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ORIGINAL ARTICLE

Estimating range disjunction time of the Palearctic Admirals (*Limenitis* L.) with *COI* and histone H1 genes

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Abstract

Three species of the genus *Limenitis* (Nymphalidae) (*L. camilla*, *L. helmanni*, *L. sydyi*) have split ranges in the Palearctic. Their disjunction was dated either to the Pleistocene or to the Subboreal time of the Holocene. This genus also exhibits an amphiberingean disjunction, *L. populi* vs four Nearctic species. To evaluate the disjunction time in Eurasia, we analysed a fragment of the mitochondrial *COI* gene and a major part of the histone H1 gene. The former was sequenced in 51 specimens of three species with Palearctic disjunctions. We detected a diverged nuclear copy of the *COI* gene in *L. camilla*. The histone H1 gene was sequenced in 64 specimens of 8 species. In five species, intra-species and intra-individual nucleotide substitutions and variation in the number of intra-genic repeats were observed and studied by cloning of individual gene copies, with individuals found with more than two variants. With 30–80 copies of histone H1 gene in *Limenitis* genomes, as estimated by real time PCR, this was interpreted as cis-heterogeneity across the histone gene cluster. No fixed differences between the western and eastern range parts were found in *L. helmanni*, *L. camilla* and *L. sydyi*, although in the former more alleles of both sequences were found in the eastern part. This suggests the range disjunctions to be too recent to be dated by molecular means and they may only be estimated to have taken place not more than 77–100 tya. This fits their provisional dating by Dubatolov and Kosterin (Entomologica Fennica 11(3), 141-161, 2000) to the Subboreal time of the Holocene.

Keywords Range disjunctions · Dating · Histone H1 · *COI* · *Limenitis* · Nemoral species

Introduction

Climate determines the assemblage of animal species inhabiting a territory both directly and indirectly, through the current vegetation and the prehistory. Climatic changes, first of all temperature and humidity, result in geographical shifts of the biota so that vegetation formations and ranges of particular plant and animal species change their borders. The climatically induced latitudinal and/or longitudinal shifts are complicated by the relief and configuration of lands and seas. Range borders of species and communities are affected by either physical impermeability of certain relief forms, like mountain ranges and straits, or peculiarities of their hydrothermal regime, like elevational zonation. Due to environment deterioration, a species range may split into pieces (undergo *range disjunction*) occupying the areas still suitable for survival (so-called *refugia*), which may be small. Range expansions often result in re-fusion of once disjunct range parts but may also lead to range fragmentation during flowing around geographical obstructions, e.g. mountains or aquatoria. Genetic isolation leads to divergence of populations to a degree depending on the isolation duration. Rejoining of once isolated population may follow three scenarios: (i) divergence resulted in reproductive barriers and hence speciation, so that the populations 'behave as good species' able of sympatric co-existence in the same territory; (ii) no sound reproductive barriers appeared, so that a secondary contact results in population fusion and genetic introgression, often with formation of geographical clines of the frequencies of diverged alleles; and (iii) incomplete reproductive isolation resulting in hybrid zones between the

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so-called 'semispecies' (Descimon & Mallet, 2009, Ebdon et al., 2021), with reinforcement of the barriers due to selection against hybrids and the so-called *parapatric speciation* (Barton et al., 2007).

During the Cenosoic, the strongest climate oscillations took place in the Pleistocene (2.58–0.0117 mya). Under condition of landmass shift to polar regions in the course of the continental drift, periodic changes in the Earth orbit eccentricity and other astronomical cycles resulted in cyclic cooling and warming with the main period of 110–130 thousand years (EPICA Community Members, 2004; Imbrie et al., 1993; Petit et al., 1999; Velichko, 1993). Importantly, this interval coincides with the characteristic time of speciation (Coyne & Orr, 2004). Hence, during the Pleistocene, the northern Eurasia served as 'an evolutionary shaker' providing conditions for repeated range disjunctions and fusions, divergence of isolated populations, introgression and speciation (Ebdon et al., 2021).

The biota of the northern Eurasia can be divided according to the predominating vegetation type to four complexes: (i) the arctoalpine complex (arctic and mountain tundras and 'deserts') occupying high latitudes and highlands and characterised by frost resistance; (ii) the boreal complex associated with coniferous (taiga) and birch forest and composed of more or less psychrophilic species; (iii) nemoral complex associated with broad-leafed forests (but the birch forests of Siberia are a boreal forest version), mostly thermo- and hygrophilic; and (iv) the steppe complex associated with grasslands (open southern mountain slopes in Siberia), rather thermo- and xerophilic. Plant and animal species with disjunctive ranges are mostly found in the arcto-alpine (i) and nemoral (iii) complexes, since the former has patchy distribution in mountains while the latter depends on the most favourable climatic conditions.

Molecular phylogeography and molecular taxonomy of alpine and arctoalpine species are actively studied in Lepidoptera (Schmitt & Hewitt, 2004; Schmitt et al., 2006; Huemer & Hausmann, 2009; Simonsen et al., 2010; Vila et al., 2011; Huemer & Hebert, 2011; Todisco et al., 2010a, b, 2012; Mutanen et al., 2012; Huemer & Mutanen, 2012; Huemer et al., 2014; Kirichenko et al., 2015; Nice et al., 2013; Kleckova et al., 2015; Caplbanq et al., 2020; Usami et al., 2021). Their ranges are fragmented during warm periods (Menchetti et al., 2021; Usami et al., 2021) when they shift to higher latitudes and elevations where their refugia persisted through interglaciations, including the current one. During the climate coolings, the arcto-alpine biota descended from the mountains, spread over plains and restore their previously split ranges. The present arcto-alpine disjunctions attract attention in two respects: (i) for taxonomical studies aimed to reveal cryptic or allopatric species rather than united species with disjunct ranges (Mutanen et al., 2012; Huemer et al., 2014, 2020; Nice et al., 2013; Kirichenko et al., 2015), and (ii) for phylogeographical studies aimed to reconstruct recent prehistory of biotas and to date range disjunctions (Schmitt & Hewitt, 2004; Schmitt, 2007, 2009, 2017; Varga & Schmitt, 2008; Todisco et al., 2010a, b, 2012; Simonsen et al., 2010; Vila et al., 2011; Solovyev et al., 2015a; Kleckova et al., 2015; Usami et al., 2021).

Many studies concerned phylogeography of boreal species with vast transpalaearctic ranges in the taiga zone, paying most attention to range fragmentation during the climate coolings in the Pleistocene, to reconstructing locations of 'glacial refugia' and ways of reoccupation of the recent ranges during the Holocene (de Lattin, 1967; Uimaniemi et al., 2000; Hundertmark et al., 2002; Zink et al., 2002; Babik et al., 2004; Goropashnaya et al., 2004; Joedicke et al., 2004; Oshida et al., 2005; Ursenbacher et al., 2006; Fedorov et al., 2008; Saitoh et al., 2010; Bernard et al., 2011; Schmitt & Varga, 2012; Yakovlev et al., 2017; Dincă et al., 2019; Bartoňová et al., 2021). The boreal biota, intermediate in its cold tolerance between the arcto-alpine and nemoral ones, was less subject to fragmentation so that the permanently contiguous taiga belt persisted in North Eurasia. Some boreomontane species tend to form longitudinal disjunctions; e.g. the leaf beetle *Gonioctena intermedia* (Helliesen, 1913) (Coleoptera: Chrysomelidae) feeding on the bird cherry (*Prunus padus* L.) and rowan tree (*Sorbus aucuparia* L.) occurs in Scandinavia, the Alps and Carpathians (Mardulyn et al., 2009; Quinzin & Mardulyn, 2014; Quinzin et al., 2017). There are cases of disjunct boreal species not associated with mountains; e.g. the damselfly *Nehalennia speciosa* (Charpentier, 1840) (Odonata: Coenagrionidae) mostly inhabiting peat-moss mires has an amphipalearctic disjunctive range (Bernard et al., 2011; Kosterin, 2005). As a rule, range disjunctions occurring in boreal species are relatively small, e.g. for the distance between adjacent mountain systems etc., and caused solely by relief. Studies concerning molecular phylogeography of disjunct boreal species are few (Bernard et al., 2011; Mardulyn et al., 2009; Quinzin & Mardulyn, 2014; Quinzin et al., 2017; Suvorov, 2011). Some of them concern butterflies: the amphiberingean skipper *Hesperia comma* Linnaeus, 1758 (Forister et al., 2004) (Lepidoptera: Hesperiidae), the boreomontane *Lycaena helle* Denis & Schiffermuller, 1775 (Lepidoptera: Lycaenidae) with a fragmented range (Finger et al., 2009; Habel et al., 2010, 2011a, b).

During the strongest coolings of the Pleistocene, the northern Europe and western North America were covered by ice shields. The northern Asia was not covered with ice except for the extreme north because of scarce precipitation, but was mostly occupied with the so-called tundrosteppe landscapes formed in cold and dry climate (Belova, 1985; Kozhevnikov & Ukraintseva, 1992; Velichko, 1993; Arkhipov & Volkova, 1994; Ukraintseva, 1996), the recent relics of which are found in East Yakutia (Yurtsev, 1981). The more thermophilic nemoral, subnemoral and boreal

biotas withdrew to lower latitudes. However, the contiguous, latitudinally oriented belt of the mountains of South Siberia appeared an impermeable barrier for them, so these biotas were able to retreat only to the Mediterranean Refugum (Babik et al., 2004; de Lattin, 1967; Dubatolov & Kosterin, 2000; Quinzin et al., 2017; Ursenbacher et al., 2006), the Manchurian Refugium (Nazarenko, 1982; Dubatolov & Kosterin, 2000; Zink et al., 2002; Oshida et al., 2005; Fedorov et al., 2008; Saitoh et al., 2010; Dudko, 2011) or the lesser and not so important Siberian-Central Asian Refugium supposedly situated in Heptapotamia (Zhetysu; SE Kazakhstan) (Dubatolov & Kosterin, 2000). Some nemoral species retreated to more than one refugium that resulted in range disjunctions (Dubatolov & Kosterin, 2000; Uimaniemi et al., 2000; Schmitt & Seits, 2001; Hundertmark et al., 2002; Goropashnaya et al., 2004; Joedicke et al., 2004; Bernard et al., 2011; Schmitt & Varga, 2012; Solovyev et al., 2015a, Menchetti et al., 2021). Oppositely, in warm periods, thermophilic species could reunite the earlier split range.

Thus, in the Pleistocene/Holocene, the ranges of the arcto-alpine and nemoral species had their split-reunion cycles in counter-phase (Menchetti et al., 2021) and differed in the predominant orientation of disjunctions, respectively latitudinal (between the Arctic and mountains) versus longitudinal (between Europe and the Far East).

At present the nemoral broad-leafed forests are absent from Siberia except for extreme west and east and a small linden refugium in Kemerovo Province (birch forests are not nemoral but are either a version of boreal forests or, in their southern range, can be classified as subboreal) (Ermakov, 1998; Dubatolov & Kosterin, 2000, 2015). Paleopalinological data summarised in the referenced work suggest that during the Atlantic period or the Climatic Optimum of the Holocene (5–7.5 thousand years ago), the contiguous belt of nemoral broad-leafed forests was restored in North Eurasia, so the range disjunction of some nemoral Lepidoptera species should have formed relatively recently, during a moderate cooling of the following Subboreal period (2.5–5 thousand years ago).

