

Restricted geographic distribution and low genetic distinctiveness of steppic *Crioceris quinquepunctata* (Coleoptera: Chrysomelidae) populations in Central East Europe

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Crioceris quinquepunctata is a European leaf beetle, rare and strictly associated with steppe-like habitats in Central and Eastern Europe. We sampled suitable localities in Central East Europe to determine the current distribution and to verify whether populations isolated by the Carpathian Mountains (within Pannonian and Pontic area) show genetic differentiation. Sequences from the beetle COI and ITS1 were amplified and compared. Furthermore, *ftsZ* and *hcpA* genes of the endosymbiont *Wolbachia* were analysed as additional genetic markers. We found only two populations of *C. quinquepunctata* (in Moravia and Podolian Upland). Unusually low genetic differences between these populations were revealed, which is in contrast to previous studies on other steppe beetles. The reasons for such low diversity are speculative and probably related to recent natural expansion or man-made translocation of *C. quinquepunctata*.

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1. Introduction

The genus *Crioceris* Geoffroy, 1762 (Coleoptera: Chrysomelidae) consists of 62 species found all over the world (except for Neotropical and Australian regions) (Monrós 1960) and 22 species found in the Palearctic, including eight species known in Europe (Löbl & Smetana 2005). All of them are oligophagous or monophagous, feeding on members of the genus *Asparagus* (Asparagaceae) (Clark et al. 2004).

In the past, *Crioceris quinquepunctata* (Scopoli, 1763) was distributed in Europe in Austria, Bosnia and Herzegovina, Bulgaria, Croatia, central and southern European parts of Russia, the Czech Republic, France, Germany, Hungary, Italy, Moldova, Poland, Romania, Slovakia, Ukraine, Serbia and Montenegro (Löbl & Smetana 2005: 361). Most records are old and were not repeated so they mostly have historical value and hence there is a need for new data. For example, only two localities are known from Ger-

Table 1. List of investigated xerothermic localities by countries, with codes of UTM squares, years and numbers of collected specimens (from most localities none was found). Species codes: CA, *Crioceris asparagi*; C5, *C. quinquepunctata*; C12, *C. duodecimpunctata*; C14, *C. quatuordecimpunctata*.

Country & Locality	UTM	Years	CA	C5	C12	C14
Poland						
Nida vic.	DB62	2005–2013	>10	–	10	6
Gogolin vic.	BA89	2003–2012	>10	–	5	–
Górażdże	BB80	2008–2012	7	–	8	–
Prudnik	XR87	2008–2012	5	–	2	–
Kamień Śląski	BB90	2003–2013	7	–	–	–
Chęciny	DB62	2011	–	–	7	–
Duszniki Zdrój	WR98	2008, 2012	–	–	–	–
Dziewcza Góra	FB77	2006, 2008, 2011	–	–	–	–
Gołębia Góra	WR95	2008	–	–	–	–
Góra Bukowa	FB30	2012	–	–	–	–
Gródek ad Hrubieszów	GB02	2012	–	–	–	–
Hebdów	DA55	2012	–	–	–	–
Jagienieckie Łąki	XS70	2008–2011	–	–	–	–
Kajasówka	DA04	2010, 2013	–	–	–	–
Kalwaria Paławska	FV29	2011	–	–	–	–
Kąty II	FB41	2006, 2013	–	–	–	–
Kietrz	BA85	2011–2013	–	–	–	–
Kików	DA98	2010, 2012	–	–	–	–
Klucze	CA97	2012	–	–	–	–
Kraków Skotniki	DA13	2006–2013	–	–	–	–
Ligota Dolna	BA99	2004–2013	–	–	–	–
Morsko	DA75	2008–2013	–	–	–	–
Niewiatrowice	DA58	2010	–	–	–	–
Rybna	XS83	2007–2012	–	–	–	–
Sandomierz	EB51	2006–2012	–	–	–	–
Stobrawa	XS83	2008–2012	–	–	–	–
Szymiszów	CB09	2004–2013	–	–	–	–
Wały ad Miechów	DA48	2012	–	–	–	–
Wieprzecka Góra	FB51	2006, 2012	–	–	–	–
Winna Góra	YS04	2007–2012	–	–	–	–
Zubrzyce	XR95	2005–2008	–	–	–	–
Czech Rep.						
Pouzdrany	XQ12	2012	–	7	7	7
Sedlec	VQ69	2012	–	–	3	7
Popice	XQ22	2012	–	–	–	6
Mohelno	WQ83	2012	–	–	–	–
Slovakia						
Cenkovska Step	CT28	2012	–	–	>10	6
Bratysława	XP54	2011	–	–	–	7
Devinska Kobyla	XP43	2011	–	–	–	6
Dlha n. Orava	CV85	2011	–	–	–	–
Dunajske Luhy	XP80	2011	–	–	–	–
Plášťovce	CU43	2012	–	–	–	–
Skalica-Veternik	XQ61	2012	–	–	–	–
Velke Vrchy	CU19	2011	–	–	–	–
Ukraine						
Miodobory	MQ46	1993–1998	>10	–	>10	8
Lomachyntsi	NQ22	2010	–	6	–	6
Ustia n. Dniestrem	MP77	2010	–	–	5	8
Subicz n. Dniestrem	MP88	2010	–	–	3	9
Bilche Zolote	MQ10	2010	–	–	5	–
Kryve	MQ27	2010	–	–	>10	–

