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ORIGINAL ARTICLE

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

Gayane KARAGYAN[1], Vladimir LUKHTANOV[2], Meri MAZMANYAN[1], Ilona STEPANYAN[1], Tigran GHREJYAN[1], Alena ABAKUMOVA[3] and Oxana NESTEROVA[3]

[1] Scientific Center of Zoology and Hydroecology, NAS RA, P. Sevak 7, Yerevan 0014, Armenia;
e-mails: gaykaragyan@yahoo.com, meriliv33@gmail.com, ilonastepanyan37@gmail.com, tkredjyan@gmail.com

[2] Zoological Institute of Russian Academy of Sciences, Universitetskaya nab. 1, St. Petersburg 199034, Russia;
e-mail: lukhtanov@mail.ru

[3] Belarusian State University, Faculty of Biology, Kurchatova 10, Minsk 220045, Belarus; e-mails: helen.a.2013@mail.ru, oxanesterova@gmail.com

Key words. Coleoptera, Chrysomelidae, *Leptinotarsa decemlineata*, Colorado potato beetle, invasion, Old World, chromosome, *COI*, DNA barcoding, taxonomy

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 pericentromeric 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 Eurasian 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 (Ushatinskaya, 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 *Doryphora 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; Cohen et al., 2021). These methods are promising for the investigation of intra- and interpopulation polymorphism, as well as migration routes and microevolutionary processes accompanying the formation of the current CPB range (Udalov & Benkovskaya, 2011). Nonetheless, currently there is little data on mitochondrial DNA barcodes, the standardized gene regions (658 bp long, 5' segment of mitochondrial *cytochrome oxidase subunit I*, *COI*) in GenBank (Table 2). This is strange, since over the past 15 years, DNA barcodes have become a universal tool not only for identifying species (Hebert et al., 2003), but also for understanding the evolution and ecology of biodiversity (Kress et al., 2015).

The aim of this study was to reveal possible similarities or differences between Armenian and Belarusian populations of Colorado potato beetle based on karyological and DNA barcode data. In addition, we were interested in testing the prediction that due to the founder effect (Barton & Charlesworth, 1984), invasive populations have a lower genetic diversity than native populations of the same species (Dlugosch & Parker, 2008; Holm et al., 2018; but see also: Roman & Darling, 2007). To test this prediction, a chromosomal and DNA barcode comparison of native (North America) and introduced (Europe) populations of CPB was carried out.

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 ($AgNO_3$) solution and 2 drops of colloidal developer solution (0.2 g gelatin, 10 ml distilled water and 0.1 ml concen-

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

NN	Locality	Date of collection	Karyologically examined specimens	DNA examined specimens
1.	Armenia, Armavir Prov. env. Arshaluys vill.	13.06.2019	15♂, 19♀	1
2.	Armenia, Kotayk Prov., env. Aghavnadzor vill.	18.07.2019	3♂, 3♀	1
3.	Armenia, Lori Prov., env. Hartagyugh vill.	6.08.2019	4♂, 4♀	1
4.	Armenia, Gegharkunik Prov., env. Chambarak vill.	11.07.2019	4♂, 3♀	1
5.	Belarus, Vitebsk Reg., Vitebsk District, Pushkari vill.	30.07.2019	2♂, 2♀	1
6.	Belarus, Vitebsk Reg., Polotsk District, env. Polotsk city	31.07.2019	2♂, 2♀	1
7.	Belarus, Grodno Reg., Ashmyany District, Zhuprany vill.	10.08.2019	3♂, 1♀	1
8.	Belarus, Gomel Reg., Svietlahorsk District, Rakshin vill.	19.07.2019	1♂, 3♀	1
9.	Belarus, Vitebsk Reg., Orsha District, Krapivno vill.	29.07.2019	3♂, 1♀	1
10.	Belarus, Vitebsk Reg., Vitebsk District, env. Tulovo town	30.07.2019	1♂, 3♀	–
11.	Belarus, Brest Reg., Zhabinka District, env. Zhabinka city	8.07.2019	1♂, 3♀	–
12.	Belarus, Gomel Reg., Kalinkavichy District, env. Kalinkavichy town	17.07.2019	1♂, 3♀	–
13.	Belarus, Brest Reg., Luninets District, env. Luninets town	14.07.2019	3♂, 1♀	–
14.	Belarus, Mogilev Reg., Mogilev District; Kuty vill.	23.07.2019	3♂, 1♀	–
15.	Belarus, Mogilev Reg., Babruysk District, Telusha vill.	24.07.2019	3♂, 1♀	–
16.	Belarus, Minsk Reg., Minsk District, Shchomyslitsa vill.	3.06.2019	4♀	–