Earlier some authors non-critically dated the continuous range of nemoral forests and species to the Tertiary, that is not less 2.5 mya, and their disjunction to the Pleistocene (Matyushkin, 1976; Belyshev & Haritonov, 1981; Polozhiy & Krapivkina, 1985; Mikkola, 1987; Logunov, 1996; Ermakov, 1998; for review see Dubatolov & Kosterin, 2000). Such a long isolation would result in allopatric speciation so that a species may turn out to be two (or more) sibling species. The Pliocenic dating was proposed for the amphipalearctic disjunction of the Azure-winged Magpie *Cyanopica cyanus* (Pallas, 1776) (Haring et al., 2007; Kryukov et al., 2004) and for isolates of the Steppe Viper *Vipera ursinii* (Bonaparte, 1835) complex (Ferchaud et al., 2012; Zinenko et al., 2015). However, the climate cyclicity of the Pleistocene could result in similar range disjunctions associated with different particular coolings such as the Subboreal moderate cooling (0.5–3.7 tya) and the Late Wurm (10–23 tya), Early Wurm (100–130 tya), Riss (170–200 tya) and earlier glaciations (Dubatolov & Kosterin, 2000). Since the present pattern resulted from superimposition of climatic and biotic events which took place in different times of the past, we cannot date any disjunction solely by its shape without objective, e.g. molecular data.

Dubatolov and Kosterin (2000) recognised three types of disjunct ranges of nemoral and subnemoral species of Lepidoptera in North Eurasia:

(i) *Amphipalearctic ranges*, split into two large parts: the European (east up to the Ural Mts.) and the Far Eastern (from the eastern Transbaikalia or Amurland to Japan);
(ii) *European-West Siberian-Far Eastern ranges*, differing from the former type by existence of a small West Siberian isolate between the large European and Far Eastern parts;
(iii) *Altai-Far Eastern ranges*, split between an isolate in SE West Siberia (including Altai and in one case also Tian Shan Mts.) and the main Far Eastern part.

Earlier we estimated the disjunction time of the European-West Siberian-Far Eastern range of the subnemoral epiplemid *Eversmannia exornata* (Eversmann, 1837) (Dubatolov & Kosterin, 2000; Dubatolov et al., 1994) as relatively recent (not more than 100 tyr) (Solovyev et al., 2015a). The present work is aimed to date the range disjunctions in the palearctic representatives of a genus from another infraorder of Lepidoptera, *Limenitis* L. (Papilionoidea: Nymphalidae) (Fig. 1).

There are about two dozen species of *Limenitis* in the Palearctic, including species with split ranges. One of these is *Limenitis camilla* (Linnaeus, 1758) with an amphipalearctic range, which inhabits the western Eurasia–in Europe north up to 59° N, the Caucasus, Transcaucasia, the southern Ural and the western part (near Tyumen' City) of the West Siberian Lowland (*L. camilla camilla* L.) and, after a huge range gap, in the southern Far East of Russia including Amur Province, Jewish Autonomous Region, southern Khabarovskiy Kray, Primorye, South Kuriles, in NW China, Korea and Japan (*L. camilla japonica* Ménétriés, 1857) (Dubatolov & Kosterin, 2000; Gorbunov & Kosterin, 2007). Two other species have Altai-Far Eastern disjunct ranges: *Limenitis helmanni* Kindermann in Lederer, 1853 has a western isolate at the Kusnetsk Elevation, Altai (in the territories of Russia, Kazakhstan and China) and Tian Shan (in Kazakhstan) (*L. helmanni helmanni*), and the main range part in the south-eastern Transbaikalia, the southern Far East of Russia including Amur Province, Jewish

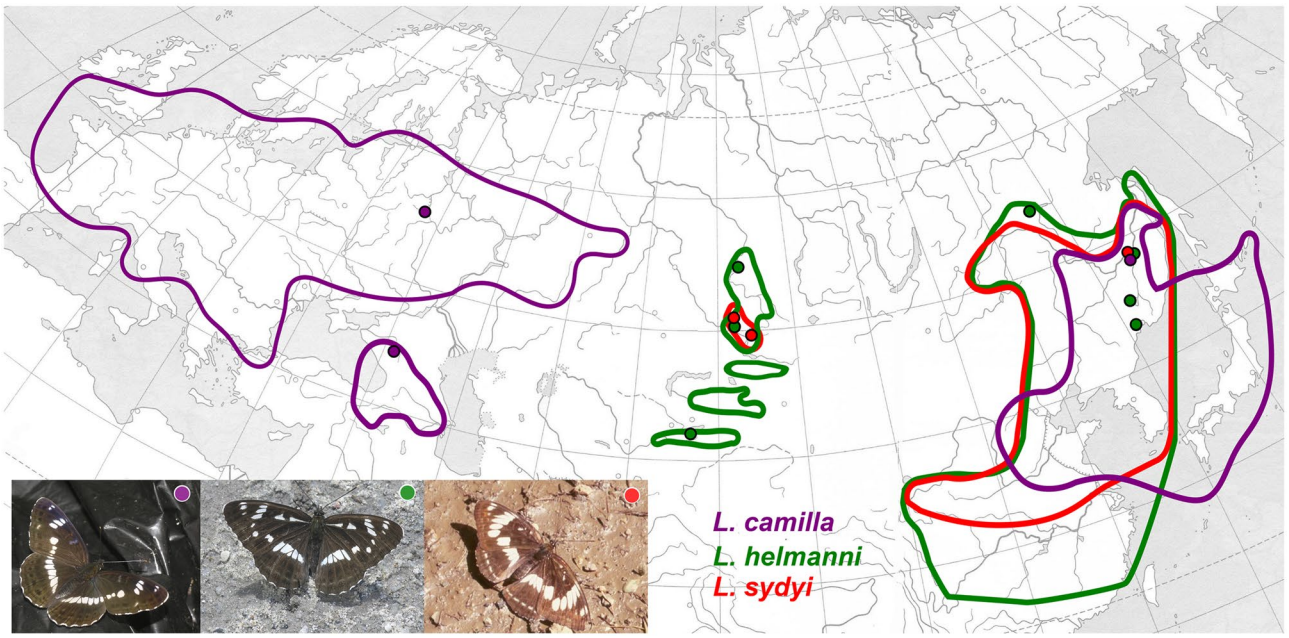

Fig. 1 Disjunction of the *Limenitis* spp. ranges in the Palearctic. The borders of the ranges of *L. camilla*, *L. helmanni* and *L. sydyi* are marked with purple, green and red lines, respectively; the dots indicate sampling localities

Autonomous Republic, southern Khabarovskiy Kray and Primorye (but not the islands) in the southern Far East of Russia, NE, Central and E China and Korea (*L. helmanni duplicata* Staudinger, 1892). *Limenitis sydyi* Kindermann in Lederer, 1853 has a similar range but is absent from Tian Shan, with the western isolate situated in W and N Altai Mts. (*L. sydyi sydyi*); the range of the eastern subspecies *L. sydyi latefasciata* Ménétriés, 1859 is similar to that in the previous species (Dubatolov & Kosterin, 2000; Gorbunov & Kosterin, 2007). The differences between the subspecies in all three pairs are scarce.

It is noteworthy that the genus *Limenitis* also provides an example of an amphiberingean disjunction. There are four Nearctic species of *Limenitis*, namely *L. arthemis* Drury, 1773, *L. archippus* Cramer, 1775, *L. lorquini* Boisduval, 1852, and *L. weidemeyerii* Edwards, 1861, strongly differing in appearance from the palearctic representatives because of the Batesian mimicry with the unpalatable butterflies *Battus philenor* (Linnaeus, 1771) and *Danaus plexippus* (Linnaeus, 1758) as models (Mullen, 2006). However, the Nearctic *Limenitis* species and the Palearctic *L. populi* (Linnaeus, 1768) share their foodplants belonging to the family Salicaceae while all other palearctic species use *Lonicerea* (Caprifoliaceae). According to the estimations by Mullen (2006; Mullen et al., 2010), the last common ancestor of *L. populi* and the Nearctic species which shifted to Salicaceae existed about 9 mya, while the last common ancestor of the genus *Limenitis* existed ca 11 mya (Ebel et al., 2015). Thus, we may estimate the time of the Palearctic range disjunctions in the genus as relative to the time of the Beringian disjunction.

Materials and methods

Material

Eight species of the genus *Limenitis* were studied. Most specimens were collected by V.V. Dubatolov and O.E. Kosterin with the net and frozen immediately or preserved dry on cotton layers. Specimens of *L. camilla* from the western part of the range (Moscow Province) were kindly provided by M.G. Kovalenko (Bush), Moscow State University. Specimens of *L. helmanni* and *L. sydyi* from the nothern Kazakhstan were collected by V.K. Zinchenko and V.V. Lukhtanov. A leg preserved in pure alcohol of a specimen of *L. arthemis* was kindly provided by Kim Mitter, University of Maryland, ATOLep Collection, USA. Details of the specimens examined are provided in Table 1.

Markers chosen

We choose the following molecular markers:

– The popular mitochondrial DNA fragment *COI-Leu tRNA- COII* (Folmer et al., 1994; Simon et al., 1994) already used for the genus *Limenitis* earlier (Mullen, 2006; Mullen et al., 2010);

Table 1 Studied specimens of *Limenitis* spp

Species	Subspecies	Specimen	Sex	Date	Locality	Collector	Accession # for histone H1 gene	Accession # for *COI*
L. camilla	*japonica*	Lc19	♂	13.06.2012	Russia, Khabarovskiy Kray, 28 km SE of Khabarovsk, Bychikha village, 48.29° N, 134.83° E	Dubatolov, V.V	OU723957, OU723958	OU723912 (OU724030–numt)
		Lc30	♂	06.07.2011	- " -	- " -	OU723959-OU723962	OU723913
		Lc31	♂	06.07.2011	- " -	- " -	OU723963, OU723964	OU723914
		Lc38	♂	17.06.2012	- " -	- " -	OU723965	OU723915
		Lc39	♂	17.06.2012	- " -	- " -	OU723966, OU723967	OU723916
		Lc40	♂	25.06.2012	Russia, Khabarovskiy Kray, 44 km SE of Khabarovsk, Chirki Cordon, 48.19° N, 134.68° E	Dubatolov, V.V	OU723968	OU723917 (OU724031-numt)
	camilla	Lc119	♂	15.07.2012	Russia, Moscow Province, Odintsovo District, Volkovo village env., 55.68° N, 36.68° E	Bush, M.G	OU723969, OU723970	OU723918 (OU724032-numt)
		Lc120	♂	15.07.2012	- " -	- " -	OU723971-OU723974	OU723919
		Lc121	♀	17.07.2014	- " -	- " -	OU723975, OU723976	OU723920 (OU724033-numt)
		Lc122	♀	20.07.2014	- " -	- " -	OU723977, OU723978	OU723921 (OU724034-numt)
		Lc152	♂	8.07.2019	Russia, N Caucasus, Karachay-Cherkes Republic, 6.3 km NE Dombay Town, 43.34° N, 41.67° E,	Kosterin, O.E	OW493383	-
L. helmanni	*duplicata*	Lh7	♀	3–5.08.2005	Russia, Khabarovskiy Kray, 44 km SE of Khabarovsk, Chirki Cordon, 48.19° N, 134.68° E	Dubatolov, V.V	OU723979	OU726520
		Lh15	♂	26.07.2010	Russia, Khabarovskiy Kray, 28 km SE of Khabarovsk, Bychikha village, 48.29° N, 134.83° E	Dubatolov, V.V	OU723980, OU723981	-
		Lh16	♂	26.07.2010	- " -	- " -	OU723982	OU726522
		Lh17	♂	15.06.2012	- " -	- " -	OU723983	OU726507
		Lh18	♂	17.06.2012	- " -	- " -	OU723984, OU723985	OU726526
		Lh22	♂	11.07.2011	Russia, Amur Province, Zeya Nature Reserve, Bol'shaya Ekaringra Cordon, 54.09° N, 126.87° E	Dubatolov, V.V	OU723986, OU723987	-
		Lh23	♀	11.07.2011	- " -	- " -	OU723988	-
		Lh28	♂	13.07.2011	Russia, Khabarovskiy Kray, 28 km SE of Khabarovsk, Bychikha village, 48.29° N, 134.83° E	Dubatolov, V.V	OU723989, OU723990	OU726525
		Lh29	♀	06.07.2011	- " -	- " -	OU723991, OU723992	-
		Lh35	♂	13.06.2012	- " -	- " -	OU723993	OU726519
		Lh36	♂	13.06.2012	- " -	- " -	OU723994	OU726521
		Lh37	♂	13.06.2012	- " -	- " -	OU723995, OU723996	OU726523