Table 1, continued

Country & Locality	UTM	Years	CA	C5	C12	C14
Novodnistrovsk	NP33	2010	–	–	8	–
Zvenihorod	LQ83	2010	–	–	>10	–
Biala Gora ad Zolochiv	LR31	2007	–	–	–	–
Czartowa Góra ad Rohatyn	LQ27	2010, 2011	–	–	–	–
Dzwinogród	CB67	2010	–	–	–	–
Halicz	LQ34	2011	–	–	–	–
Huciska ad Lviv	LR45	2011	–	–	–	–
Kasova Góra ad Bursztyn	LQ25	2011	–	–	–	–
Suraż	FD36	2012	–	–	–	–
Złata Gora ad Brody	LR53	2007	–	–	–	–
Austria						
Edelstahl-Spitzenberg	VQ00	2011	–	–	–	–
Hainburg	XP43	2012	–	–	–	–
Leitha Gebirge	XP10	2012	–	–	–	–
Mannersdorf	XP21	2012	–	–	–	–
Hungary						
Vackisujfalu	CT78	2012	–	–	5	7
Nagyvisnyó	DU53	2012	–	–	7	–
Verpelét	DT49	2012	–	–	5	–
Latorpuszta	DU71	2012	–	–	–	–
Lénárdaróc	DU53	2012	–	–	–	–
Noszvaj	DU60	2012	–	–	–	–
Pénzesgyőr	YN13	2010	–	–	–	–
Topolca	XM88	2010	–	–	–	–
Velence	CT23	2010	–	–	–	–

many and at least one of them refers to artificially settled populations on *Asparagus* plantations (Schmitt & Rönn 2011). In Poland, *C. quinquepunctata* was recorded in the 19th century and at the beginning of the 20th century (Borowiec *et al.* 2011), but it has not been found since then. It raises the possibility that its populations, especially in the north of the Carpathians and the Alps are presently extinct. Schmitt and Rönn (2011) reported about 20 localities for this species in the Pannonian Basin. However, these data are from a broad time-span (spanning the 19th and 20th century), so the present distribution of *C. quinquepunctata* in the south-west of the Carpathians is poorly known. In Ukraine this species was found only around the Black Sea (e.g. in Crimea), whereas in central and western part of the country (e.g. in Podolian Upland) it was found only in a single locality in the Dniester valley (Kubisz D., unpublished).

Crioceris quinquepunctata is strongly associated with the steppe and similar dry grasslands in Central and Eastern Europe. Despite having

abundantly host plants (*Asparagus* spp.), the species has only been collected from specific dry and warm localities, mainly the steppes, except for a single locality in Germany (Schmitt & Rönn 2011). These habitats probably originated in the Pleistocene steppe-tundra—an environment comprised of dry grasslands and wet bogs (Nehring 1890).

Currently, steppes are distributed in the continental zones of Eastern Europe (Pontic steppes), the Pannonian Basin and the Iberian Peninsula; however, they have been highly fragmented. The steppes are presently restricted to “warm-stage” refugia (Stewart *et al.* 2010). These three natural steppe areas in Europe are highly isolated from each other by mountains, forests and anthropogenic barriers.

Steppes contain plant communities rich in rare and endemic species of plants and animals (mainly insects). Despite the protection of steppe-like habitats within the framework of Natura 2000 network and many nature reserves, these habitats are still seriously threatened. In many lo-

calities, populations of steppe species of plants and animals have been lost, and many other are on the brink of extinction (Michalik & Zarzycki 1995).