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	Armavir 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. Hartagyugh vill.	This study
L. decemlineata	SCZHE-19-004	MW346683	Armenia	Gegharkunik Prov., env. Chambarak vill.	This study
L. decemlineata	SCZHE-19-140	MW346682	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-19-194	MW348766	Belarus	Grodno Reg., Ashmyany District, Zhuprany vill.	This study
L. decemlineata	SCZHE-19-075	MW348765	Belarus	Gomel Reg., Svietlahorsk District, Rakshin vill.	This study
L. decemlineata	SCZHE-19-127	MW348764	Belarus	Vitebsk Reg., Orsha District, Krapivno vill.	This study
L. decemlineata	USDABRL Roehrdanz 1868	HQ605769	USA	Fargo, ND	Yocum et al., 2011
L. decemlineata	USDABRL Roehrdanz 1313	HQ605768	USA	MD	Yocum et al., 2011
L. decemlineata	BIOUG<CAN>:AY165708	AY165708	Canada	Ontario, Wellington County, Puslinch Township	Hebert et al., 2003
L. decemlineata	—	XM_023169376	USA	Long Island New York	GenBank
L. decemlineata	ZFMK-TIS-5917	KU915233	Germany	Thuringia, Eisenach, Wilhelmsthal, Fischteiche	Rulik et al., 2017
L. decemlineata	ZFMK-TIS-2000691	KU914658	Poland	Woiwodschaft Westpommern, Powiat Slawienski, Landg, Campingplatz Wicie	Rulik et al., 2017
L. decemlineata	ZFMK-TIS-2003262	KU911127	Germany	Saxony-Anhalt, Halberstadt, Landkreis Harz, Athenstedt	Rulik et al., 2017
L. decemlineata	ZFMK-TIS-2000692	KU909921	Poland	Woiwodschaft Westpommern, Powiat Slawienski, Landg, Campingplatz Wicie	Rulik et al., 2017
L. decemlineata	ZFMK-TIS-2003261	KU907193	Germany	Saxony-Anhalt, Halberstadt, Landkreis Harz, Athenstedt	Rulik et al., 2017
L. decemlineata	GBOL_Col_FK_8406	KM439182	Germany	North Rhine-Westphalia, Niederrheinische Bucht, Rhein-Sieg, Bornheim-Hemmerich, Ortslage	Hendrich et al., 2015
L. decemlineata	BFB_Col_FK_10263	KM439498	Austria	Burgenland, Neusiedlersee, Neusiedl am See, Illmitz, Biologische Station	Hendrich et al., 2015
L. decemlineata	GBOL_Col_FK_8725	KM439249	Germany	Baden-Wuerttemberg, Suedliches-Oberrhein-Tiefl, Breisgau-Hochschwarzwald, Boetzingen, Ortslage	Hendrich et al., 2015
L. decemlineata	BC ZSM COL 02288	JF889843	Germany	North Rhine-Westphalia, Niederrheinische Bucht, Rhein-Sieg, Bornheim-Hemmerich, Ortslage	GenBank, iBOL