Table 1 (continued)

Species	Subspecies	Specimen	Sex	Date	Locality	Collector	Accession # for histone H1 gene	Accession # for *COI*
		Lh107	♂	01.07.2014	Russia, Primorye, Barabash village env., 43.18° N, 131.48° E	Kosterin, O.E	OU723997, OU723998	OU726524
		Lh115	♂	04.07.2014	Russia, Primorye, Khanka District, 1 km N of Platono-Aleksandrovskoe village, Lake Khanka W bank, 45.06° N, 131.99° E	Kosterin, O.E	OU723999	OU726527
		Lh116	♂	04.07.2014	- " -	- “ -	OU724000, OU724001	OU726529
		Lh117	♂	04.07.2014	- " -	- " -	OU724002-OU724004	OU726528
	helmanni	Lh-3	♂	29.07.2006	Russia, Novosibirsk Academy Town, 54.86° N, 83.10° E	Kosterin, O.E	OU724005	OU726506
		Lh-1	♂	05.07.2009	Kazakhstan, Almaty, Zailiyskiy Alatau Mt. Range N foot, Kamenskoe Plateau, 43.16° N, 76.78° E	Kosterin, O.E	OU724006	OU726518
		Lh43	♀	21.06.2000	Kazakhstan, East-Kazakhstan Province, Glubokoe village env., 50.14° N, 82.31° E	Zinchenko, V.K	OU724007	-
		Lh52	♂	19.07.2013	Russia, Novosibirsk Academy Town, the Zyryanka River valley, 54.82–83° N, 83.10–12° E	Kosterin, O.E	OU724008, OU724009	OU726517
		Lh53	♂	19.07.2013	- “ -	- “ -	OU724010, OU724011	OU726508
		Lh54	♂	21.07.2013	- “ -	- “ -	OU724012	OU726509
		Lh55	♂	21.07.2013	- “ -	- “ -	OU724013-OU724016	OU726510
		Lh124	♂	21.06.2015	Russia, Novosibirsk Province and District, the Nizhniy 1.5 km WSW Koyon village at the Volchikha River mouth, 54.81° N, 83.37° E	Kosterin, O.E	OU724017	OU726511
		Lh125	♂	21.06.2015	- “ -	- “ -	OU724018	OU726512
		Lh126	♂	21.06.2015	- “ -	- “ -	OU724019	OU726513
		Lh127	♂	21.06.2015	- “ -	- “ -	OU724020	OU726514
		Lh128	♂	21.06.2015	- “ -	- “ -	OU724021	OU726515
		Lh129	♂	21.06.2015	- “ -	- “ -	OU724022	OU726516
L. sydyi	*latifasciata*	Ls4	♀	07.07.2005	Russia, Khabarovskiy Kray, Bol’shekhekhtsirskiy Nature Reserve, ~5 km SW of Chirki Cordon, 48.2° N, 135.0° E	Dolgikh, A.M	OU723939	OU723922
		Ls5	♀	07.07.2005	- “ -	- “ -	OU723940	OU723923
		Ls13	♂	26.07.2010	Russia, Khabarovskiy Kray, 28 km SE of Khabarovsk, Bychikha village, 48.29° N, 134.83° E	Dubatolov, V.V	OU723941	OU723924
		Ls14	♀	26.07.2010	- “ -	- “ -	OU723942	OU723925
		Ls32	♂	06.07.2011	- “ -	- “ -	OU723943, OU723944	OU723926
		Ls41	♀	25.06.2012	Russia, Khabarovskiy Kray, 44 km SE of Khabarovsk, Chirki Cordon, 48.19° N, 134.68° E	Dubatolov, V.V	OU723945-OU723947	OU723927
		Ls118	♀	15.07.2014	- “ -	- “ -	OU723948	OU723928

Table 1 (continued)

Species	Subspecies	Specimen	Sex	Date	Locality	Collector	Accession # for histone H1 gene	Accession # for *COI*
		Ls135	♀	02.07.2015	Russia, Khabarovskiy Kray, 28 km SE of Khabarovsk, Bychikha village, 48.29° N, 134.83° E	- " -.	OU723949	OU723929
		Ls136	♂	02.07.2015	- " -	- " -	OU723950	OU723930
	sydyi	Ls142	♂	16.06.2017	Russia, Altayskiy Kray, 15 km SE of Staroaleyskoe village, the Aley River at the Taraskina Mt. foot, 50.92° N, 82.15° E	Zinchenko, V.K	OU723951, OU723952	OU723931
		Ls143	♂	16.06.2017	- " -	- " -	OU723953	OU723932
		Ls002k	♂	20.06. 2004	Kazakhstan, East Kazakhstan Province, Landman village env., 49.77° N, 84.33° E	Lukhtanov, V.A	OU723954	OU723933
		Ls004k	♂	20.06. 2004	- " -	- " -	OU723955, OU723956	OU723934
L. amphyssa		Lam12	♀ ?	26.07.2010	Russia, Khabarovskiy Kray, 28 km SE of Khabarovsk, Bychikha village, 48.29° N, 134.83° E	Dubatolov, V.V	OU230924	-
L. moltrechti		Lm105	♂	01.07.2014	Russia, Primorye, Barabash village env., 43.18° N, 131.48° E	Kosterin, O.E	OU230932	-
		Lm103	♂	01.07.2014	- " -	- " -	OU230933-OU230934	-
L. populi		Lp45	♂	14.07.2013	Russia, Novosibirsk Province and District, the Shadrikha River valley 1.5 km NE of Shadrikha village, 54.82° N, 83.22° E	Kosterin, O.E	OU230941-OU230942	-
		Lp46	♂	14.07.2013	- " -	- " -	OU230943	-
		Lp47	♂	14.07.2013	- " -	- " -	OU230944-OU230945	-
		Lp48	♂	14.07.2013	- " -	- " -	OU230946	-
		Lp49	♂	14.07.2013	- " -	- " -	OU230947-OU230949	-
		Lp50	♂	15.07.2013	Russia, Novosibirsk Academy Town, the Zyryanka River valley, 54.82–83° N, 83.10–11° E	Kosterin, O.E	OU230950	-
		Lp51	♂	15.07.2013	- " -	- " -	OU230951-OU230957	-
L. reducta		Lr133	♀	29.07.2015	Russia, Krasnodarskiy Kray, Kabardinka village NW suburbs, 44.66° N, 37.91° E	Kosterin, O.E	OU230925	-
L. arthemis		La0			ATOLep tag number AV-91–0116	Provided by Kim Mitter, ATOLep collection, USA	OU230970-OU230973	-

– The nuclear histone H1 gene (partial, but most part of its coding region). Unlike core histones, histone H1 is one of the most variable eucaryotic proteins because of its variable C-terminal hydrophilic domain (Berdnikov et al., 1993; Doenecke et al., 1997; Happel & Doenecke, 2009; Kosterin et al., 1994; Ponte et al., 2003; Caterino et al., 2011). This allowed us to use histone H1 gene(s) for phylogenetic reconstructions at inter- and even intraspecies level (Solovyev et al., 2015a; Zaytseva et al., 2012, 2015). In insects, hundreds of copies of the so-called *histone repeat*, containing the genes of histones H1, H2A, H2B, H3 and H4 and spacers between them, usually form the *histone cluster* (Eirín-López et al., 2009; Solovyev et al., 2015a) (in some species, e.g. *Drosophila virilis* Sturtevant, 1916, *Chironomus thummi* (Kieffer, 1911), the histone H1 gene is situated beyond the histone repeat, see Eirín-López, et al., 2009).

This combination of the popular and new markers allowed to clarify the relatively recent age of the Palaearctic disjunctions in *Limenitis*.

DNA extraction, amplification, cloning and sequencing

Genomic DNA from frozen, preserved in alcohol or dried samples was extracted following Solovyev et al. (2015a).

The mtDNA fragment including a 3′ part of the *COI* gene and 5′ part of the *tRNA-LEU* was amplified with the published universal insect forward primer Eva and reverse primer Pat (Simon et al., 1994). To improve performance, we worked out a version of the latter primer, PatM, to fit the corresponding sequence of *L. helmanni* available in public databases (accession NC_034754). This primer pair worked well and was used for sequencing *L. helmanni* and *L. camilla* mtDNA but gave unstable results with *L. sydyi*, so that we managed to sequence the studied fragment only in one specimen, *Ls41*. Basing on this sequence we worked out a specific reverse primer Syd, which was used to sequence a shorter fragment in other specimens. For sequencing the studied fragment in *L. camilla* and *L. sydyi*, additional reverse primer HCO (Folmer et al., 1994) and forward primer Cam were used, the latter worked out by us to fit the sequence DQ205111 of *L. camilla*. Primers were designed using the Primer3 software (http://frodo.wi.mit.edu/ (Rozen & Skaletsky, 2000) and produced by Biosset firm (Novosibirsk). Details of the primers are provided in Table 2 and their disposition in Supplement Fig. 1s.

The histone H1 has highly variable terminal domains (Ponte et al., 2003) which make impossible working out primers fitting to its gene in broad range of objects. But since the genes of histones H4, H1 and H2a are disposed in the histone repeat in this order, and the histone H4 and H2a genes and the region of the histone H1 gene coding for its central globular domain are evolutionary

Table 2 Primers used in the study

Designation	Sequence (5′-3′)	Direction	Match sequence	Match species
Eva	GGAGGATTTGGAAATTGATTAGTTCC	f	*COI*	Invertebrata (Simon et al., 1994)
Pat	TCCAATGCACTAATCTGCCATATTA	r	*tRNA-LEU*	Invertebrata (Simon et al., 1994)
HCO	TAAACTTCAGGGTGACCAAAA	r	*COI*	Invertebrata (Folmer et al., 1994)
Cam	GCCACACTTCACGGAACTCAA	f	*COI*	*L. camilla*
PatM	TCCATTACATATARTCTGCCATATTA	r	*tRNA-LEU*	*L. helmanni*
Syd	ATGAATGTTCAGCTGGCGGT	r	*COI*	*L. sydyi*
CCMi	CGTTATAAGAATGTTCAGCTGGA	r	*COI*	*L. camilla*
CCNu	CATTATAAGAATGTTCAGCTGGG	r	*COI*	*L. camilla*
LH4-2f	ATGACCGGTCGCGGTAAAGGAGGC	f	H4	*B. mori* (Solovyev et al., 2015a)
m40H2ar	GTTTTCCCAGTCACGACCCTTMACTTTKC-CVCCTTT	r	H2A	*H. erato*, *B. mori*, *Oreta* sp., *Eversmannia exornata*, *Leptidea* sp.
m40H1f	CAGGAAACAGCTATGACATWCARACYA AGGGCAAGGG	f	H1	*H. erato*, *B. mori*, *Oreta* sp., *Eversmannia exornata*, *Leptidea* sp.
m20H1f2	GTAAAACGACGGCCAGTGCKATCAAGAAA TAYATMGCSGC	f	H1	*H. erato*, *B. mori*, *Oreta* sp., *Eversmannia exornata*, *Leptidea* sp.
m40H1f3	GTTTTCCCAGTCACGAAGACCTCC GAKATGGT 143	f	H1	*L. helmanni*, *H. erato*
m24H1r	AACAGCTATGTCCATGCCTTGCCCTTRGTY TGWAT	r	H1	*H. erato*, *B. mori*, *Oreta* sp., *Eversmannia exornata*, *Leptidea* sp.
Lsp1f	AATGGCTGATACCGCTGTCG	f	H1	*L. helmanni*
Lsp2r	GACGCCGCCGCTTTCTT	r	H1	*L. helmanni*, *L. arthemis*
HeH1C-2r	CTTCTTTGGCTTGGGCGCTTT	r	H1	*H. erato*

conservative, at the first step we used the approach by Solovyev et al. (2015a) and amplified and sequenced, in specimens *Lh-1*, *Lh-3* and *Lh*-29 of *L. helmanni*, two DNA fragments, one spanning from the 3′-part of the histone H4 gene to the conservative part of the histone H1 gene, and the other from the H1 gene conservative part to the 5′-part of the histone H2a gene, each including a non-coding spacer between genes. We followed the approach by Wahlberg and Wheat (2008) and worked out the following hybrid degenerate primers on the basis of the H1 and H2a gene sequences of *Heliconius erato* (Linnaeus, 1758), *Bombyx mori* (Linnaeus, 1758) and *Leptidea* spp.: m40H1f, m20H1f2, m40H1f3, m24H1r, m40H2ar (Table 2); the primer LH4-2f was adopted from our earlier work (Solovyev et al., 2015a).

