Genetic implications of a biological invasion: Chromosomal and DNA barcode monomorphism in Old World populations of Colorado potato beetle *Leptinotarsa decemlineata* (Coleoptera: Chrysomelidae)

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Abstract. Once introduced into new area, invasive species can be expected to have low genetic diversity due to the founder effect. Here we tested this prediction using cytogenetic and molecular analysis of Armenian and Belarusian populations of Colorado potato beetle *Leptinotarsa decemlineata* (Say, 1824) and by comparing the results with those of native (North America) and those introduced into Europe. This revealed that the karyotype of males from Armenia and Belarus is remarkably conserved with 2n = 35 (34 + X0), n = 17Aa + X0; and includes a pair of large acrocentric chromosomes. Thus, these populations belong to the so-called acrocentric chromosome race of the Colorado potato beetle. At diakinesis there are clearly visible argentophilic signals, probably NORs (the nucleolus organizer regions) present on some autosomal bivalents, while the X chromosome was homogenously argentophilic during different stages of meiosis. C-banding revealed a small amount of constitutive heterochromatin weakly visible in the pericentric regions of some chromosomes. Analysis of the DNA-barcode fragment of the gene *cytochrome c oxidase subunit I* (COI) revealed a single haplotype (we call it “the European haplotype”) and lack of inter-population variability in all the samples collected from different locations in Armenia and Belarus. The comparison of our karyological and molecular data with that available in the literature and GenBank shows that all the populations studied from the Old World are monomorphic with respect to karyotype and the mitochondrial DNA-barcode. We assume that (1) the presence of acrocentric chromosomes in the karyotype and (2) the European haplotype of mitochondrial genome are the ancestral states for all populations in the Old World and inherited from the New World invaders who colonized Europe 100 years ago. New World populations are polymorphic with respect to karyotype and mitochondrial genes; however, the European haplotype has not yet been found in America. We believe that in the future it will be found in North America, which will shed light on the origin of populations of this dangerous pest in Eurasia.

INTRODUCTION

Colorado potato beetle *Leptinotarsa decemlineata* (Say, 1824) is one of the best known invasive insect species. Despite significant progress in plant protection, Colorado potato beetle (CPB) is still the main pest of potato (*Solanum tuberosum*) worldwide. However, the beetles may damage tomatoes (*Solanum lycopersicum*), eggplant (*Solanum melongena*) and tobacco (*Nicotiana tabacum*), as well as feed and survive on the other plants of the family Solanaceae (Alyokhin, 2009; Liu et al., 2012).

CPB spread across the United States and Canada during the second half of the 19th century and then invaded Europe at the beginning of the 20th century (France, Bordeaux) and is currently present almost throughout the European continent as far as China in Asia (De Wilde & Hsiao, 1981; Jolivet, 1991; Capinera, 2001; Udalov & Benkovskaya, 2011). Within the former Soviet Union, this species was first recorded in Byelorussian SSR in 1947 (Markovets, 1950) and in Armenian SSR – in 1976 (Nalbandyan, 1984). CPB is highly fecund, highly intraspecifically polymorphic, ecologically plastic and adaptable, and quick to develop resistance to insecticides, which enables it to successfully adapt to different environmental factors (Usatinskaya, 1981; Udalov & Benkovskaya, 2011).

CPB is well studied with respect to morphology, phenotypic structure, life-cycle, ecology, distribution and behaviour (for references, see Fasulati, 1993; Boiteau, 1994;
Udalov & Benkovskaya, 2011; Brechko et al., 2016). However, there are not many cytogenetic and molecular studies on *L. decemlineata*.

The karyotype of *L. decemlineata* (as *Dorcyphora decemlineata*) was first described by Stevens (1906) and the male chromosome number was misinterpreted as 2n = 36 (34 + XY). Further karyological studies showed that the diploid chromosome number of this species is 2n = 35 (34 + X0) in the male and 2n = 36 (34 + XX) in the female, with a large submetacentric X chromosome and mostly submetacentric autosomes (Guénin & Scherler, 1951; Smith, 1953; Hsiao & Hsiao, 1982, 1983; Hsiao, 1985; Petitpierre et al., 1988).

