raucus* 2
					Hutir Vilny	Panfily
R1	112	118 / 136 / 136	100	100	1	1
R2				110	2	
R3		136	100	100	1	
R4				100	5	
R5			100/108	100 / 110	1	
R6				110	2	
R7	119	136	100	100	2	5
Sample volume					14	6
Nclon					7	2
Neff					4,90	1,38
Het					0.86	0.33
Geff					0.30	0.08

Note. Abbreviations see table 1.

Table 3. The variation of some clonal diversity indices within populations of the three weevil species genus *Otiorhynchus*

Species	Index				Source
	Nclon	Neff	Hexp	Geff	
O. ligustici	2–7	1.92–3.64	0.60–0.76	0.14–0.23	This article
O. raucus	2–7	1.38–4.90	0.33–0.86	0.08–0.30	This article
O. scaber	2–16	1.29–7.25	0.24–0.88	0.01–0.15	(Suomalainen, Saura, 1973; Stenberg et al., 2000)

Note. Abbreviations see table 1.

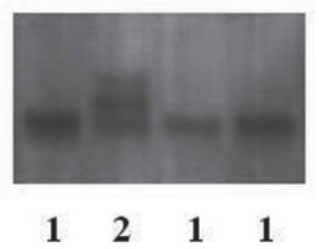

Fig. 3. The electrophoretic spectra of the malate dehydrogenase in the muscle of *Otiorhynchus raucus* specimens: 1 — phenotype Mdh^{136}; 2 — phenotype $Mdh^{118/136/136}$.

A different inter-population differentiation pattern is revealed for the second studied species, *O. raucus*. All 7 clones (R1–R7) identified for this species were found in the population No. 1, R1 and R7 clones are present also in the population No. 2 (fig. 4). This inter-population differentiation being smaller than in the first species is surprising because *O. raucus* populations are separated by a twice longer distance of about 140 km.

Discussion

The polyclonal structure of the studied species. The number of clones and the clonal diversity level of the both species studied are at least not inferior to those of the previously studied *O. scaber* (table 3). As it was mentioned above, none of the populations examined by us is monoclonal. That opens up broad perspectives of the future investigations of both species populations outside the primary research region. In addition, polyclonality of the studied species populations may be a proof of their reticulate (polyphyletic) origin (Mateos, Vrijenhoek, 2002). This may potentially have a strong impact on the systematics of these species.

The constant heterozygosity (revealed by us) of a sample is often a result of its hybrid origin (Suomalainen, Saura, 1973). In our case, we have a reason to predict the allotriploid genetic constitution of the both species examined. A similar genetic constitution is found

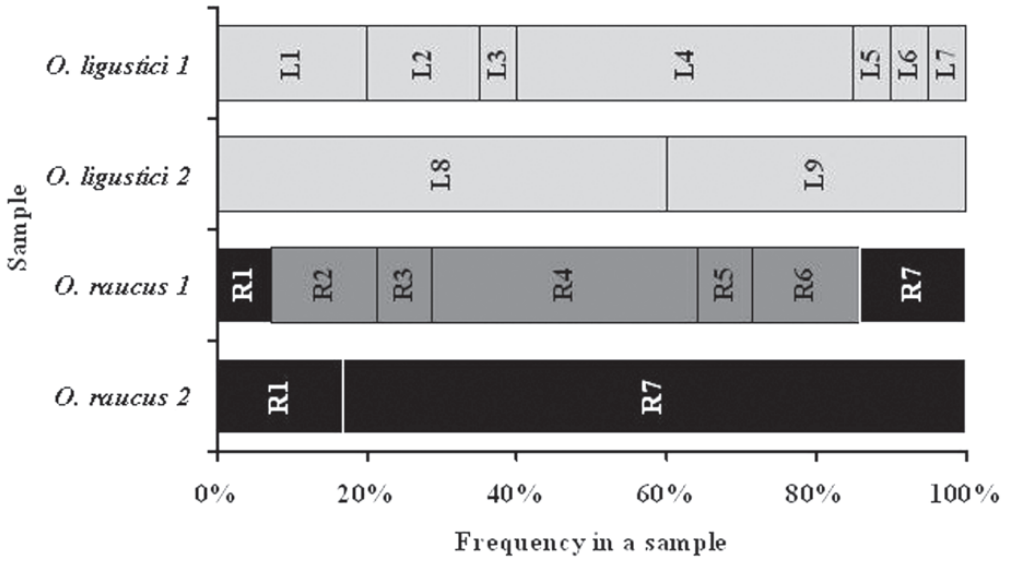

Fig. 4. The polyclonal structure of two species genus *Otiorhynchus* samples from Kyiv vicinities: L1–L9 — *O. ligustici* clones; R1–R7 — *O. raucus* clones.

in a vertebrate hybrid form, where allotriplody caused the alternative reproduction mode (gynogenesis) (Stenberg et al., 1997; Quattro et al., 1992).

Discordance between the studied protein electrophoretic spectra pattern and the supposed species ploidy level. It was noted above that not all examined weevil specimens have asymmetric heterozygous protein spectra. This phenomenon requires an explanation.

It should be noted that at the beginning of the *O. scaber* clonal structure study the symmetry of some heterozygous electrophoretic spectra was detected (Suomalainen, Saura, 1973). The same fact was also demonstrated in the study of the *Pelophylax esculentus-ridibundus* hybrid form (Amphibia, Ranidae). In some allotriploid hybrid frogs, heterozygous protein spectra are symmetrical, which is unexpected (Günther, Hähnel, 1976). There are 3 possible explanations for this discordance.

Firstly, the gene dosage compensation effect is possible.

The second — the duplication of genes encoding the studied proteins is also possible. The presence of genes duplication in the family Curculionidae has been previously demonstrated for certain nuclear genes (Yan et al., 2016; Binu et al., 2016) and for some sequences of mitochondrial DNA (Boyce et al., 1989).

But the most likely explanation, as we think, is the ploidy level variation. We suppose that two species studied are not only triploids but contain a tetraploids admixture, as was demonstrated earlier for *O. scaber* (Stenberg et al., 1997). At the moment, it is known that the ploidy level of the genus *Otiorhynchus* parthenogenetic forms varies from 2 n to 10 n (Stenberg, Saura, 2013). For this reason, the hypothesis of the presence in the population species we studied allotetraploids admixture is seemed to be quite probable. The final answer to this question can be obtained during the further studies covering a wider region and also including the karyological analysis of specimens from the geographically remote populations.

The studied species geographic distribution and inter-population genetic differentiation. Both studied species are known to be wingless (Wanat, 2011). So, the geographical distances and wide rivers are an effective isolation barrier for them. In this case, we could expect more explicit genetic differentiation between the *O. raucus* populations separated by a greater distance and located on the different Dnieper banks. However, we see the absence of local clones in remote populations of this species. This discrepancy between the expected and observed inter-populational differentiation can be explained most likely by artificial introduction — a phenomenon that is currently widespread (Vorburger, Reyer, 2003).

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