PCR mixtures (30 µl) contained 0.2 mM of each dNTP, 1.5 mM $MgCl_2$, 0.5 µM of each primer, 1 µl (30–60 ng) of genomic DNA, 1X polymerase buffer and 1 U of Taq DNA polymerase (Lab. Medigen, Novosibirsk, Russia) or 1 U of Smart-Taq DNA Polymerase (Lab. Medigen). PCR was performed using a thermal cycler MyCycler (Bio-Rad, USA) with the following program: (1) 94 °C–2 min 30 s, 1 cycle; (2) 95 °C–15 s; 55–57 °C (adjusted for specific cases)–30 s; 68 °C–3 min, 35 cycles; (3) 68 °C–4 min, 1 cycle. For amplification of difficult matrices were used KAPA3G Plant PCR kit (KAPABiosystems, USA) following the manufacturer's instructions.

Sanger reaction was conducted in 30 µl volume of mixture containing 1 µl BrightDye Terminator version 3.1 (Nimagen, Netherlands), 200–400 ng of DNA, 5 pmol of primer and 6 µl of buffer solution for BrightDye 3.1. MyCycler (Biorad, USA) was used with the following program: 95 °C–45 s; 50 °C–30 s; 60 °C–4 min; 26 cycles. Analyses of the products were carried out at the SB RAS Genomics Core Facility, Novosibirsk.

To distinguish paralogous copies of the histone H1, the amplicons obtained from *La0*, *Lc30*, *Lc120*, *Lh55*, *Lh117*, *Lp49* and *Lp51* specimens (see the section "Intra-species and intra-specimen variation of the H1 gene in Limenitis spp." for the explanation) were cloned into pGEM-T Easy vector (Promega, USA). One Shot Max Efficency DH5α-T1 competent cells (Invitrogen, USA) were used for transformation; plasmid DNA from app. 30 different clones was extracted by alkaline lysis and directly sequenced. In other cases, polymorphism in sequences was detected as superimposition of signals in Sanger reaction sequenograms.

The real-time qPCR was preformed to estimate the number of copies of histone H1 genes. A single-copy GPADH gene was used as endogenous control. Specific primers for histone H1 and GAPDH genes were designed using Primer 3 (http://frodo.wi.mit.edu) and OligoAnalyzer 3.1 (http://eu.idtdna.com/calc/analyzer). The forward and reverse primer sequences are listed in Table 1s (supp). The qPCR cycling conditions were 95 °C for 5 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 20 s. Data were collected at 60 °C. A melt curve analysis was performed at the end of each qPCR experiment. All real-time qPCRs were performed on the Bio-Rad CFX platform (Bio-Rad, USA) using qPCR set with Eva Green I (Syntol, Russia). The qPCR data were analysed using the ΔCt method, cycles of quantification (*Cq*) defined for GAPDH and histone H1 genes, to define numbers of copies histone H1 genes the difference of *Cq* values (ΔCt) used as power of 2.

Sequence alignment and analyses

For sequence alignments, calculating genetic distances and constructing phylogenetic trees using the neighbour joining and maximum likelihood methods, the MEGA 6.0 software package (http://www.megasoftware.net) (Tamura et al., 2013) was used, with the regions involved into indels excluded (the complete deletion mode).

The Bayesian approach implemented in the BEAST v. 2.4.3 software (Bouckaert et al., 2014) was used for the following: (i) the reconstruction of a phylogenetic tree and estimation of the substitution rate in the histone H1 gene of the studied *Limenitis* spp.; (ii) the formal Bayesian estimation of the time of divergence of in the western and eastern part of the range of *L. helmanni* based on *COI* sequences. Jmodeltest 2.1.10 program (Darriba et al., 2012) was used for the model choice. For both purposes we used the HKY + G model of nucleotide substitution, the base frequences estimated from the sequences, and parameters estimated in runs of 10 million generations with a pre-run burn-in of 1 million generations. For the former purpose (i) we used the time of existence of the most recent common ancestor of the *Limenitis* spp. (10.9 mya, $\sigma = 1.95$) estimated by Ebel et al. (2015). We used the Yule model of branching and the log-normal distribution of rate substitution in one run for the estimation of the substitution rate. For the second purpose (ii) of the divergence time estimation we used the estimation of the substitution rate of the COI gene by Quek et al. (2004) and the Yule and the birth-and-death models in 6 independent runs. Tracer 1.6 software (Rambaut et al., 2014) was used for combining independent runs and FigTree 1.4.3 software (http://tree.bio.ed.ac.uk/software/figtree/) by A. Rambaut and DensiTree (Bouckaert, 2010) software were used for visualisation of the consensus tree. Haplotype networks were visualised in Network 5 software (http://www.fluxus-engineering.com/sharenet.htm).

Table 3 qPCR results for the genes *GAPDH* and histone H1 in *Limenitis* sp

Species	Specimen	Cq GAPDH	Cq histone H1	ΔC_t	$2^{\Delta Ct}$	Repetitions[1]
L. helmanni	Lh29	28.52	23.43	5.09	34.01	1
	Lh55	33.05	27.55	5.50	45.22	2
		36.50	30.92	5.58	47.75	2
		24.98	19.34	5.65	50.09	1
	Lh116	25.31	19.75	5.56	47.20	1
	Lh126	23.28	18.32	4.97	31.24	1
L. camilla	Lc121	36.69	30.63	6.06	66.75	2
		26.24	20.10	6.14	70.60	1
	Lc40	26.41	20.19	6.22	74.43	1
L. sydyi	Ls5s	27.57	21.27	6.17	72.11	2
		29.39	23.23	6.16	71.50	2
		27.12	20.98	6.14	70.29	1
L. populi	Lp50	26.81	20.50	6.31	79.26	2
		30.00	23.63	6.37	82.93	2

[1]Value 1 means that the Cq values were estimated in one qPCR experiment for both *GAPDH* and histone H1 genes, value 2 means that they were estimated in two independent experiments for each gene and averaged

Results

Interspecies variation of the histone H1 gene in *Limenitis*

We sequenced most part of the histone H1 gene for in 64 specimens of nine species of *Limenitis* spp. and found intraspecies and even intra-individual variation in some of them (see the next section). Therefore, the inter-species comparison of the primary structure of this gene (Supplementary Fig. 2s) was made using a single sequence representing each species. These are indicated below in parentheses as follows ([specimen designation in Table 1]_[sequence variant designation according to Tables 3, 4, 5, 6, 7, 8, 9, 10, 11, and 12, where necessary], [the number of specimens investigated in a given species]): *L. arthemis* (*La0*_B, 1), *L. populi* (*Lp49*_B0, 7), *L. helmanni* (Lh29_A0, 29), *L. camilla* (*Lc39*_D0, 11), *L. sydyi* (*Ls118*_D0, 13), *L. amphyssa* Ménétriés, 1859 (*Lam12*, 1), *L. moltrechti* Kardakoff, 1828 (*Lm105*_B, 2), *L. reducta* Staudinger, 1901 (*Lr133*, 1). The sequence of specimen *Lh29*_A0 of *L. helmanni* was used as the basis for comparison and as a reference for the position numbers in interspecies comparison (Supplementary Fig. 2s). Further on nucleotide position numbers are given outside parentheses while amino acid position numbers inside parentheses.

The nucleotide sequence coding for the central globular domain of H1 was invariably 219 bp long. In this sequence, there were 28 positions polymorphic between species, all substitutions being synonymous and confined to the third codon positions. The histone H1 gene sequence corresponding to the globular domain of *L. populi* was most diverged from other species.

In *L. helmanni*, the sequence coding for the N-terminal domain of histone H1 was 117 bp (corresponding to 39 amino acid residues). In other species, indels and few nucleotide substitutions were found as compared to this sequence. *L. populi* has from 1 to 6 additional alanine codons inserted after position 63 while in *L. moltrechti*, *L. arthemis* and *L. amphissa*, one, two and three alanine codons respectively are inserted after position 93. After

Table 4 Examples of histone H1 gene variants in *L. camilla*

After position 402
Lc120_A TCC**GCCGCCGCC**----------GCT
Lc30_B TCC**GCCGCCGCCGCC**---------GCT
Lc31_C TCC**GCCGCCGCCGCCGCC**------GCT
Lc120_D TCC**GCCGCCGCCGCCGCCGCC**---GCT
Lc120_E TCC**GCCGCCGCCGCCGCCGCCGCC**GCT
After position 606
Lc19_D GCCGCC**GCGGCGGCGGCGGCG**---TCG
Lc19_F GCCGCC**GCGGCGGCGGCGGCGGCG**TCG

position 81 an insertion of the glycine codon was found in *L. sydyi* and of the threonine codon in *L. arthemis*, and in the latter species also the glycine codon was found to be deleted after position 84. Of 21 polymorphic positions found in this domain, only 7 positions were associated with five amino acid substitutions: G67A (amino acid substitution A23T) in *L. camilla*, G34C, C35A (A12Q) in *L. sydyi*, A27C (E9D) and G79A (A27T) in *L. arthemis*, and G94A and C95A (both leading to amino acid substitution A32N) in *L. moltrechti*.

In the histone H1 molecule, the most variable is the C-terminal domain which undergoes frequent indels due to its repetitive nature (Ponte et al., 2003). Expectedly, we found indels in the corresponding part of the histone H1 gene in *Limenitis* spp., which for the sequences compared (Supplementary Fig. 2s) are as follows. After position 402, one of the 3 alanine codons was replaced with a double threonine codon in *L. populi* and with a valine codon in *L. reducta*. Additional alanine codons after this position were inserted in *L. amphyssa* (one codon), *L. moltrechti* (two codons), *L. camilla* and *L. athemis* (4 codons in each), and *L. sydyi* (5 codons). In *L. sydyi*, a threonine codon was inserted after position 417. The number of tandemly repeated polyalanine and polyserine codons after position 483 was variable: 4–7 alanine codons and 2–6 serine codons, in several cases serine codons were replaced with threonine codons. After position 519, a serine codon was inserted in *L. sydyi* and double alanine and triple threonine codons (coding for AATTT) were inserted in *L. reducta.* After position 537, a glycine codon was inserted in *L. populi.* After position 543, a valine codon was inserted in *L. arthemis*. After position 603, glycine and alanine codons were inserted in *L. sydyi*; a proline codon was replaced by two alanine codons and two serine codons (AAS) in *L. camilla*; and the proline codon was duplicated in this position in *L. arthemis*. After position 621, the serine codon was deleted in *L. populi*, *L.arthemis* and *L. sydyi* while the second proline codon was inserted in *L. arthemis*. In *L. populi*, *L. sydyi* and *L. arthemis*, after the alanine codon tract (position 645), one more alanine codon and serine codon were inserted. An alanine codon was inserted after position 726 in *L. arthemis*. The gene region coding for the C-terminal domain contained many nucleotide substitutions, including at least 14 non-synonymous ones among the species studied (in some cases the differences in repeated codon tracks could equivocally be interpreted either as substitutions or indels).

The number of histone H1 gene copies in the *Limenitis* genome was estimated by qPCR as several dozen: in the studied specimens of *L. sydyi*, *L. camilla* and *L. populi* as ca 70–80 and ca 30–50 in *L. helmanni* (Table 3).