More recent studies report the first data obtained using C-banding staining and in situ restriction enzyme digestion combined with the analysis of repetitive DNA for studying the organization of heterochromatin in chromosomes of *L. decemlineata* (Baus Lončar et al., 2005). Repetitive DNA in nuclear DNA of CPB and pericentromerically located small heterochromatic blocks on all chromosomes in its karyotype. The chromosome location of two different satellite-DNA families on mitotic and meiotic chromosomes of *L. decemlineata* was studied by fluorescence in situ hybridization using LEDE-I and LEDE-II satellite DNAs as probes (Lorite et al., 2013). Positive hybridization signals in the pericentromeric region on some chromosomes, including X chromosome, is recorded.

There are several studies on CPB populations that used molecular markers (Jacobson & Hsiao, 1983; Azeredo-Espin et al., 1991, 1996; Zehnder et al., 1992; Sidorenko et al., 2000; Hawthorne, 2001; Sidorenko & Berezovska, 2002; Grapputo et al., 2005; Grapputo, 2006; Lorite et al., 2013; Zhang et al., 2013; Przybylska et al., 2014; Izzo et al., 2018; Yang et al., 2020), including analyses based on sequencing the whole genome (Crossley et al., 2017; Zhang et al., 2013; Grapputo et al., 2013). More recent studies report the first data obtained using C-banding staining and in situ restriction enzyme digestion combined with the analysis of repetitive DNA for studying the organization of heterochromatin in chromosomes of *L. decemlineata* (Baus Lončar et al., 2005). Repetitive DNA in nuclear DNA of CPB and pericentromerically located small heterochromatic blocks on all chromosomes in its karyotype. The chromosome location of two different satellite-DNA families on mitotic and meiotic chromosomes of *L. decemlineata* was studied by fluorescence in situ hybridization using LEDE-I and LEDE-II satellite DNAs as probes (Lorite et al., 2013). Positive hybridization signals in the pericentromeric region on some chromosomes, including X chromosome, is recorded.

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**MATERIAL AND METHODS**

**Material and sampling**

Adults of *Leptinotarsa decemlineata* were collected in Armenia and Belarus in 2019 (Table 1) on *Solanum tuberosum*. For the karyological study male and female abdomens were dissected, immersed in 0.9% sodium citrate solution at room temperature for 40 min. Then the gonads were fixed in 3:1 fixative (96% ethanol: glacial acetic acid). The remaining bodies of the same specimens were fixed in 96% ethanol for DNA study. The fixed samples were frozen and stored at −20°C until processed.

**Preparation of karyological slides**

The dissected gonads were placed on slides in a drop of 70% acetic acid. Squashed chromosomal preparations were obtained using the dry ice quick-freezing technique (Conger & Fairchild, 1953). Ag-banding was done according to the method proposed by Howell & Black (1980), with minor modifications. The slides were exposed to hydrolysis in 2N formic acid for 10 min, rinsed in running water and dried. Then 4–5 drops of 50% aqueous silver nitrate (AgNO₃) solution and 2 drops of colloidal developer solution (0.2 g gelatin, 10 ml distilled water and 0.1 ml

| NN | Locality | Date of collection | Karyologically examined specimens | DNA examined specimens |
|----|----------|--------------------|----------------------------------|-----------------------|
| 1  | Armenia, Arnavir Prov. env. Arshaluys vill. | 13.06.2019 | 15, 19, 25 | 1 |
| 2  | Armenia, Kotayk Prov., env. Aghavnavdzor vill. | 18.07.2019 | 3, 5, 19 | 1 |
| 3  | Armenia, Lori Prov. env. Hartagugy vill. | 6.08.2019 | 4, 5, 19 | 1 |
| 4  | Armenia, Gegharkunik Prov., env. Chambarak vill. | 11.07.2019 | 4, 5, 19 | 1 |
| 5  | Belarus, Vitebsk Reg., Vitebsk District, Pushkari vill. | 30.07.2019 | 2, 5, 19 | 1 |
| 6  | Belarus, Vitebsk Reg., Polotsk District, env. Polotsk city | 31.07.2019 | 2, 5, 19 | 1 |
| 7  | Belarus, Grodno Reg., Ashmiany District, Zhupraniv vill. | 10.08.2019 | 3, 5, 19 | 1 |
| 8  | Belarus, Gomel Reg., Svetlahorsk District, Rakshin vill. | 19.07.2019 | 1, 5, 19 | 1 |
| 9  | Belarus, Vitsebsk Reg., Orsha District, Krapivno vill. | 29.07.2019 | 3, 5, 19 | 1 |
| 10 | Belarus, Vitsebsk Reg., Vitsebsk District, env. Tulovo town | 30.07.2019 | 1, 5, 19 | 1 |
| 11 | Belarus, Brest Reg., Zhabinka District, env. Zhabinka city | 8.07.2019 | 1, 5, 19 | 1 |
| 12 | Belarus, Gomel Reg., Kalinkavichy District, env. Kalinkavichy town | 17.07.2019 | 1, 5, 19 | 1 |
| 13 | Belarus, Brest Reg., Luninets District, env. Luninets town | 14.07.2019 | 3, 5, 19 | 1 |
| 14 | Belarus, Mogilev Reg., Mogilev District; Kuty vill. | 23.07.2019 | 3, 5, 19 | 1 |
| 15 | Belarus, Mogilev Reg., Babruysk District, Telusha vill. | 24.07.2019 | 3, 5, 19 | 1 |
| 16 | Belarus, Minsk Reg., Minsk District, Schomylitsa vill. | 3.06.2019 | 4 | 1 |