L. decemlineata	O.V.Sukhorukova 296 (LBIA)	DQ649100	Russia	Bashkortostan, Beloreckiy, Mezhgorie	Udalov & Benkovskaya, 2010
L. decemlineata	O.V.Sukhorukova 306 (LBIA)	DQ649101	Russia	Bashkortostan, Beloreckiy, 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, Fedorovskiy, Dedovo	Udalov & Benkovskaya, 2010
L. decemlineata	M.B.Udalov 379 (LBIA)	DQ649096	Russia	Bashkortostan, Fedorovskiy, Dedovo	Udalov & Benkovskaya, 2010
L. decemlineata	M.B.Udalov 14 (LBIA)	DQ649099	Russia	Bashkortostan, Ufimskiy, Dmitrievka	Udalov & Benkovskaya, 2010
L. decemlineata	M.B. Udalov 35 (LBIA)	DQ127906	Russia	Bashkortostan, Ufimskiy; Dmitrievka	Udalov & Benkovskaya, 2010
L. decemlineata	R.A. Ilyasov 332 (LBIA)	DQ649095	Russia	Bashkortostan, Tatyshlinskiy, Schulganovo	Udalov & Benkovskaya, 2010
L. decemlineata	R.A. Ilyasov 355 (LBIA)	DQ649094	Russia	Bashkortostan, Tatyshlinskiy, Schulganovo	Udalov & Benkovskaya, 2010
L. decemlineata	M.B. Udalov 421 (LBIA)	DQ127909	Russia	Bashkortostan, Fedorovskiy; Dedovo	Udalov & Benkovskaya, 2010
L. decemlineata	O.V. Sukhorukova 305 (LBIA)	DQ127907	Russia	Bashkortostan, Beloreckiy; Mezhgorie	Udalov & Benkovskaya, 2010
L. decemlineata	Yu.M.Nikonorov 923 (LBIA)	DQ011111	Russia	Bashkortostan, Miyakinskiy, Kirgiz-Miyaki	Udalov & Benkovskaya, 2010
L. decemlineata	M.B. Udalov 363 (LBIA)	DQ127908	Russia	Bashkortostan, Tatyshlinskiy; Schulganovo	Udalov & Benkovskaya, 2010
L. decemlineata	BCZSM_COLA_1040	KM448111	Austria	Burgenland	Hendrich et al., 2015
L. haldemani	BIOUG<CAN>:09BBCOL-0248	HM433598	USA	Oklahoma, Marshall Co., UOBS, near Lake Texoma	GenBank, iBOL
L. haldemani	BIOUGCAN:09BBCOL-0247	HM433597	USA	Oklahoma, Marshall Co., UOBS, near Lake Texoma	GenBank, iBOL
L. haldemani	—	DQ459377	USA	AZ, Benson	Greenstone et al., 2007
L. haldemani	BIOUG<CAN>:10BBCOL-0771	HQ984330	USA	Texas, Caverns of Sonora Nature Trail	GenBank, iBOL
L. juncta	BMNH:704381	KC255422	USA	WV, Randolph Co., Elkwater	Nie et al., 2018
L. juncta	USDABRL Roehrdanz FPB-A	HQ605770	USA	MD	Yocum et al., 2011
L. juncta	USDABRL Roehrdanz 2181SB	HQ605771	USA	MD	Yocum et al., 2011
L. texana	USDABRL Roehrdanz 1914	HQ605774	USA	Weslaco, TX	Yocum et al., 2011
L. texana	WWAI ww21931	MK288007	Sth Africa	—	GenBank
L. texana	USDABRL Roehrdanz 1915	HQ605775	USA	Weslaco, TX	Yocum et al., 2011
L. texana	WWAI ww21948	MK288008	Sth Africa	—	GenBank