Intra-species and intra-specimen variation of the histone H1 gene in *Limenitis* spp

The histone H1 gene fragment in question appeared homogeneous in the only studied specimen of *L. reducta* and homogeneous and identical in both studied specimens of *L. amphissa.* Other species, namely *L. arthemis*, *L. helmanni*, *L. camilla*, *L. sydyi*, *L. populi* and *L. moltrechti*, manifested heterogeneity for indels not only among specimens but also within some of them. The heterogeneity was noticed while analysing sequenograms of the relevant fragment amplified from genomic DNA and was later investigated by cloning in some specimens. Cloning revealed some additional nucleotide substitutions in paralogous copies of the studied histone H1 gene fragment, not recorded by genome DNA sequencing where only versions present in many copies were seen. When two variants of the histone H1 gene sequence were found in one specimen in comparable quantities (manifested as overlapping peaks in sequenograms resulted from sequencing from genomic DNA or in comparable numbers of clones), we could not distinguish heterozygotes for cis-homogeneous histone clusters from cases of cis-heterogeneous clusters. If more than two variants were revealed, we surely faced cis-heterogeneity for paralogous histone H1 gene copies.

Variants of the studied sequence were designated (independently for each species) by a letter and digit. The sequence length variants correspond to letters in alphabetic order, starting with A for the shortest; the digits denote variants differing in nucleotide substitutions.

Limenitis arthemis

The studied histone H1 gene fragment was cloned in the only studied specimen of *L. arthemis* (La0) at our disposal. It exhibited heterogeneity in two regions of the histone H1 gene: (i) in the GCC-tract (this triplet codes for alanine) after position 402 enclosed between the serine codons TCC (at 5′-end) and TCG (at 3′-end); (ii) in the TCC-tract (this triplet codes for serine) after position 504 between the alanine codon GCA at 5′-end and serine codons at 3′-end. Cloning revealed four variants of the sequence. The most frequent between the clones was variant B ($(GCC)_7(TCC_5)$); less frequent were variants A ($(GCC)_7(TCC)_4$), C ($(GCC)_7(TCC)_6$) and D($(GCC)_8(TCC)_5$). Besides, there was a synonymous polymorphic position 84 occupied by either C or T.

Limenitis moltrechti

In *L. moltrechti*, position 402 was followed by either 3 or 4 tandem repeats of the GCC alanine codon, with both variants revealed in specimen *Lm103* but only the latter in *Lm105*.

Limenitis camilla

The histone H1 gene of *L. camilla* after position 402 had a tract of 3 to 7 alanine codons GCC (variants A–E) (Table 4). It was cloned in specimens *Lc30* and *Lc120*. The number of sequence variants found in a specimen varied from one to three (in *Lc120*). Besides, specimen *Lc19* (from which the histone H1 gene was not cloned) exhibited heterogeneity of the tract after position 606 which contained 5 (variant D) or 6 (variant F) alanine codons GCG. Variants A0, B0, D0 and E0 had A in position 603; variants B1 and D1 had C in this position. Variant B2 differs from B0 by synonymous substitutions T234C and A603C and a non-synonymous substitution A592G (corresponding to K198E). Variant D2 differs from D0 by synonymous substitutions T234C and C408T and a non-synonymous substitution T349C (S117P); variant E2 differed from E0 by a synonymous substitution A729G and non-synonymous substitutions C58T (P20S) and G448A (A136T). Altogether, 9 different sequences of the histone H1 gene were revealed in *L. camilla* (Table 5).

Limenitis helmanni

In *L. helmanni*, position 483 was followed by a heterogeneous region with a polyalanine and polyserine tract $(GCC)_k(GCA)_l(GCC)_m(TCC)_n$, where $k = 0–1$, $l = 3–4$, $m = 3–5$ and $n = 2–4$. Nucleotide substitutions increase diversity of the paralogous H1 gene copies. Taking them into account, 17 sequence variants were revealed (Tables 6 and 7). The length polymorphism (variants A–J) was observed both between and within specimens. The studied histone H1 gene fragment was cloned for specimens *Lh55* and *Lh117*.

Position 99 was heterogeneous (A or G) in specimen *Lh23*, position 474 (G or T) in specimen *Lh128*, and position 388 (A or G) in specimens *Lh23*, *Lh57* and *Lh127* (Table 7). The substitutions were revealed in specimens where the H1 gene was only sequenced from genomic DNA, so they were not put into correspondence to certain length variants. The variants with index '0' (A0, C0, E0, F0, G0, H0, J0) had A in position 388; the variants with index '1' (A1, B1, D1) had G there. As compared to A0, the variant A2 differed by substitutions A99G (synonymous) and A388G (A130T); the variant A3 by two synonymous substitutions A99G and A388G and a non-synonymous substitution A669T (K223N); the variant A4 by a non-synonymous substitution A167G (K56R). As compared to the variant B0, the variant B2 had synonymous substitutions A99G and A388G; the variant B3–a non-synonymous substitution G76A (A26T). As compared to the variant C0, the variant C2 had a synonymous substitution G429A and the variant C3–a non-synonymous substitution G730A (A244T).

Limenitis sydyi

The most part of the histone H1 gene sequenced in *L. sydyi* had a variable length of 669–681 bp because of indels; no cloning was made. The variation concerned the polyalanine tracts: 4 to 9 codons GCC after position 402 with, and 3 or 4 codons GCG after position 606. Six variants (A–F) for the sequence length were revealed (Table 8). Four nucleotide substitutions were registered: G or T in position 144 in *Ls13*; T or G in the same position in *Ls142*; G684T in *Ls4*; A372T in *Ls143* (the histone H1 gene sequences of this specimen was heterogeneous for this substitution since the sequenogram contained in this position overlapping peaks of A and T) (Table 9).

Limenitis populi

L. populi exhibited the greatest diversity of histone H1 gene variants among the species studied. This gene was cloned in specimens *Lp49* and *Lp51*. As different from other species, indels were found (by sequencing from genomic DNA samples) also in the region coding for the N-terminal domain. The position 63 (according to the reference sequence of *L. helmanni*) was followed by a GCC (alanine) tract of 3, 6, 7 or 8 copies (length variants A, B, C, D, respectively) (Table 10). Most frequent was the variant B having $(GCC)_6$.

Besides, sequencing from genomic DNA revealed indels in the region coding for the C-terminal domain (length variants E, B1, F, G). As compared to the variant A, the variant G contained after position 465 a large 75-bp long deletion corresponding to the amino acid sequence KAAT AAAAAATSPAKSKPTKGAATK. The variant F (specimen *Lp50*) after the same position had a 30-bp deletion corresponding to the decapeptide KAATAAAAAA; in addition there was a duplication of a threonine codon ACC after position 504. The variants E and H differed from the variant B by insertions in the region coding for the C-terminal domain, GCCACC (coding for AT) after position 504 in the former and GCAGCC (AA) after position 486 in the latter. Several non-synonymous substitutions were found in the variant B family: G628T (A210S) in the variant B1, A101G (K34R) in B2, A467G (K156R) in B3. The variant C1 differed from the variant C by a non-synonymous substitution A112G (K38E) (Table 11). Interestingly, such a diversity was found in the same population from Novosibirsk environs, where all analysed specimens were collected.

Variation of the histone H1 gene in different range parts of *Limenitis* spp. with disjunct ranges

Occurrence of the histone H1 variants expectedly appeared uneven in different parts of the disjunct ranges of three species of *Limenitis*.

The eastern and western disjunct parts of the range of *L. helmanni* strongly differed in a predominating type of the H1 gene variants. In the Far Eastern specimens, the variants C predominated (found in 11 of 16 specimens, ~ 70%) while in the western part of the species range they were found only in 3 specimens of 13 (~ 23%). In the western part, variants B predominated (found in 10 specimens of 13; ~ 77%) while in the eastern part, this variant was found in only one specimen (~ 6%). The variants A were found in 5 specimens from each part of the range. Other variants were rare: variants F, G and E were found in two specimens while variants H and J in one specimen each. These rare variants were found in either of the range parts only: variant D in the western part while variants of E, F, H, G and J in the eastern part. So, 5 of 8 variant types, here denoted with letters, were found only in the eastern part, while the number of specimens analysed from the western and eastern range parts was comparable (although yet small): 13 and 16, respectively.

All specimens of *L. camilla* were found to contain variant D (Table 5, Fig. 3a). The variants B were found in both range parts; the variants A and E were found only in the western part of the range, in three and one specimens respectively; the variant C was found in one specimen in the eastern part.

In *L. sydyi* the variants C and D were found in both range parts, while the variants A, B and E only in the eastern part, but the western sample of four specimens was too small to make any conclusions (Table 9).

Neither of the nucleotide substitutions found in the histone H1 gene in the three studied species with disjunct ranges was diagnostic for any range part, that is found in all specimens from a given part but not elsewhere. In *L camilla*, two substitutions were found in one specimen *Lc12* from the western range part and three substitutions were found, also in one specimen *Lc30*, from the eastern range part (Table 5). In *L. sydyi* four substitutions were revealed in different specimens, two from the western range part and two from the eastern range part (Table 9). In *L. helmanni*, the polymorphic position 388 was occupied by either G or A (A130T) in both western and eastern part of the species' range (Table 7) but with different frequences: G was more frequent (77% of specimens) in the West Siberian part of the range while A was more frequent (75%) in the Far Eastern part. In addition, three nucleotide substitutions were revealed in the western range part and four in the eastern range part. All but one of them were found in one specimen only, while a synonymous substitution A99G was met in four specimens from the eastern range part.

Substitution rate in the histone H1 gene in *Limenitis*

Figure 2 shows a phylogenetic tree of the studied *Limenitis* species reconstructed on the basis of the histone H1 gene with the use of Bayesian approach. In this tree, *L. sydyi* represents a long early diverged branch, the Palaearctic *L. populi* clusters together with the Nearctic *L. arthemis*, and *L. camilla* and *L. helmanni* form a tight cluster as recently diverged sister species. Based on the estimate of the time of existence of the most recent common ancestor (MRCA) of *Limenitis* spp. by Ebel et al. (2015) of 10.9 mya (7.06–13.77 HPD95%), we estimated the average substitution rate in the histone H1 gene as 6.2×10^{-9} per site per year (HPD $3.5–9.2 \times 10^{-9}$). This rate expectedly varies in different branches being the highest in *L. camilla* (10.5×10^{-9}) and *L. reducta* (10.7×10^{-9}) and the lowest in *L. amphyssa* (3.1×10^{-9}).

Variation of the *COI* fragment in *Limenitis* species with disjunct ranges and an attempt to estimate the disjunction age

A ca 1.2-kb-long mtDNA fragment containing the 3′ region of the *COI* gene was sequenced in most available specimens of the three *Limenitis* species with disjunct Palearctic ranges. In *L. helmanni* the region shared by all individual sequences was 1217 bp long, corresponding to positions 1734–2950 of the mitochondrial genome of *L. helmanni* available in public databases (accession NC_034754). This numeration will be used below, while the amino acid positions will be given starting from the first amino acid residue in the *COI* protein.

In 24 specimens of *L. helmanni*, 10 polymorphic sites were found. The sequence from specimen *Lh28* differed from the consensus by a substitution G2370A; *Lh124*–by a substitution G2595A. Specimens *Lh115*, *Lh116*, *Lh117* and *Lh18* shared 6 substitutions as compared to the consensus sequence: T1914C, C2001T, T2046C, T2064C, C2313T, G2370T; besides, *Lh 115*, *Lh117* and *Lh18* had two more substitutions A2163G and T2805C, while *Lh52* had a substitution T2817C (Table 12).