Table 1. *Leptinotarsa decemlineata* used in the chromosome and DNA-barcode analyses.
Table 2. Specimens of the *Leptinotarsa* used in the DNA-barcode analysis.

| Species         | Specimen voucher | GenBank accession no. | Country                  | Locality                  | Reference                      |
|-----------------|------------------|-----------------------|--------------------------|---------------------------|--------------------------------|
| *L. decemlineata* | SCZHE-19-001     | MW346685              | Armenia                  | Aramiv Prov., env. Arshaluys vill | This study                     |
| *L. decemlineata* | SCZHE-19-002     | MW346681              | Armenia                  | Kotayk Prov., env. Aghavnadzor vill | This study                     |
| *L. decemlineata* | SCZHE-19-003     | MW346686              | Armenia                  | Lori Prov., env. Hartayugh vill | This study                     |
| *L. decemlineata* | SCZHE-19-004     | MW346683              | Armenia                  | Ghegharunik Prov., env. Chambarak vill | This study                     |
| *L. decemlineata* | SCZHE-19-127     | MW348764              | Belarus                  | Vitebsk Reg., Vitebsk District, Pushkari vill | This study                     |
| *L. decemlineata* | SCZHE-19-141     | MW346684              | Belarus                  | Vitebsk Reg., Polotsk District, env. Polotsk city | This study                     |
| *L. decemlineata* | SCZHE-19-140     | MW346682              | Belarus                  | Gredno Reg., Ashtamny District, Zhuprany vill | This study                     |
| *L. decemlineata* | SCZHE-19-097     | MW348765              | Belarus                  | Gomel Reg., Svetlahorsk District, Rakshin vill | This study                     |
| *L. decemlineata* | SCZHE-19-101     | MW348764              | Belarus                  | Vitebsk Reg., Orsha District, Krapivno vill | This study                     |
| *L. decemlineata* | SCZHE-19-127     | MW346685              | Belarus                  | Gomel Reg., Svetlahorsk District, Rakshin vill | This study                     |
| *L. decemlineata* | SCZHE-19-141     | MW346684              | Belarus                  | Vitebsk Reg., Polotsk District, env. Polotsk city | This study                     |
| *L. decemlineata* | ZFMK-TIS-2000692 | KU909921              | Poland                   | Woiwodschaft Westpommern, Powiat Slawienski, Landg, Campingplatz Wicie | Rulik et al., 2017 |
| *L. decemlineata* | ZFMK-TIS-2000691 | KU914685              | Poland                   | Woiwodschaft Westpommern, Powiat Slawienski, Landg, Campingplatz Wicie | Rulik et al., 2017 |
| *L. decemlineata* | ZFMK-TIS-20003262 | KU911127              | Germany                  | Saxony-Anhalt, Halberstadt, Landkreis Harz, Ahlenstedt | Rulik et al., 2017 |
| *L. decemlineata* | ZFMK-TIS-2000692 | KU910921              | Poland                   | Woiwodschaft Westpommern, Powiat Slawienski, Landg, Campingplatz Wicie | Rulik et al., 2017 |
| *L. decemlineata* | GBOL_Col_FK_4806 | KM439182              | Germany                  | North Rhine-Westphalia, Niederrheinische Bucht, Rhein-Sieg, Bornheim-Hemmerich, Ortslage | Hendrich et al., 2015 |
| *L. decemlineata* | GBOL_Col_FK_8725 | KM439249              | Austria                  | Burgenland, Neusiedlersee, Neusied am See, Illmitz, Biologische Station | Hendrich et al., 2015 |
| *L. decemlineata* | GBOL_Col_FK_4806 | KM439182              | Germany                  | North Rhine-Westphalia, Niederrheinische Bucht, Rhein-Sieg, Bornheim-Hemmerich, Ortslage | Hendrich et al., 2015 |