The main threats include natural succession, leading to sites being overgrown by common plant species nonspecific to steppes, and changes in land use, with many natural steppe plant communities being replaced by farmlands, orchards or wood plantations. Moreover, steppes are extra-zonal elements in Central Europe; also, they are highly susceptible to natural and man-made climatic and environmental changes. All these threats lead to fragmentation and isolation of steppe areas. For these reasons, steppes require active protection, such as regular cutting and removal of plants (especially shrubs and trees) or grazing by sheep or goats.

Despite having many suitable habitats *C. quinquepunctata* populations in Central and Eastern Europe are strongly isolated, with the Carpathian range forming a distinctive barrier between the north-eastern and south-western populations (Fig. 1). Previous genetic studies of similarly distributed species, e.g. leaf-beetles *Crioceris quatuordecimpunctata* (Scopoli), *Cheilotoma musciformis* (Goeze), weevil *Centricnemus leucogrammus* (Germar) (Curculionidae) and buprestid *Coraebus elatus* (Fabricius) (Buprestidae), reveal clear differences between populations from the southern and northern arch of the Carpathian mountains (Kajtoch & Lachowska-Cierlik 2009, Kajtoch 2011, Kubisz *et al.* 2012a,b, Kajtoch *et al.* 2013a,b). Apart from molecular analyses based on DNA markers isolated from beetle genomes, a helpful tool in estimation of population differentiation could also be genotyping of endosymbionts or parasites. Notably, genotyping of the bacterium *Wolbachia* was recently used as an additional marker for genetic population studies of insects, e.g. for steppe species *Crioceris quatuordecimpunctata* and *Polydrusus inustus* Germar (Kajtoch *et al.* 2012, Kubisz *et al.* 2012a).

Our study seeks to determine current distribution of *C. quinquepunctata* in Central East Europe, notably in the Carpathian Basin and its surroundings. Moreover, we aim to verify whether genetic differences exist between populations of *C. quinquepunctata* currently localized in two

areas, namely in Ukraine and Pannonia (Moravia). We used also endosymbiotic bacterium *Wolbachia* genotyping as an additional marker for estimation of population differentiation.

2. Material and methods

2.1. Sampling

There is no detailed information on the present distribution of *C. quinquepunctata* in Ukraine, it is absent in Poland (Mazur & Kubisz 2013), and there is only one map of the distribution of the species in the Pannonian Basin (Schmitt & Rönn 2011). However, this map is based on data from a broad time-span (the 19th and 20th centuries). Consequently, current distribution of this species is poorly known in Central and Eastern Europe.

Specimens of *Crioceris* were sought during several field trips in Central East Europe in 1993–2013 (Table 1). In the course of this research, we investigated a few dozen steppe and dry grassland sites, where this species was detected in the past, as well as in many other steppe localities which potentially could sustain their populations (Table 1). We paid special attention to searching beetles in *Asparagus* plants (checking all found plants). All field trips were performed during the middle of the growing season (15 May–15 July) and in good weather conditions. Beetles were caught using sweep-nets and by hand from their host plants.

We used the same number of specimens (5) from each of the two known populations of *C. quinquepunctata* for further genetic analyses. The rest of the available specimens were left for museum collections, because DNA isolation damaged bodies of these beetles. This is because we isolated DNA from the whole specimens as only this procedure allows one to sequence *Wolbachia* genes (as well as host-plant barcodes from insect hemolymph, which are not presented in this study). The specimens were first preserved in 99% ethanol and afterwards stored at –22°C.

All specimens, also the slightly damaged by DNA isolation, were deposited at the Department of Collections, Institute of Systematics and Evolution of Animals, Polish Academy of Sciences in Cracow, Poland.