In 10 specimens of *L. camilla*, 1245 bp were sequenced (positions 1698–2945 according to NC_034754). Interestingly, specimens *Lc39*, *Lc40*, *Lc119*, *Lc121* and *Lc122* demonstrated heterogeneity for this sequence, with several nucleotides simultaneously found in some positions. All 43 polymorphic positions found were in the *COI* gene coding sequence; 39 of them were in the first or third codon positions and synonymous. The non-synonymous nucleotide substitution A2270G resulted in the amino acid substitution Y272C; T2903C resulted in L483S; two more nucleotide substitutions, G2923A and A2925G, were in the same codon and resulted in the amino acid substitution E490K. Such an extraordinary variation was explained by *L. camilla* having a copy of the *COI*-containing mitochondrial DNA fragment in its nuclear genome (NUMT). Sequences of the mitochondrial *COI* in other *Limenitis* spp. available in public databases unequivocally suggested that position 272 was invariably occupied by tyrosine (in *L. amphissa*, *L. archippus*, *L. arthemis*, *L. doerriessi*, *L. chosensis*, *L. ciocolatina*, *L. glorifica*, *L. helmanni*,

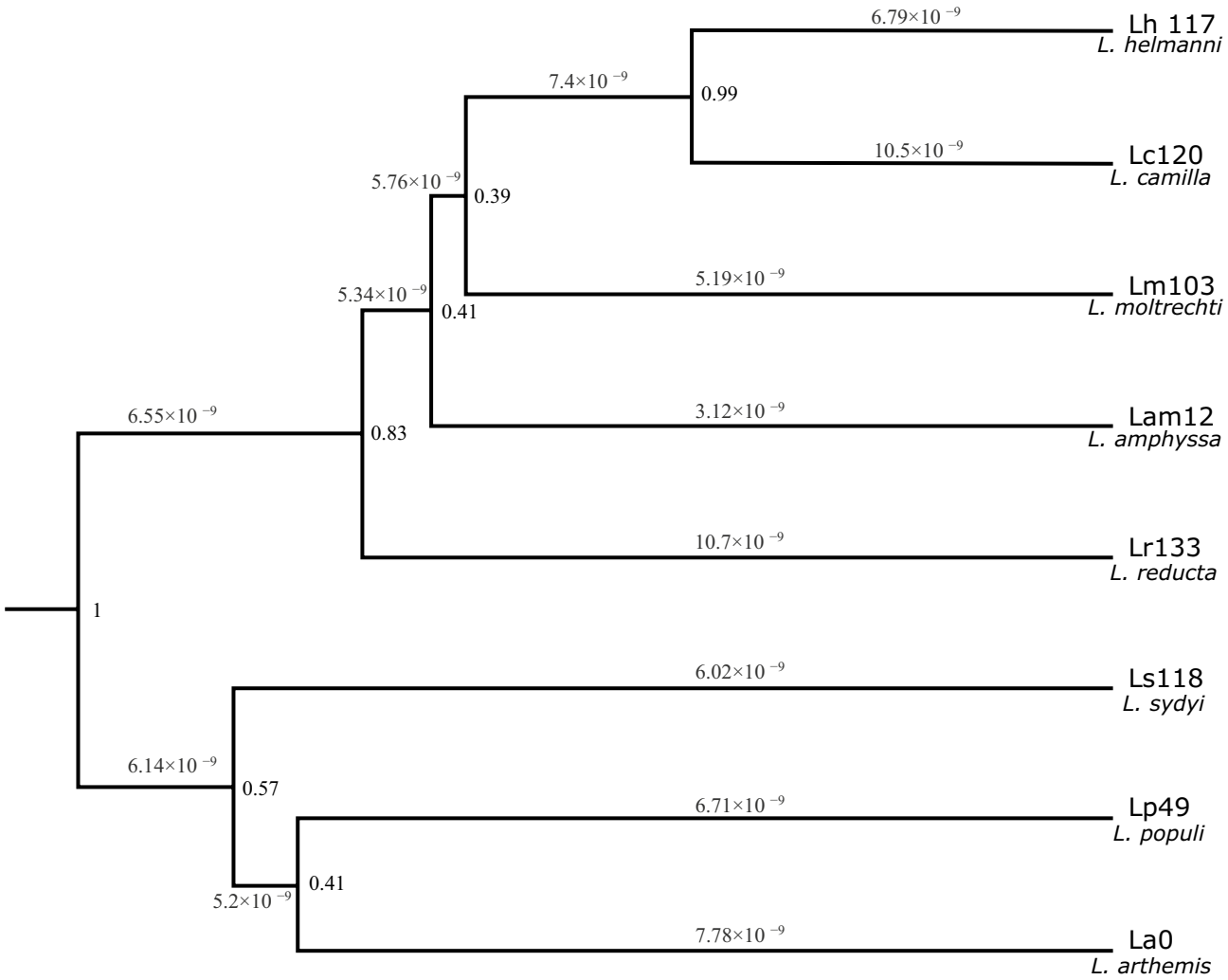

Fig. 2 The consensus Bayesian tree for *Limenitis* spp. based on 699 bp of the histone *H1* gene sequence. Posterior probabilities are indicated at nodes; mean substitution rates (sites/mya) are indicated above branches. Designations of specimens are as in Table 1

L. homeyeri, *L. lepechini*, *L. moltrechti*, *L. lorquini*, *L. populi*, *L. reducta*, *L. sydyi*, *L. sulpitia*, *L. weidemeyerii*), position 483 by leucine and position 490 by glutamate (in *L. arthemis*, *L. amphissa*, *L. doerriessi*, *L. glorifica*, *L. helmanni*, *L. homeyeri*, *L. moltrechti*, *L. populi*, *L. sydyi*, *L. pausanias*, *L. weidemeyerii*). This allowed us to assume the nucleotides A, T, G and A in respective positions 2270, 2903, 2923 and 2925 as belonging to the mitochondrial *COI* gene and other nucleotides to the NUMT. Using the specific reverse primers CCMi for the mitochondrial sequence and CCNu for the NUMT allowed us to selectively amplify and sequence them. The mitochondrial sequence appeared identical in all studied specimens of *L. camilla* regardless of their provenance.

In 16 specimens of *L. sydyi*, 1242-bp-long sequence of *COI* (positions 1698–2939 according to NC_034754) was sequenced. A synonymous nucleotide substitution C1950T was found in 4 of 11 specimens from the eastern range part (*Ls13*, *Ls41*, *Ls135*, *Ls136*), while in 3 specimens from both range parts (*Ls002k*, *Ls004k*, *Ls118*) both variants were distinctly revealed in sequenograms. A synonymous substitution T2829C was found in the specimen *Ls44*.

Among the three species studied, *L. helmanni* demonstrated the greatest, although still low, nucleotide variation in the *COI* fragment. Therefore, we made an attempt to formally estimate the age of its range disjunction using the Bayesian approach implemented in the BEAST software (Bouckaert et al., 2014), with the Yule and birth-and-death models of branching. The respective estimates obtained for the mean divergence time of the eastern and western range parts of *L. helmanni* were 194 kya (HPD95% of 70–337 kya) and 247 kya (HPD 95% 85–443 kya). The enormous highest probability density (HPD) intervals obtained suggest them rather uninformative and indicate that this method actually failed to adequately estimate the divergence time that was expected for the situation of low variation and absence of substitutions fixed in different range parts.

Table 5 Histone H1 gene sequence variants revealed in *L. camilla*. Letters A–F correspond to length variant; digits denote variants differing in nucleotide substitutions. Nucleotide substitutions (with the corresponding amino acid substitutions, or '-' for synonymous substitutions, in parenthesis) are indicated as compared to the reference sequence of the specimen *Lc40*; positions are indicated according to the sequence of the specimen *Lh29A*

Range part	Specimen	The number of length variants of histone H1 gene	The number of GCC after position 402	The number of GCG after position 606	Polymorphism in position 30	Polymorphism in position 603	Substitutions	Variants of histone H1 gene
East	*Lc19*	2	6	5, 6	K	M		D?, F?
	*Lc30**	2	4	5	G	C		**B1**
							T234C (-), A592G (K198E)	**B2**
			6	5	G	C		**D1**
					G	A	T234C (-), C408T (-), T349C (S117P)	**D2**
	Lc31	2	5, 6	5	K	C		C?, D?
	Lc38	1	6	5	G	A		D0
	Lc39	2	4, 6	5	G	A		B0,D0
	Lc40	1	6	5	G	A		D0
West	*Lc119*	2	4, 6	5	G	C		D1,B1
	*Lc120**	3	3	5	G	A		**A0**
			6	5	G	C		**D1**
			7	5	G	A		**E0**
					G	A	C58T (P20S), G448A (A136T), A729G (-)	**E2**
	Lc121	2	3, 6	5	K	A		A?, D?
	Lc122	2	3, 6	5	G	A		A0, D0
	Ls152	1	4	5	G	C	-	B1

*In these specimens, the variants were revealed by cloning with further Sanger sequencing of clones (these variants are boldfaced in the last column)

Discussion

Inter-individual heterogeneity of the histone H1 gene in *Limenitis*

According to the RealTime qPCR data, the histone H1 gene is repeated 30–50 times in *L. helmanni*, and ca 80 times in *L. camilla*, *L. sydyi* and *L. populi*, which is the same repetition magnitude as in *Drosophila melanogaster* Meigen, 1830 which has ca 100 copies (Lifton et al., 1978). Taking into account repetitions of histone genes, the presence of different histone H1 gene variants in the same individual can result from the following: (i) heterozygocity for the entire histone gene cluster, when different variants are present in

Table 6 The length polymorphism of histone H1 genes revealed in *L. helmanni*.: indels after position 438

```
Lh53_A  TCCTCCGCCGCAGCAGCA---GCCGCCGCC------TCCTCC------TCG
Lh52_B  TCCTCCGCCGCAGCAGCA---GCCGCCGCC------TCCTCCTCC---TCG
Lh53_C  TCCTCCGCCGCAGCAGCA---GCCGCCGCCGCC---TCCTCC------TCG
Lh52_D  TCCTCCGCCGCAGCAGCA---GCCGCCGCC------TCCTCCTCCTCCTCG
Lh117_E TCCTCCGCCGCAGCAGCA---GCCGCCGCCGCC---TCCTCCTCC---TCG
Lh15_F  TCCTCCGCCGCAGCAGCAGCAGCCGCCGCCGCC---TCCTCC------TCG
Lh28_G  TCCTCCGCCGCAGCAGCA---GCCGCCGCCGCCGCCTCCTCC------TCG
Lh15_H  TCCTCC---GCAGCAGCAGCAGCCGCCGCCGCC---TCCTCC------TCG
Lh22_J  TCCTCC---GCAGCAGCAGCAGCCGCCGCC------TCCTCC------TCG
```

Table 7 Histone H1 gene sequence variants revealed in *L. helmanni*. For the designation principle see the text. Positions are indicated according to the sequence of specimen *Lh29A* as a reference; nucleotide substitutions (with the corresponding amino acid substitutions, or '-' for synonymous substitutions, in parenthesis) are indicated as compared to the reference sequence

Range part	Specimen	The number of length variants of histone H1 gene	The number of certain codons after position 483				Polymorphism in position 388	Other substitutions	Variants of histone H1 gene
			GCC	GCA	GCC	*TCC*			
East	*Lh7*	1	1	3	3	2	G	A99G **(-)**	A2
	Lh15	2	1, 0	4	4	2	A		F0, H0
	Lh16	1	1	3	4	2	A		C0
	Lh17	1	1	3	4	2	A		C0
	Lh18	2	1	3	3,4	2	R		A?, C?
	Lh22	1	1	4	4	2	A		F0
		1	0	4	3	2			J0
	Lh23	1	1	3	4	2	R	A99R (-)	A?
	Lh28	1	1	3	5	2	A		G0
		1	1	3	4	2	A	G429A (-)	C2
	Lh29	2	1	3	3, 4	2	A		A0, C0
	Lh35	1	1	3	4	2	A		C0
	Lh36	1	1	3	4	2	A	C730A (A244T)	C3
	Lh37	2	1	3	3	2, 3	G	A99G (-)	A2, B2
	Lh107	2	1	3	4, 5	2	A		C0, G0
	Lh115	1	1	3	4	2	A		C0
	Lh116	2	1	3	4	2, 3	A		C0, E0
	*Lh117**	3	1	3	3	2	G	A99G (-), A669T (K223N)	**A3**
			1	3	4	2, 3	A		**C0, E0**
West	*Lh-3*	1	1	3	3	3	G		B1
	Lh-1	1	1	3	3	2	G		A1
	Lh43	1	1	3	4	2	A		C0
	Lh52	2	1	3	3	3, 4	G		B1, D1
	Lh53	2	1	3	3, 4	2	R		A0, C0
	Lh54	1	1	3	3	3	G		B1
	*Lh55**	2	1	3	3	2	G		**A1**
								A167G (K56R)	**A4**
			1	3	3	3			**B1**
								G76A (A26T)	**B3**
	Lh124	1	1	3	3	3	G		B1
	Lh125	1	1	3	3	3	G		B1
	Lh126	1	1	3	3	3	G		B1
	Lh127	2	1	3	3, 4	3, 2	R		B, C
	Lh128	1	1	3	3	2	G	G474K (-)	A
	Lh129	1	1	3	3	3	G		B1

*In these specimens, the variants were revealed by cloning with further Sanger sequencing of clones (these variants are boldfaced in the last column)

different homologous chromosomes but are shared by all copies within a cluster (the variants are orthologous), or (ii) cis-heterogeneity across a cluster in the chromosome (the variants are paralogous). We assume the second interpretation since in *L. arthemis*, *L. populi*, *L. helmanni*, *L. camilla*, and *L. sydyi*, individuals were found (*La0*, *Ls41*, *Lp51*, *Lh117*, *Lc120*) which possessed more than two histone H1 gene variants. Existence of individuals homogeneous for this gene (*Ls13*, *Lh17*, *Lc40*, *Lp46*) may be explained by the so-called concerted evolution when some paralogous variant expands to occupy the entire cluster (Nei & Rooney, 2005).