| *L. decemlineata* | BCZSM COL 02288  | JF88843               | Germany                  | North Rhine-Westphalia, Niederrheinische Bucht, Rhein-Sieg, Bornheim-Hemmerich, Ortslage | GenBank, iBOL |
| *L. decemlineata* | O.V.Sukhorukova 296 (LBIA) | DQ649100              | Russia                   | Bashkortostan, Belorecky, Mezhgorie | Udalov & Benkovskaya, 2010 |
| *L. decemlineata* | O.V.Sukhorukova 306 (LBIA) | DQ649101              | Russia                   | Bashkortostan, Belorecky, Mezhgorie | Udalov & Benkovskaya, 2010 |
| *L. decemlineata* | M.B. Udalov 23 (LBIA) | DQ649098              | Russia                   | Bashkortostan, Ufimskiy, Dmitrievka | Udalov & Benkovskaya, 2010 |
| *L. decemlineata* | M.B. Udalov 607 (LBIA) | DQ649097              | Russia                   | Bashkortostan, Fedorovsky, Dedovo | Udalov & Benkovskaya, 2010 |
| *L. decemlineata* | M.B. Udalov 379 (LBIA) | DQ649096              | Russia                   | Bashkortostan, Fedorovsky, Dedovo | Udalov & Benkovskaya, 2010 |
| *L. decemlineata* | M.B. Udalov 14 (LBIA) | DQ649099              | Russia                   | Bashkortostan, Ufimskiy, Dmitrievka | Udalov & Benkovskaya, 2010 |
| *L. decemlineata* | R.A. Ilyasov 332 (LBIA) | DQ649095              | Russia                   | Bashkortostan, Tatyshlinsky, Schulganovo | Udalov & Benkovskaya, 2010 |
| *L. decemlineata* | R.A. Ilyasov 355 (LBIA) | DQ649094              | Russia                   | Bashkortostan, Tatyshlinsky, Schulganovo | Udalov & Benkovskaya, 2010 |
| *L. decemlineata* | M.B. Udalov 421 (LBIA) | DQ127909              | Russia                   | Bashkortostan, Fedorovsky, Dedovo | Udalov & Benkovskaya, 2010 |
| *L. decemlineata* | O.V. Sukhorukova 305 (LBIA) | DQ127907              | Russia                   | Bashkortostan, Belorecky, Mezhgorie | Udalov & Benkovskaya, 2010 |
| *L. decemlineata* | Yu.M.Nikonorov 923 (LBIA) | DQ011111              | Russia                   | Bashkortostan, Myakinsky, Kirgiz-Miyaki | Udalov & Benkovskaya, 2010 |
| *L. decemlineata* | M.B. Udalov 363 (LBIA) | DQ127908              | Russia                   | Bashkortostan, Tatyshlinsky, Schulganovo | Udalov & Benkovskaya, 2010 |
| *L. decemlineata* | BCZSM COL 02288  | JF88843               | Germany                  | North Rhine-Westphalia, Niederrheinische Bucht, Rhein-Sieg, Bornheim-Hemmerich, Ortslage | Hendrich et al., 2015 |

The slides were treated for 1–3 min in 0.2 N HCl at room temperature, then rinsed with distilled water. The slides were covered with a coverslip and incubated on a hotplate for 3–4 min at 60°C in a moist chamber (warmed beforehand). The slides were dried after rinsing in distilled water. C-ending was revealed using the protocol of Rozeck (2000). The slides were treated for 1–3 min in 0.2 N HCl at room temperature then rinsed in distilled water. Thereafter, the slides were placed in 5% Ba(OH)2 solution at 20°C for approximately 4 min, then rinsed with distilled water. Then the slides were incubated in 2× SSC solution (0.3 M sodium chloride containing 0.03 M trisodium citrate) at 60°C for 1 h. After rinsing in distilled water, the slides were dried and stained using 4% Glemsa solution in phosphate buffer (pH 6.8) for 8 min.
DNA extraction, PCR amplification and sequencing

Total DNA was extracted from the wing muscle, using the Qia-gen DNeasy Blood & Tissue Kit (QIAGEN, Hilden, Germany) following the protocol for Animal Tissue.