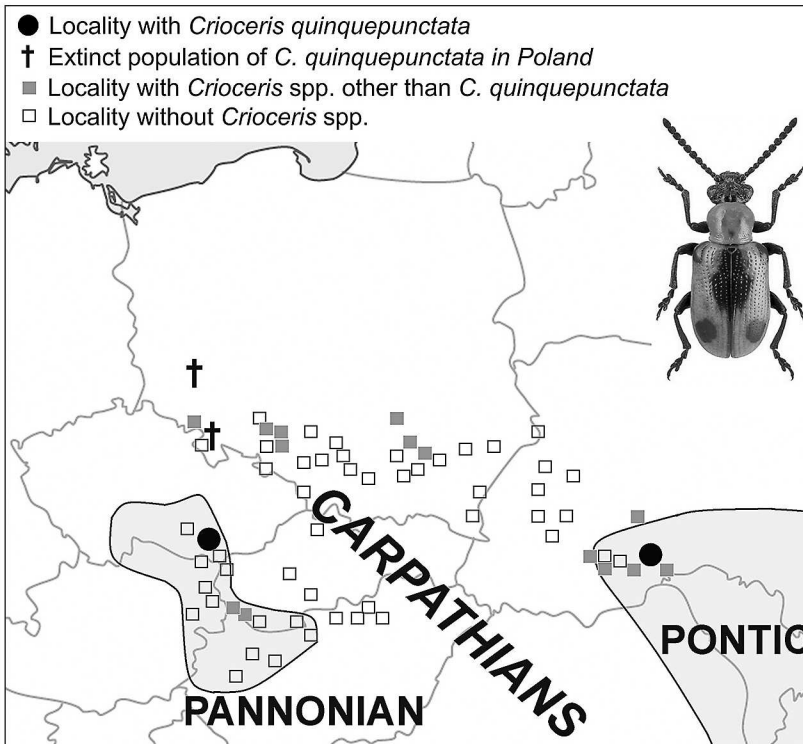


Fig. 1. *Crioceris quinquepunctata* and other *Crioceris* spp. in Central East Europe. Picture of *C. quinquepunctata* provided kindly by Professor L. Borowiec (Iconographia Coleopterorum Poloniae: <http://coleoptera.ksib.pl/kfp/search.php?img=38958>).

2.2. Laboratory procedure

Two DNA markers were used for population studies of *C. quinquepunctata*: Cytochrome Oxidase I (COI) mitochondrial gene and Internal Transcribed Spacer of nuclear rDNA (ITS1). Additionally, two DNA markers were used to detect the presence of *Wolbachia*: cell division protein gene (*ftsZ*) and hybrid cluster protein gene (*hcpA*) give reference for these. *Wolbachia* was used here as an additional, unlinked genetic marker for population genetic studies of its host (like in Kajtoch et al. 2012 and Kubisz et al. 2012a,b). All laboratory procedures were carried out exactly like in the studies of *Crioceris* barcoding and *C. quatuordecimpunctata* population genetics (Kubisz et al. 2012a,b). All newly obtained sequences were deposited in GenBank (Accession nos. KC123330–KC123344).

2.3. Data analysis

We used standard method which allow for comparison of genetic diversity and differentiation between studied populations. Pairwise genetic

distances were calculated using the Kimura 2 parameter model (K2P). Haplotype network preparation and *Wolbachia* strain identification were carried out as in the study of *C. quatuordecimpunctata* (Kubisz et al. 2012a) using the following programs: DnaSP v.5 (Librado & Rozas 2009), MEGA v.5 (Tamura et al. 2011), TCS 1.21 (Clement et al. 2000) and BLASTn algorithm (Altschul et al. 1990).

Phylogenetic analyses were omitted in this work as the phylogeny of *Crioceris* species (among others, the relationship of *C. quinquepunctata* with other European congeners) was the topic of a previous research by Kubisz et al. (2012b) and the phylogeny of *Wolbachia* within infected *Crioceris* species was presented by Kubisz et al. (2012a).

3. Results

3.1. *Crioceris quinquepunctata* distribution

We caught several dozen *Crioceris* beetles representing four species, mostly *C. duodecim-*