trated formic acid – HCOOH) were placed on each slide. 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-banding was revealed using the protocol of Rożek (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% Giemsa solution in phosphate buffer (pH 6.8) for 8 min.

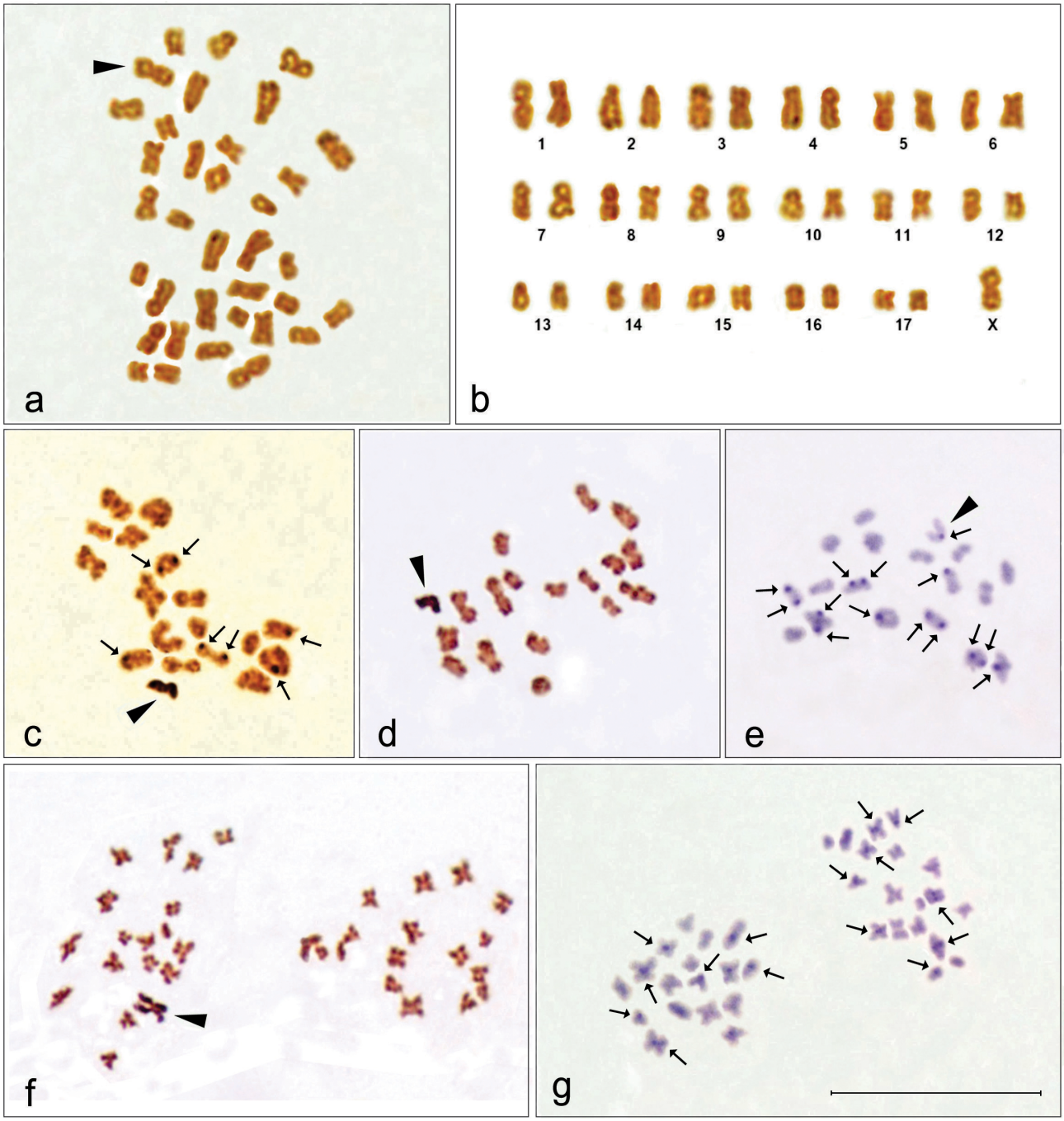

Fig. 1. Karyotype of males of *Leptinotarsa decemlineata* from Armenian populations. a – mitotic metaphase; b – karyogram; c – diakinesis; d, e – prometaphase I; f, g – metaphase II. Arrowheads indicate X sex chromosome. c – Ag-banding, arrows indicate argentophilic signals (NORs); e, g – C-banding, arrows indicate C-blocks. Bar equals 10 μm.

DNA extraction, PCR amplification and sequencing

Total DNA was extracted from the wing muscle, using the Qiagen 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 $MgCl_2$, 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.