Deletions and insertions of short repeats inside the histone H1 gene may be ascribed to slippage mispairing during DNA replication (Ponte et al., 2003). Earlier, indels in the sequences

Table 8 The length polymorphism of histone H1 genes revealed in *L. sydyi*

The length variant of the histone H1 gene	The number of GCC after position 402	The number of GCG after position 606
A	6	3
B	7	3
C	7	4
D	8	4
E	9	3

coding for the N- and C-terminal domains of the H1 molecule were found in *Lepidoptera* at the intra-generic, inter-species level, e.g. in *Oreta rosea*, *O. paki* and *O. pulchripes* (Solovyev et al., 2015a), but hitherto were not reported at intra-species level and, more-over, in the same organism.

Most probably, the revealed paralagous variants originated relatively recently and independently in *Limenitis* species since they differ in sites of indels. At the same time, the length polymorphism of the polyalanine tracts after amino acid position 134 in *L. camilla*, *L. sydyi* and *L. arthemis* could be inherited from the common ancestor. *L. helmanni* possesses extraordinary polymorphism for the histone H1 gene variants found within specimens (Table 7). This polymorphism is unlikely to be inherited from the species' common ancestor since such long persistence of alleles in the species' history would result in segregation equilibrium among them. But if so, diverse variants as we found in *L. helmanni* would be in segregation equilibrium and we would hardly find homozygotes among specimens. However, 6 individuals *Lh1*, *Lh3*, *Lh17*, *Lh35* and *Lh115* were found homogeneous for the histone H1 gene (at least as far as our sequencing from total

Table 9 Histone H1 gene sequence variants revealed in *L. sydyi*. Positions are indicated according to the reference sequence *Lh29A* of *L. helmanni*, nucleotide substitutions (all synonymous) are indicated as compared to the consensus sequence of *L. sydyi*

Range part	Specimen	The number of length variants of gene histone H1	The number of GCC after position 402	The number of GCG after position 606	Substitutions	Variants of histone H1 gene
East	*Ls4*	1	7	3	G684T	B1
	Ls5	1	7	4		C0
	Ls13	1	7	3	G144T	B2
	Ls14	1	8	4		D0
	Ls32	2	6	3		A0
			8	4		D0
	Ls41	3	7	3		B0
			7	4		C0
			9	3		E0
	Ls118	1	8	4		D0
	Ls135	1	8	4		D0
	Ls136	1	8	4		D0
West	*Ls142*	2	7, 8	4	G144K	C?, D?
	Ls143	1	8	4	A372W	D0, D1
	Ls002k	1	8	4		D0
	Ls004k	2	7, 8	4		C0, D0

Table 10 The length polymorphism of the histone H1 gene revealed in *L. populi*. Positions are indicated according to the reference sequence *Lh29A* of *L. helmanni*

Variant	The number of GCC after position 63	After position 465	After position 486	After position 504
A	3	-	-	-
B	6	-	-	-
C	7	-	-	-
D	8	-	-	-
E	6	-	-	Insertion GCCACC
F	6	Deletion 30 bp	-	Duplication ACC
G	3	Deletion 75 bp	-	-
H	6	-	Insertion GCAGCC	-

Table 11 Histone H1 gene sequence variants revealed in *L. populi*. Positions are indicated according to the reference sequence *Lh29A* of *L. helmanni*; nucleotide substitutions are indicated (with corresponding amino acid substitutions in parenthesis) as compared to the consensus sequence of *L. populi*

Specimen	The number of length variants of histone H1 gene	The number of GCC after position 63	Indels	Substitutions	Variants of the gene histone H1
Lp45	2	6			B0
			Insertion GCCACC after position 504		E0
Lp46	1	6			B0
Lp47	2	6, 8			B0, D0
Lp48	1	6		G628T (A210S)	B1
*Lp49**	3	3, 6, 8			A0, B0, D0
Lp50	1	6	Deletion 30 bp after position 465 Duplication ACC after 504		F0
*Lp51**	5	6		A101G (K34R)	B2
				A467G (K156R)	B3
		7			C0
				A112G (K38E)	C1
		8			D0
		3	Deletion 75 bp after position 465		G0
		6	Insertion GCAGCC after 486		H0

*In these specimens, the variants were revealed by cloning with further Sanger sequencing of clones

genomic DNA could detect), and 3 of them (*Lh17*, *Lh35*, *Lh115*) had the same variant C (Table 7). Most probably this variant is ancestral and permanently generates other variants by indels due to slippage mispairing: indels in the GCC/TCC tracts resulting in the variants A, G and E while duplication of the GCA resulting in the variants F. This assumption is supported by the case of specimen *Lh117* which contains three variants altogether, which cannot be interpreted through mere heterozygosity. Polymorphism for the histone H1 gene in *L. camilla* can be more easily interpreted in the same way, since the variant D was found in all specimens studied, in some cases accompanied by variants of different lengths (Table 5), most probably derived from it through indels.

The histone H1 gene simultaneously reveals two modes of molecular variation: the regions coding for the conservative N-terminal and, especially, central globular domains and some parts of the C-terminal domain are under strict purifying selection where mostly synonymous nucleotide substitutions are allowed while most of the variable hydrophilic C-terminal domain is allowed to vary in size due to indels. Heterogeneity in the histone cluster exerts difficulties to sequencing, but at the same time, short intra-genic repeats apt to slippage mispairing are analogous to microsatellite loci and so provide some pitfalls of population history and theoretically may help in phylogeographic reconstructions. Indels may be useful for phylogeography as unequivocally marking some evolutionary lineages. However, the histone H1 gene in *Limenitis* seems to be too variable for this purpose, since frequent indels in its region coding for the C-terminal domain are prone to homoplasy, that is to multiple independent origins of apparently identical indels.

Table 12 Alleles of the *COI* gene fragments revealed in *L. helmanni*

West		East	
Lh-3, *Lh53–55*, *Lh125–129*	A	*Lh7*, *Lh16-17*, *Lh36-37*, *Lh107*	A
Lh124	A2	*Lh28*	A1
Lh52	A3	*Lh115*, *Lh117*, *Lh18*	B
Lh-1, *Lh35*	A (partially sequenced)	*Lh116*	B1

Nuclear copy (NUMT) of the *COI* region in *L. camilla*

The sequences of the *COI* region available in public databases for *L. camilla* form two groups differing for ca 2.7% (uncorrected *p*-distance), so that Dincă et al. (2015) considered a possibility of existence of two sympatric cryptic species. In our study, sequences of both groups were co-amplified from some specimens. This fact can be interpreted either as heteroplasmy for mitochondrial genomes or presence of a copy of the relevant mtDNA in the nuclear genome of *L. camilla*, a case traditionally abbreviated as NUMT (for 'nuclear mitochondrial DNA'; Richly & Leister, 2004). The former explanation looks very improbable since a gene drift under strict maternal

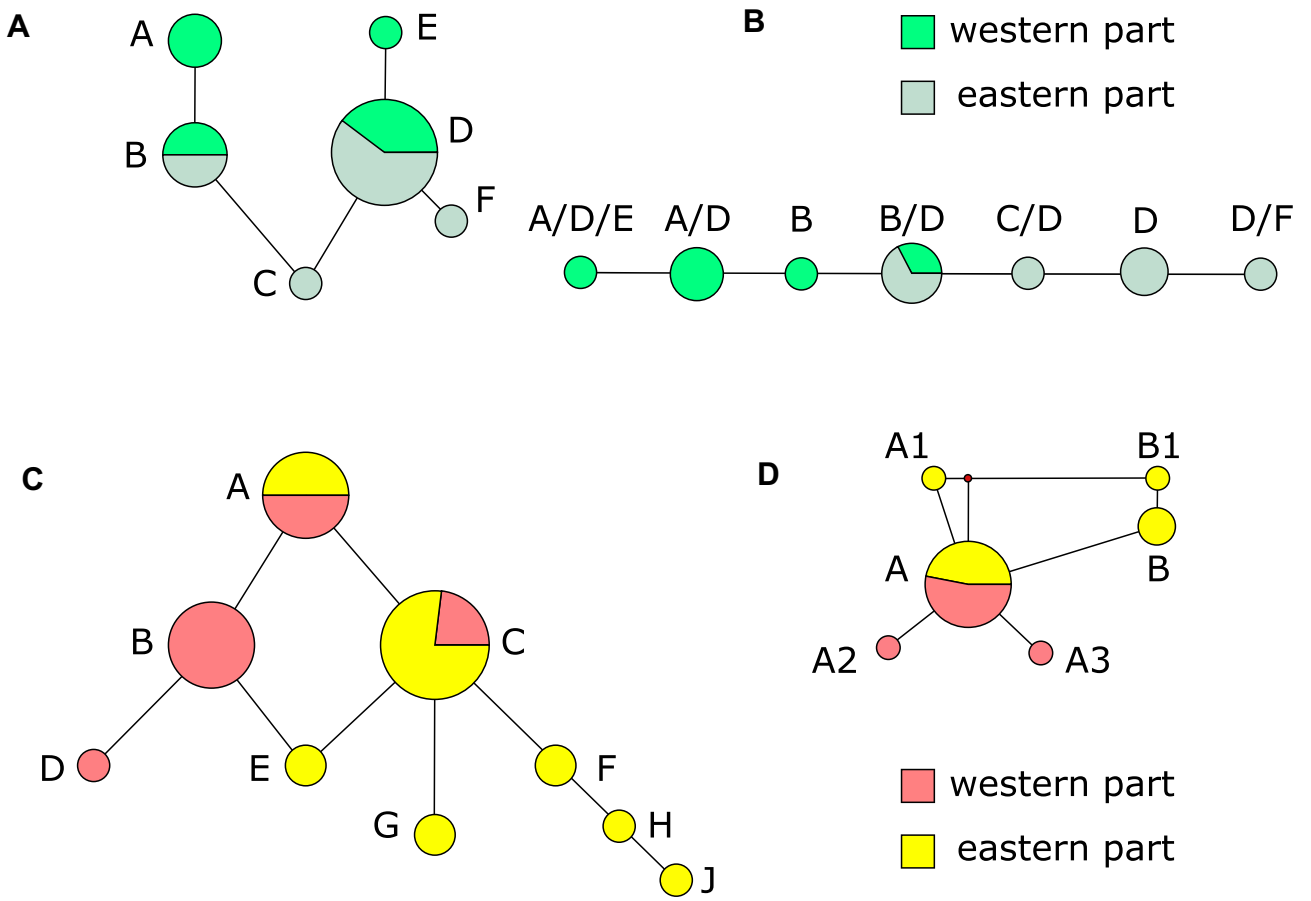

Fig. 3 Haplotypic networks for the studied histone H1 gene (**a**–**c**) and *COI* fragment (**d**) of *L. camilla* (**a**–**b**) and *L. helmanni* (**c**–**d**) from the eastern and western parts of their ranges; **b** shows representation of combinations of histone H1 gene haplotypic variants in individuals of *L. camilla*

transfer of mitochondria would result in a loss of one copy in a series of generations. At the same time, NUMTs involving *COI* were found in different insects: Hemiptera (Sunnucks & Hales, 1996), Orthoptera (Bensasson et al., 2000, 2001; Song et al., 2008; Zhang & Hewitt, 1996), Hymenoptera (Magnacca & Brown, 2010; Pamilo et al., 2007), Coleoptera (Cai et al., 2011; Jordal & Kambestad, 2014; Koutroumpa et al., 2009), Diptera (Hlaing et al., 2009). Few cases of NUMT were reported for Lepidoptera, where they were found in *Astraptes fulgerator* (Brower, 2006), *Bombyx mori* (Hazkani-Covo et al., 2010) and *Ephestia kuehniella* (Lepidoptera: Pyralidae) (Lämmermann et al., 2016).