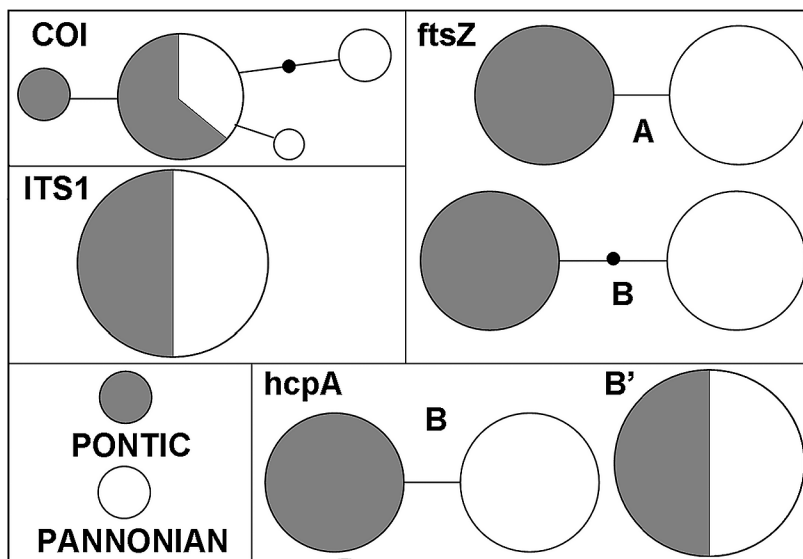


Fig. 2. Haplotype networks of *C. quinquepunctata* populations (Pontic and Pannonian) drawn for COI and ITS1 beetle markers and for *ftsZ* and *hcpA* *Wolbachia* genes. Size of the circle corresponds to number of specimens sharing particular haplotype. Number of circles in particular genetic markers corresponds to number of haplotypes found in studied populations in those markers. Small black dots indicate haplotypes missing in sampled individuals (these are intermediate haplotypes joining haplotypes found in sampled individuals). A, B and B' – names of *Wolbachia* supergroups to which identified gene variants belong.

punctata (Linnaeus) and *C. asparagi* (Linnaeus), sometimes also *C. quatuordecimpunctata*; all localities of particular species are presented in Table 1. On the other hand, we managed to find *C. quinquepunctata* in only two localities, one situated to the north-east of the Carpathian range in the Podolian Upland (south-western Ukraine–Pontic steppes; 48°35'10.14"N and 27°08'2.39"E, 6 specimens) in 2010, and the other found to the south-west of these mountains in Moravia (the Czech Republic–Pannonian steppes; 48°47'36.80"N and 16°41'40.20"E, 7 specimens) in 2012 (Fig. 1). We did not find the species in other localities in western Ukraine, Slovakia, Austria and Hungary, although we found other *Crioceris* species there (Table 1). We also tried to find *C. quinquepunctata* in south-western and south-eastern Poland, where the species was collected over 100 years ago, and in other areas with suitable habitats. However, these populations now appear to be extinct (SW Poland) or they have never been present there (SE Poland).

3.2. Genetics of *Crioceris quinquepunctata* populations

No indels were detected in the 788 bp of the COI gene and in the 606 bp of ITS1 gene.

ITS1 sequences were identical in all the individuals from the both regions while COI sequence from the Pannonian and Pontic individuals differed by 0.0–0.4%. COI sequences for individuals within the Pannonian population differed by 0.0–0.1% and within the Pontic population by 0.0–0.1%. Three variable sites in the COI gene fragment were found, two parsimony informative were identified within the Pannonian population (haplotype diversity $h = 0.800 (\pm 0.164 \text{ SD})$; nucleotide diversity $\pi = 0.00183 (\pm 0.00129 \text{ SD})$), while the Pontic population had only one variable and parsimony informative site ($h = 0.600 (\pm 0.175 \text{ SD})$; $\pi = 0.00076 (\pm 0.00061 \text{ SD})$). As ITS1 was monomorphic in both populations, there was only one haplotype in this marker.

The most common haplotype of COI was found in two Pannonian and three Pontic individ-

uals. Two other haplotypes were found in the Pannonian population and one in Pontic. As ITS1 was monomorphic in both populations, there was only one haplotype in this marker (Fig. 2).

3.3. *Wolbachia* screening

No indels were observed in 472 bp of the *ftsZ* gene and in 453 bp of the *hcpA* gene.

All specimens from both *C. quinquepunctata* populations were found positive for infection by *Wolbachia* (Fig. 2). Two *Wolbachia* strains were found in both populations and most individuals had double infections (all specimens from the Pontic population and four out of five from the Pannonian population). *Wolbachia* strains from the Pannonian and Pontic populations had similar but not identical *ftsZ* sequences of two distinct types, one differing by one mutation and another by two mutations (substitutions). *Wolbachia* strains had identical one type of *hcpA* sequences in both populations and another *hcpA* type differing by one mutation (substitution) between the Pannonian and Pontic populations. According to *ftsZ*, one of the *Wolbachia* strains belonged to supergroup A and the other to supergroup B. However, for the *hcpA* marker this was not so clear, as one strain belonged to supergroup B while the other seemed to be an intermediate between supergroups A and B, but rather closer to supergroup B (called B').