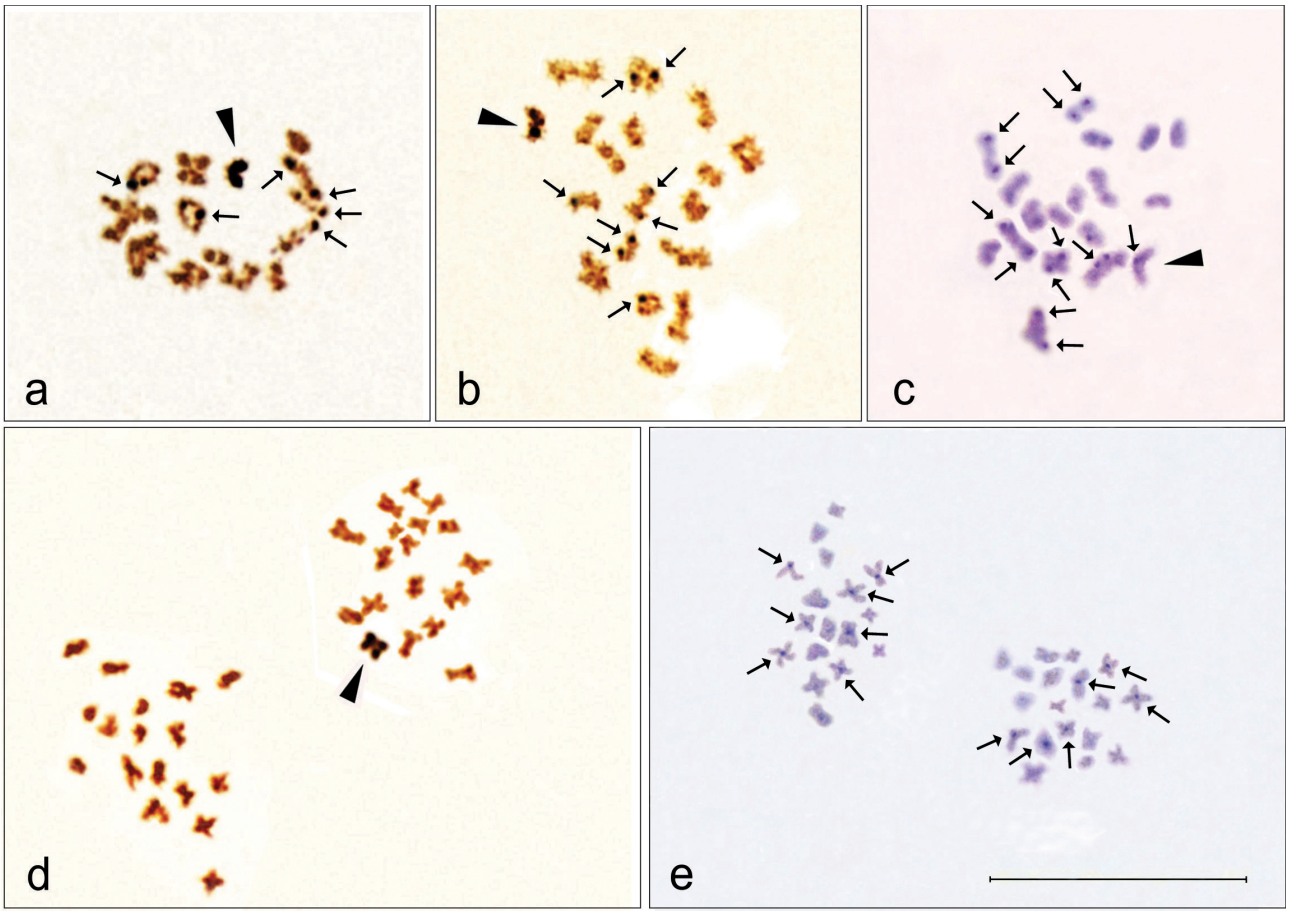

Fig. 2. Karyotype of males of *Leptinotarsa decemlineata* from Belarusian populations. a – early diakinesis; b – late diakinesis; c – metaphase I; d, e – metaphase II. Arrowheads indicate X- sex chromosome. a, b – Ag-banding, arrows indicate argentophilic signals (NORs); c, e – C-banding, arrows indicate C-blocks. Bar equals 10 μm.

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 AY165708 Canada	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 MW346684 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 KM439182 Germany	G	G	C	T
L. decemlineata KM439498 Austria	G	G	C	T
L. decemlineata KM439249 Germany	G	G	C	T
L. decemlineata DQ649101 Russia Bashkortostan	G	G	C	T
L. decemlineata DQ649098 Russia Bashkortostan	G	G	C	T
L. decemlineata DQ649097 Russia 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 DQ649100 Russia Bashkortostan	G	G	C	T
L. decemlineata DQ649096 Russia Bashkortostan	G	G	C	T
L. decemlineata DQ649099 Russia Bashkortostan	G	G	C	T
L. decemlineata JF889843 Germany	G	G	C	G
L. decemlineata DQ127907 Russia Bashkortostan	G	G	C	T
L. decemlineata DQ011111 Russia Bashkortostan	G	G	C	-
L. decemlineata DQ127908 Russia Bashkortostan	G	G	C	T
L. decemlineata KM448111 Austria	G	G	C	T
L. haldemani HM433598 USA	A	A	T	G
L. haldemani HM433597 USA	A	A	T	G
L. haldemani DQ459377 USA	–	A	T	T
L. haldemani HQ984330 USA	A	A	T	T
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, at 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 pericentromeric regions of some chromosomes that did not form distinct blocks (Figs 1e, g; 2c, e). At prometaphase-metaphase I (Figs 1e, 2c), small pericentromeric 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