Amplification of a fragment of the COI gene was done using PCR and the following pair of primers (Folmer et al., 1994): LCO1490 5'-GGTCAACAAATCATAAAGATATTGG-3' and HCO2198 5'-TAAACTTCAGGGTGACCAAAAAATCA-3'.

PCR was performed in a 25 μl reaction volume using 2.5 μM MgCl₂, 1.2 μl BSA (concentration 1 mg/ml), 0.8 μM GeneAmp dNTPs Mix (Applied Biosystems, Beverly, MA, USA), 0.6 μM of each primer, 2.5 μl 1 × PCR buffer II (Applied Biosystems), 1 U of ABI AmpliTaq DNA Polymerase (Applied Biosystems) and 3 μl of the respective genomic DNA extract.

PCR conditions for COI amplification were as follows: initial denaturation period of 2 min at 94°C was followed by 30 cycles of 1 min at 94°C, annealing for 30 s at 45°C and extension for 1 min 30 s at 72°C, with a final extension step of 10 min at 72°C.

PCR products were purified with ExoStar (GE Healthcare, Little Chalfont, UK) in accordance with the manufacturer’s manual and sequenced in both directions externally by StarSEQ GmbH (Mainz, Germany).

DNA extraction, PCR amplification, gel electrophoresis, PCR products purification were carried out in the DNA laboratory of the Natural History Museum, University of Oslo.
All sequences obtained in this study were submitted to GenBank (accession numbers MW346681–MW346686 and MW348764–MW348766). Their accession numbers and specimen vouchers are presented in Table 2. The voucher specimens and all DNA extracts were deposited in the Scientific Center of Zoology and Hydroecology, NAS RA.

Samples and sequence alignment

Nucleotide sequences obtained in this study were edited and aligned using BioEdit software (Hall, 1999). 27 additional DNA barcodes of *L. decemlineata* (3 from USA, 1 from Canada, 2 from Austria, 6 from Germany, 2 from Poland and 13 from Bashkortostan, Russia) were obtained from GenBank and added to the alignment. We edited the GenBank sequence JF889843 (Germany) by extracting its terminal part that was not properly aligned. DNA barcodes of *L. haldemani*, *L. juncta* and *L. texana* (11 samples) were also obtained from GenBank and added to the alignment as outgroups to root the trees (Table 2).

An additional alignment was created to study mitochondrial polymorphism in American populations. For this purpose, 82 haplotypes of the mitochondrial genome fragment that included the terminal part of the *COI* gene and the initial part of the *COII* gene were downloaded from GenBank (Crossley et al., 2017). Since this fragment does not overlap the standard DNA barcode, phylogenetic trees for it were built separately. The species *Diabrotica undecimpunctata* was used as an outgroup to root the trees.

Phylogenetic tree construction

The evolution model test for DNA substitutions was performed in MEGA X (Kumar et al., 2018). For the DNA barcode dataset, Tamura-Nei TN93+G was the optimal model. For the fragment that included the terminal part of the *COI* gene and the initial part of the *COII* gene, Tamura three-parameter (T92+G) was the optimal model. Maximum Likelihood phylogenetic trees were constructed for the two datasets using the substitution models found. The standard nonparametric bootstrap (Felsenstein, 1985) (100 replicates) was used to evaluate the statistical nodal support of the trees.

The Bayesian phylogenetic analysis was performed using the program MrBayes v.3.2.7 (Ronquist et al., 2012). Two runs of 10,000,000 generations with four chains (one cold and three heated) were performed for both datasets. The consensus of the obtained trees was visualized using FigTree v.1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/).