4. Discussion

Presently *Crioceris quinquepunctata* is an extremely rare species known only from few populations in the Pannonian and Pontic refugias (Fig. 1). Its distribution is probably even more restricted than it can be deduced from the Fauna Europaea web-site (<http://www.faunaeur.org>) and Schmitt and Rönn (2011). In many historical localities, the species has not been found for many years, despite intensive search, and it is probably extinct there (Poland, Slovakia). In other Pannonian countries, it is probably very rare species and in at least half of the localities specified by Schmitt and Rönn (2011) in the Czech Republic, Austria and Hungary it is pres-

ently extinct. In western Ukraine, it is still found in only one locality in the Dniester valley. This information strongly suggests that this species should be treated as vulnerable or endangered in all Central European countries, and it is possible that in some areas it is extinct. Moreover, the species should be included in the Red List of animals threatened with extinction developed in Slovakia, Austria and Hungary – it is included in the Red List of the Czech Republic as endangered; it is critically endangered in Germany; in Poland, it is labelled as probably extinct (Binot *et al.* 1998, Pawłowski *et al.* 2002, Farkač *et al.* 2005). Finally, this species, similar to other steppe leaf-beetles (e.g. *Cheilotoma musciformis* (Goeze), Kajtoch *et al.* 2013b), should be added to the Habitat Directive of the European Union and Special Areas of Conservation should be designated for its conservation.

The two found populations are approximately 700 km distant from each other in a straight line, approx. 800 km distant around the northern Carpathians and approx. 1,300 km distant around the southern Carpathians. They are separated by unfavorable mountainous environments in the Carpathians (high altitude and the lack of both suitable habitats and host plants). In spite of that isolation and the rarity of the species, there are no considerable genetic differences between the populations found on different sides of the Carpathians. In both populations, specimens with the same mtDNA haplotypes were found (four from the Pontic area and two from the Pannonian area), and there is only limited evidence for correlation of mtDNA variation with the geographic localization of the samples: one haplotype from one individual is unique to the Pontic population and two haplotypes from three individuals are unique to the Pannonian population; however, these haplotypes differ by only 1–2 mutations from the most common and widespread haplotype. Variability within nuclear DNA was not detected, which may be due to either lower polymorphism of the ITS1 marker, which may not have had enough time to accumulate mutations, or its homogenization (ITSs are tandemly repeated units belonging to the family of rDNA genes and undergo concerted evolution, Nei & Rooney 2005). The same pattern (the lack of differences in ITS1 despite COI variability) was found in *C.*

quatuordecimpunctata (Kubisz *et al.* 2012a). Furthermore, the lack of genetic isolation between the Pontic and the Pannonian populations of *C. quinquepunctata* is also corroborated by small differences between the *Wolbachia* strains. It is worth noting that *Wolbachia* strains commonly vary across populations found in different regions (e.g., in *C. quatuordecimpunctata*, Kubisz *et al.* 2012a). However, they are uniform in recently expanded species (e.g. weevil *Polydrusus inustus* (Curculionidae), Kajtoch *et al.* 2012). Highly similar strains in populations located over 700 km apart suggest that the bacteria and the mitochondrial genomes were inherited in the course of recent sweeps by each strain, each from a common source.

Interestingly, the results differ considerably from those reported in previous studies on a closely related species, *C. quatuordecimpunctata* (Kubisz *et al.* 2012a,b). This species has the same habitat, food preferences and range (they are also divided by the Carpathians), but in spite of that our study revealed distinctive differences in the mitochondrial COI and ITS1 markers as well as in the *Wolbachia* genes in the different parts of its range. All these populations have been strongly isolated for a long time and it is likely that the *Wolbachia* sweeps occurred long time ago. However, the situation of *C. quinquepunctata* is quite different despite similar habitats, the same host plants and the general distribution, the latter being more compact as it consists of only two isolated areas.

Generally, very low genetic variability of the beetle and its *Wolbachia* within and among populations is unusual. Other studied species of steppe beetles (*Crioceris quatuordecimpunctata*, *Cheilotoma musciformis*, *Centricnemus leucogrammus*, *Coraebus elatus*) have high interpopulation genetic diversity (Kajtoch 2011, Kubisz *et al.* 2012a, Kajtoch *et al.*, 2013a,b). For these species, different phylogenetic lineages were found in the north, east and south-west from the Carpathians and this pattern is common to all species studied so far.

The example of *C. quinquepunctata* shows that population genetics of steppe leaf-beetles is highly diversified and that the history and current population structure of studied species may differ from what we have known so far. That is why fur-

ther research on population genetics and molecular ecology of other steppe insects and other invertebrates is required in the future.

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