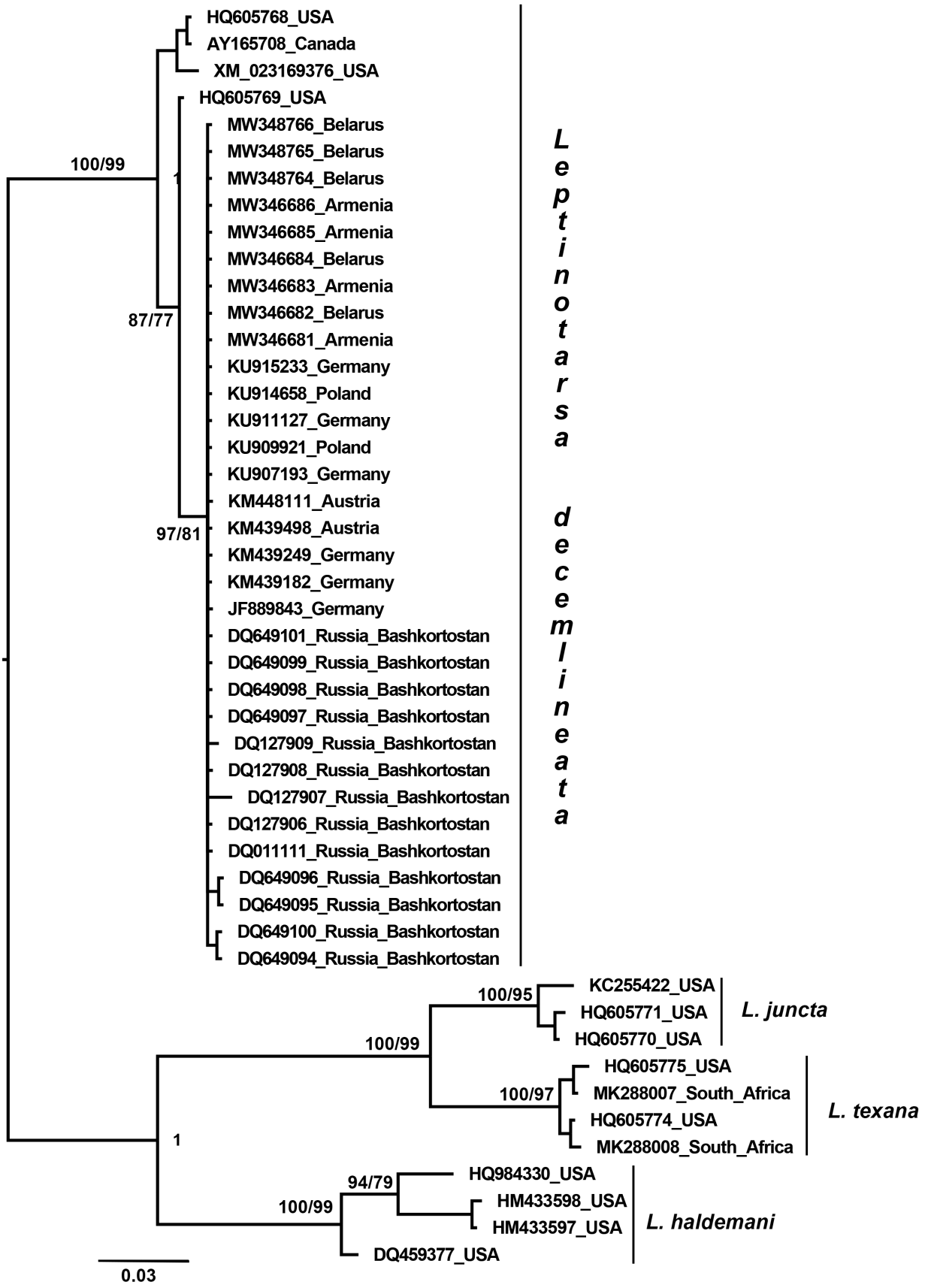

Fig. 3. Bayesian phylogenetic tree based on *Leptinotarsa* DNA barcodes. Maximum likelihood analysis revealed the same topology. The GenBank accession number and country of origin are listed for every sequence. Bayesian posterior probability for BI/Bootstrap value support for ML are indicated at nodes.