RESULTS

Chromosome analysis

Nuclear divisions were found in CPB males of both Armenian and Belarusian populations. In females the karyotype could not be determined with certainty as divisions were rare and because the morphology of the chromosomes was unclear.
Table 3. Nucleotides in four positions on the COI gene fragment differentiating the European DNA barcodes from the four available Nearctic samples.

| Position | Sequence | 166 460 538 556 |
|----------|----------|-----------------|
| L. decemlineata HQ605769 USA | A A T C |              |
| L. decemlineata HQ605768 USA | A A T C |              |
| L. decemlineata XM 023169376 USA | A A T C |              |
| L. decemlineata KU915233 Germany | G G C T |              |
| L. decemlineata MW346681 Armenia | G G C T |              |
| L. decemlineata MW346685 Armenia | G G C T |              |
| L. decemlineata MW346683 Armenia | G G C T |              |
| L. decemlineata MW346686 Armenia | G G C T |              |
| L. decemlineata MW346682 Belarus | G G C T |              |
| L. decemlineata MW348764 Belarus | G G C T |              |
| L. decemlineata MW348768 Belarus | G G C T |              |
| L. decemlineata MW348766 Belarus | G G C T |              |
| L. decemlineata MW348765 Belarus | G G C T |              |
| L. decemlineata KU914658 Poland | G G C T |              |
| L. decemlineata KU911127 Germany | G G C T |              |
| L. decemlineata KU909921 Poland | G G C T |              |
| L. decemlineata KU907193 Germany | G G C T |              |
| L. decemlineata KU349182 Germany | G G C T |              |
| L. decemlineata KU349498 Austria | G G C T |              |
| L. decemlineata KU349249 Germany | G G C T |              |
| L. decemlineata DQ649107 Bashkortostan | G G C T |              |
| L. decemlineata DQ649097 Bashkortostan | G G C T |              |
| L. decemlineata DQ127906 Russia Bashkortostan | G G C T |              |
| L. decemlineata DQ649095 Russia Bashkortostan | G G C T |              |
| L. decemlineata DQ649094 Russia Bashkortostan | G G C T |              |
| L. decemlineata DQ127909 Russia Bashkortostan | G G C T |              |
| L. decemlineata DQ649806 Russia Bashkortostan | G G C T |              |
| L. decemlineata DQ649809 Russia Bashkortostan | G G C T |              |
| L. decemlineata DQ649894 Germany | G G C T |              |
| L. decemlineata DQ127907 Russia Bashkortostan | G C C T |              |
| L. decemlineata DQ011111 Russia Bashkortostan | G C C T |              |
| L. decemlineata DQ127908 Russia Bashkortostan | G C C T |              |
| L. decemlineata KM44811 Austria | G G C T |              |
| L. haldemani HM433598 USA | A A T G |              |
| L. haldemani HM433597 USA | A A T G |              |
| L. haldemani DO450377 USA | A A T G |              |
| L. haldemani HQ984330 USA | A A T G |              |
| L. juncta KC255422 USA | A A T T |              |
| L. juncta HQ605770 USA | A A T T |              |
| L. juncta HQ605771 USA | A A T T |              |
| L. texana HQ605774 USA | A G T T |              |
| L. texana MK288007 South Africa | A G T T |              |
| L. texana MK288008 South Africa | A G T T |              |
| L. texana HQ605775 USA | A G T T |              |

Mitotic divisions were recorded only in beetles from Armenian populations. The male mitotic metaphase displayed 35 chromosomes including 17 autosomal pairs that constitute a decreasing size series and large meta-submetacentric X chromosome (Fig. 1a, b). All large and middle-sized chromosomal pairs were meta- and submetacentric, except for one acrocentric autosomal pair (AA2). The morphology of the small chromosomes was poorly visible, however, most likely, one autosomal pair (AA13) was acrocentric and the others biarmed.

Meiotic spermatocyte divisions were recorded in males from both Armenian and Belarusian populations. At diakinesis/metaphase I (MI) 17 autosomal bivalents and an unpaired meta-submetacentric X chromosome were observed (Figs 1c, d, e; 2a, b, c). The autosomal bivalents gradually decreased in size. At diakinesis and prometaphase there were two or three ring-shaped autosomal bivalents with two chiasmata, two cross-shaped bivalents with an interstitial chiasma and the remaining bivalents were rod-shaped and most likely had one terminal chiasma.

At metaphase II there were 17 and 18 chromosomes of which the majority were biarmed meta- and submetacentric. In each daughter cell, among the large meta- and submetacentric chromosomes one acrocentric chromosome was clearly visible, the morphology of the small chromosomes was unclear (Figs 1f, g; 2d, e).