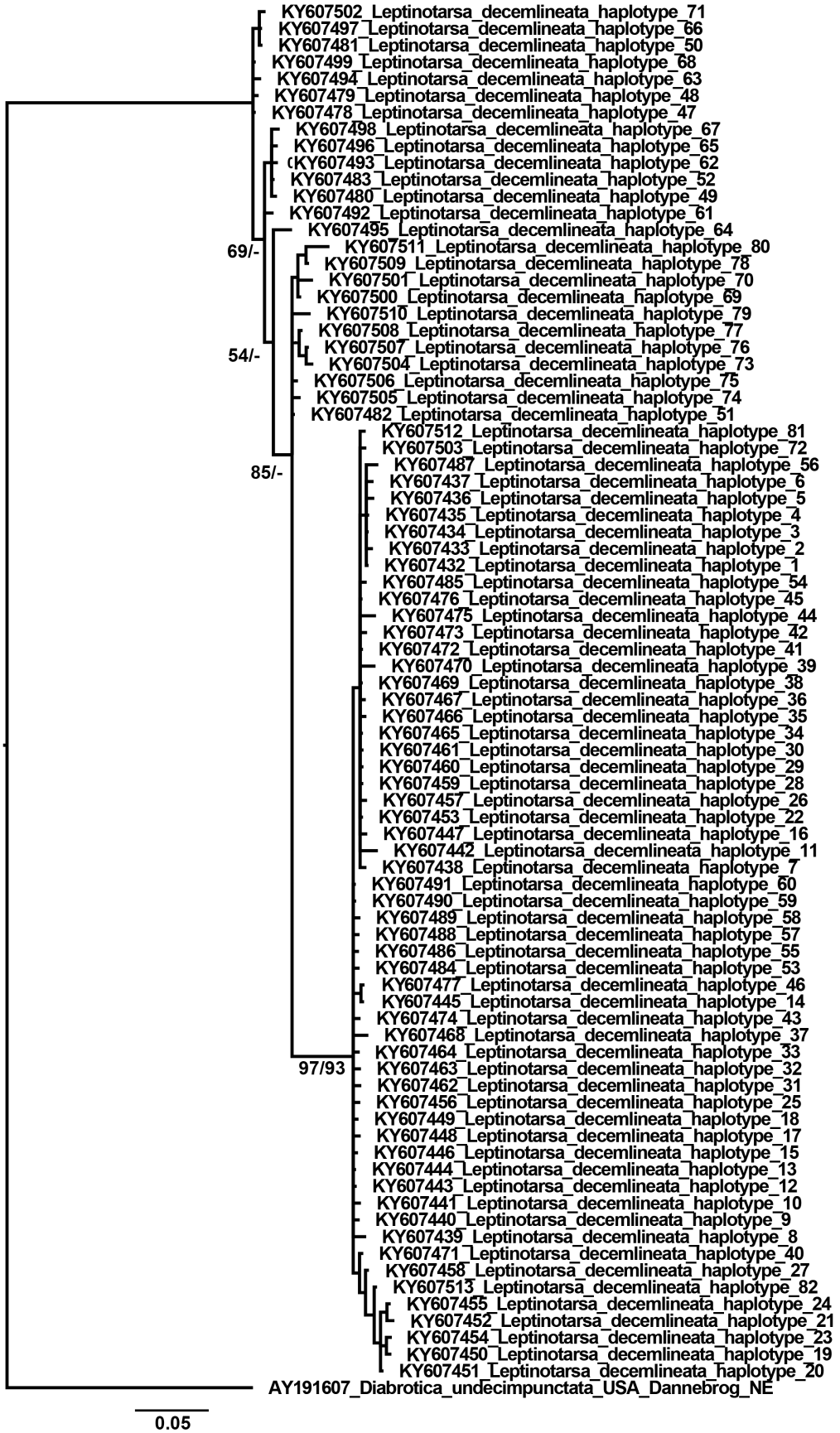

Fig. 4. Bayesian phylogenetic tree based on the North American haplotypes of the mitochondrial genome fragment that included the terminal part of the *COI* gene and initial part of the *COII* gene. The sequences of the North American *Leptinotarsa decemlineata* samples published by Crossley et al., 2017 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. juncta* and *L. texana*) formed three separate clades, of which *L. juncta* and *L. texana* were sister clades, while *L. haldemani* was another separate clade. In addition, the sequence HQ984330 of the specimen mentioned in GenBank as *Leptinotarsa* 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-banding 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 Rożek 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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