At meiosis, Ag-banding revealed that the X chromosome was brightly homogenously argentophilic (Figs 1c, d, f; 2a, b, d). In addition, diakinesis Ag-positive signals, probably NORs (the nucleolus organizer regions) were clearly visible on two ring-shaped and one rod-shaped bivalents; moreover, small and weak argentophilic signals were observed on two rod-shaped bivalents (Figs 1c; 2a, b). On the autosomal chromosomes at prometaphase-metaphase I and metaphase II there were no distinct Ag-positive signals.

The C-banding revealed a small amount of constitutive heterochromatin weakly visible in the pericentric regions of some chromosomes that did not form distinct blocks (Figs 1e, 2g; 2c, e). At prometaphase-metaphase I (Figs 1e, 2c), small pericentric block of C-heterochromatin was visible on the unpaired X chromosome, while at metaphase II (Figs 1g, 2e) it was unclear which of the large two-armed chromosomes was the X chromosome.

Thus, the karyotype of the males of Colorado potato beetle from Armenia and Belarus is 2n = 35 (34 + X0), n = 17AA + X0. In beetles from all the populations studied, a pair of large acrocentric chromosomes was present.

Comparison of the sequences and phylogenetic analyses

Comparative analysis of the sequencing results revealed no nucleotide substitutions between samples from four Armenian and five Belarusian populations of L. decemlineata. Moreover, analysis of all available DNA barcodes revealed that this variant of the DNA barcode (herein called “European haplotype”) was present in all the samples from Austria, Germany and Poland and in most (7 out of 13) samples from Bashkortostan (Russia). In 6 out of 13 Bashkortostan samples, few nucleotide substitutions are reported (Udalov & Benkovskaya, 2010); however, in our opinion, it remains unclear whether these substitutions were real or sequencing errors. Examination of the DNA barcode alignment also revealed four transitions differentiating the European samples from the four American sequences (Table 3).

Bayesian Inference (BI) and Maximum Likelihood (ML) phylogenetic analyses of the Leptinotarsa DNA barcodes revealed the topology shown in Fig. 3.

In both BI and ML trees, the sequence HQ605769 from North Dakota, USA appeared as a sister to the clade that included all the European sequences (Fig. 3). Together, the North Dakota sequence and the European sequences
Fig. 3. Bayesian phylogenetic tree based on *Leptinotarsa* DNA barcodes. Maximum likelihood analysis revealed the same topology. The GenBank accession number and country of origin are listed for every sequence. Bayesian posterior probability for BI/Bootstrap value support for ML are indicated at nodes.
Fig. 4. Bayesian phylogenetic tree based on the North American haplotypes of the mitochondrial genome fragment that included the terminal part of the COI gene and initial part of the COII gene. The sequences of the North American *Leptinotarsa decemlineata* samples published by Crossley et al., 2017 and extracted from the GenBank were analysed. For every sequence the GenBank accession number and haplotype number are listed. Bayesian posterior probability for BI/Bootstrap value support for ML are indicated at nodes. Sign “–” at the node support means that the clade was not revealed by the ML analysis.
formed a sister clade to the clade consisting of the other three North American CPB sequences. The samples from three other species of Leptinotarsa (L. haldemani, L. junc-ta and L. texana) formed three separate clades, of which L. junc-ta and L. texana were sister clades, while L. haldem-ani was another separate clade. In addition, the sequence HQ984330 of the specimen mentioned in GenBank as Lep-tinotarsa sp. belonged to the last clade and, therefore, to the same species.

Additional phylogenetic analyses were conducted to study mitochondrial polymorphism in American populations. For this purpose, 82 haplotypes of the mitochondrial genome fragment that included the terminal part of the COI gene and the initial part of the COII gene were used. BI and ML analyses of these haplotypes revealed the topology shown in Fig. 4. In contrast to European samples (Fig. 3), these analyses showed a high level of mitochondrial polymorphism in North American populations and revealed several major haplogroups (Fig. 4), with a divergence level between them of up to 4%.

**DISCUSSION**

There are more than 40 species in the genus Leptinotarsa (Jacques, 1988). Currently, there are published karyotypes for only 15 species of Leptinotarsa (for references, see Petitpierre et al., 1988, who mention 16 species, but based on the karyology of L. signaticollis Jacoby, 1883 published by Wieman, 1910 and Hsiao & Hsiao, 1983, was synonymised with L. undecemlineata (Stål, 1858) (Jacques, 1988)).

The haploid karyotype n♂ = 17AA + X0 is the modal chromosome number for the genus Leptinotarsa and recorded in 13 species (Hsiao & Hsiao, 1983; Petitpierre et al., 1988). Lower chromosome numbers are reported in two species: n♂ = 16AA + X0 in L. undecemlineata (as L. undecimlineata in Virkki, 1964; Hsiao & Hsiao, 1983) and n♂ = 11AA + X0 in L. belti Stål, 1858 (as Polygramma belti in Virkki, 1964). The karyotype with the lowest number of n = 12 could represent the plesiomorphic condition and is ancestral for the subtribe Doryphorina (Petitpierre, 2011).

According to T.H. Hsiao (1985), the Colorado potato beetle is a chromosomally polymorphic species in North America. There are three chromosomal “races”: (1) the metacentric “race”, in which all autosomes are metacentric (Mexico, USA), (2) the acrocentric “race”, derived from the metacentric “race” by the pericentric inversion in the second pair of autosomes (USA, Europe) and (3) the heterozygous meta-acrocentric “race” (USA, Canada). More recent studies revealed a large acrocentric chromosome pair in the chromosomes of a population from Canena in Spain, thus, confirming the presence of the acrocentric “race” of CPB in Europe (Lorite et al., 2013).

Our studies did not reveal chromosomal polymorphism in Armenian and Belarusian populations of CPB. The large autosomal acrocentric pair was found in the karyotypes of all the populations of L. decemlineata studied. Therefore, we assume that both Armenian and Belarusian populations of CPB belong to the acrocentric “race”, which according to literature data, is peculiar to European populations.

Up till now, karyological studies on CPB were carried out using mainly conventional staining techniques. In the present paper, Ag-banding was used to study the karyotype of the Colorado potato beetle for the first time. This revealed that the X chromosome was homogenously argentophilic during the different stages of meiosis in all the populations studied, which is most likely due to the presence of an argentophilic substance (proteins). At diakinesis there were clearly visible argentophilic signals (probably NORs) located on some autosomal bivalents: the two ring-shaped and one rod-shaped bivalents. Weak argentophilic signals were also detected on a few bivalents.

Baus Lončar et al. (2005) report for the first time the C-bandning staining of the chromosomes of L. decemlineata, which is confirmed by our study in which the chromosomes had a small amount of constitutive heterochromatin located pericentromerically. This observation is consistent with the data for other beetles. It is known that in most species of the order Coleoptera large C-blocks on chromosomes are uncommon and only recorded in a few species (for references see Rožek et al., 2004; Karagyan et al., 2012).

In the current study, analysis of the DNA barcode fragment of the COI gene revealed lack of interpopulation variability in all samples of CPB collected from different locations in Armenia and Belarus. Thus, there is only a single DNA barcode haplotype. Moreover, analysis of sequences from Austria, Germany and Poland available in the literature (Hendrich et al., 2015; Rulik et al., 2017) indicate that this DNA barcode haplotype (“European haplotype”) is the only variant known from Western Europe. A more complicated situation is found in Bashkortostan (Russia) where the European haplotype is present in 7 of 13 studied samples (Udalov & Benkovskaya, 2010). In 6 out of 13 Bashkortostan samples, there are few nucleotide substitutions (Udalov & Benkovskaya, 2010); however, in our opinion, it is unclear whether these substitutions are real or sequencing errors. Anyway, the European haplotype is the only or the predominant one in all populations of the Old World studied. Therefore, we hypothesize that the European haplotype is the ancestral state for all populations of the Old World and inherited from the New World invaders who colonized Europe 100 years ago.

In contrast to Europe, the DNA analysis of American samples, both carried out earlier (Izzo et al., 2018) and in the current study, reveal an extremely high level of polymorphism. It should be noted, however, that the analysis of American samples is based on the other fragment of the mitochondrial genome, which makes it difficult to directly compare the American and European data and prevents the phylogeographic analysis of the entire dataset (America + Europe). For this reason, we cannot identify the North American population that was the ancestor of the European lineage of the Colorado potato beetle. Nevertheless, we believe that the population-ancestor will be found in North America in the near future and will shed light on the origin of the populations of this dangerous pest in Eurasia.
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