Since NUMTs are not subject to selection, they may accumulate a considerable variation, so if two versions of supposedly mitochondrial DNA are found in the same organism, most probably a sequence more similar to related species would be the true mtDNA and the diverged one would be NUMT. In the case of *L. camilla*, the *COI* sequence version coding in the variable positions tryptophane, leycine and glutamate, as in other *Limenitis* spp., obviously represented the mitochondrial genome while the sequence encoding cysteine, serine and lysine is NUMT. Accordingly in public databases, FJ663724 (Lukhtanov et al., 2009) and related sequences represent mtDNA while GU707095 (Hausmann et al., 2011) and similar sequences are nuclear copies.

Variation of histone H1 gene and *COI* region and time of range disjunctions in *Limenitis*

Both histone H1 gene and the *COI* region of mitochondrial DNA were studied in a number of specimens from both range parts of the three *Limenitis* species with disjunct ranges. Our data revealed no nucleotide difference in both studied sequences fixed in either part of the range in these species, with the studied mtDNA sequence being identical in all specimens of *L. camilla*.

Among rare alleles, some were found in only one of the range parts which could originate in situ, but our samples are still too small to exclude their presence in the other range parts as well. These rare alleles are represented in *L. sydyi* by the mtDNA variant with the substitution C1950T and the

histone H1 gene variants A and E found only in the eastern range part. In *L. camilla*, the variants A and E of the H1 gene were found in the western part and variant C in one specimen from the eastern part.

L. helmanni showed a bit more interesting pattern. Different variant types of the histone H1 gene predominated in the eastern and western range parts, C and B, respectively, while variants D were found in the western part only. The variants B and D most probably originated by intragenic duplication from the variants A common in both range parts. The haplotypic network for the *COI* sequence (Fig. 3b) shows two main clusters differing by 8 synonymous substitutions, one occurring both in the east and west and the other one found in the west. The greater diversity of this sequence in the eastern part of the range agrees with the hypothesis of the recent, Holocenian (ca 5 tya) westward spread of this species from the eastern core of its range, because of the Holocene climatic optimum that took place earlier in the east of Eurasia and later in the west (Dubatolov & Kosterin, 2000). That spread was followed by isolation of the western range part during the climate deterioration in the Subboreal time. That recent and fast spread with subsequent isolation could result in the loss by the western populations of some haplotypes initially present in the ancestral eastern populations.

The absence of mutations fixed by the western and eastern range parts of the three considered Eurasian species of *Limenitis* suggests the range disjunctions to be too recent to be reliably dated by molecular means. We may nevertheless evaluate the upper limit of the disjunction time following the approach by Solovyev et al. (2015a) of using the amphi-Beringian disjunction in the same genus as reference.

Beringia was a land uniting the north-eastern Asia and North America since 100 mya, while formation of the Bering Strait 3–5 mya (which was twice interrupted by the so-called Beringian Bridges II and III) hindered but not excluded biota exchange between them (Sanmartin et al., 2001). In the course of climate changes, the vegetation of the Beringian area changed as well, thus opening opportunities of exchange with nemoral, boreal and arcto-alpine species between the continents. The humid versions of nemoral vegetation were generally replaced with boreal (taiga) vegetation in the late Pliocene, 3–5 mya, during the existence of Beringian Bridge II, and with the so-called tundrosteppe vegetation in the Pleistocene, 1–1.5 mya, during the existence of Beringian Bridge III (Dubatolov & Kosterin, 2000; Sanmartin et al., 2001).

The Holarctic (between Eurasia and North America) disjunctions of butterflies fit this scheme well. In the cold-tolerant species, they are intra-specific and quite recent. For instance, such disjunctions in the arcto-alpine species *Agriades glandon* (Vila et al., 2011), *Boloria freija* and *B. frigga* (Simonsen et al., 2010) were dated to 0.8, 1.1 and 1.2 mya, respectively; those in *Oeneis bore*, *O. jutta* and *O. melissa* to 1–1.5 mya (Kleckova et al., 2015), while Usami et al. (2021) revealed multiple Holarctic disjunctions in different groups of *Oeneis* during the Pleistocene. The disjunctions in the less cold-tolerant boreal species *Hesperia comma* (Forister et al., 2004), *Agriades optilete* (Vila et al., 2011), *Boloria eunomia* and *B. selene* (Simonsen et al., 2010) were dated to 1, 1, 1.7 and 2.7 mya, respectively. The intra-subgeneric disjunction of the eurytopic, rather boreal subgenus *Plebejus (Lycaeides)* spp. (the Palearctic *P. idas* and *P. argyrognomon* versus the Nearctic *P. melissa*) was dated to 2.4 mya (Vila et al., 2011).

Nemoral butterflies do not offer cases of Beringian disjunctions at the intra-species level but quite many ones (not less than 25–30) at the intra-generic level (Dubatolov & Kosterin, 2000), including the object of this study, the genus *Limenitis*. The time of existence of the last common ancestor of the genus *Limenitis* was estimated as 9 mya (Mullen et al., 2010) and 10.9 mya (7.06–13.77 HPD95%) (Ebel et al., 2015). The nemoral communities (broad-leafed forests), with which most *Limenitis* spp. (except for the boreal *L. populi*) are associated, existed at both sides of the Bering Strait not later than 3–5 mya (Fradkina, 1995; Dubatolov & Kosterin, 2000; Sanmartin et al., 2001). The thorough molecular studies of *Limenitis* suggested that the Palearctic *L. populi* and all Nearctic species form a monophyletic group (Ebel et al., 2015; Mullen, 2006; Mullen et al., 2010). This inference is supported by their specialisation to the same larval foodplants from the Salicaceae family (Mullen, 2006). Hence, North America was invaded by a representative of the willow-feeding Palearctic species which subsequently gave rise to the Palearctic *L. pop*uli and Nearctic *L. arthemis*, *L. archippus*, *L. lorquini* and *L. weidemeyeri*. The time of this event was estimated as 4 mya by Mullen (2006) who used the low estimate of the substitution rate in the *COI* gene by Brower (1994) as 11.0×10^{-9} per site per year. Currently a higher estimate of 7.5×10^{-9} per site per year is assumed more relevant (Farrel et al., 2001; Quek et al., 2004), application of which gives the time of invasion of ca 6 mya. The distance between the amphi-Beringian pair of species *L. arthemis* and *L. populi* estimated with the use of a mtDNA fragment *COI-COII* was relatively large, $p=8.3$–8.5% (Mullen, 2006). At the same time, we did not register substitutions fixed in the disjunct range parts of *L. camilla*, *L. helmanni* and *L. sydyi* in the studied part of the same sequence. Based on estimates of the substitution rate in the *COI* gene in arthropods of 7.5–11.0×10^{-9} per site per year (Brower, 1994; Farrel et al., 2001; Quek et al., 2004) and the length of the mtDNA fragment (1.2×10^{-3} bp) sequenced by us, we may very roughly estimate the maximum possible age of palaearctic disjunctions.

The probabilities of time intervals (t) between two subsequent events of fixation of substitutions are distributed exponentially: $P(t)=e^{-\lambda t}$, $t\geq0$; the expectation time t' of a

fixation event starting from any chosen time moment, e.g. the divergence event (which on average splits the time interval between subsequent event into halves) is distributed as $P(t') = e^{-2\lambda t}$; the expectation time of a substitution fixation in either of the two diverged lineages is again $P(t) = e^{-\lambda t}$, $t \geq 0$. The rate parameter λ can be calculated as a product of the estimate for the substitution rate and the length of a sequence. The expected time T for one substitution in either of two diverged lineages is 1/λ. In our case of *COI*, $\lambda \sim 9–13 \times 10^{-6}$; hence T ~ 77–110 thousand years. There is no surprise that sequence comparison was unable to trace consequences of the Holocenian disjunctions supposedly 4–5 thousands year old, while in case of their dating to the last interglacial that is just ca 100 tya, we would detect some substitutions.

Scarce nucleotide variation and absence of substitutions fixed in either western or eastern range part in either species make application of formal Bayesian approach for the estimation of the age of disjunction not so useful. We nevertheless attempted this for the *COI* fragment of *L. helmanni*, which demonstrated quite a considerable variation which, unlike the histone H1 gene, is not complicated with indels. This attempt using the Yule and birth-and-death models of branching resulted in estimates of the mean divergence time of the eastern and western range parts of *L. helmanni* as, respectively, 194 kya (with HPD95% of 70–337 kya) and 247 kya (HPD 95% 85–443 kya). This is more than twice greater than the above given simple estimate of the time necessary for the fixation of one substitution. However, the enormous highest probability density (HPD) intervals of the estimates of the divergence time obtained by the coalescence method expectedly indicate that this method does not fit well our data. Moreover, no matter how unprecise they are, such estimates should be taken with further caution since they are actually based on few substitutions found in single specimens (only variant B was found in two specimens from the east) (Fig. 3c). Naturally each single specimen is found in either of the range parts only, while the program assumes rare variants to be actually restricted to those range parts, which is by no means proved. A substantial extension of specimen sample would probably reveal some of these substitutions in both range parts, which would decrease the age estimate. So our attempt of application of the Bayesian method actually confirmed that the divergence in question was too recent to be adequately dated by molecular means, which fits well the Holocene hypothesis.

The topologies of our phylogenetic trees of *Limenitis*, reconstructed by the neighbour joining, maximum likelihood (not shown) and Bayesian methods from the *COI* fragment and histone H1 gene sequences (Fig. 2), were similar to each other and to those reconstructed based on different markers by Mullen (2006), Mullen et al. (2010) and Ebel et al. (2015). The uncorrected *p*-distance between *L. populi* and *L. arthemis* of 8.6% based on the histone H1 gene appeared almost the same as in the *COI-COII* fragment; so we evaluate the average substitution rate in the histone H1 gene in *Limenitis* as 6.2×10^{-9} per site per year that is close to the rate of 7.5×10^{-9} in *COI* gene according to Farrel et al. (2001) and Quek et al. (2004). Hence, these two markers can be used in *Limenitis* in similar time scale. It should be noted that our previous studies of two other groups of Lepidoptera revealed lower relative substitution rates in the histone H1 gene: twice as slow as in the *COI* gene in *Oreta* (Drepanidae) (Solovyev et al., 2015a) and five times as slow in *Leptidea* (Pieridae) (Solovyev et al., 2015b), the latter belonging to the same superfamily Papilionoidea as *Limenitis*. The similar substitution rates of the mitochondrial *COI* fragment and the nuclear histone H1 gene in *Limenitis* correspond to the similar results with respect to the disjunct Palearctic ranges: no substitution has been fixed in the histone H1 gene in the western and eastern range parts of all the three studied species with disjunct ranges.

The presented results on *Limenitis* well correspond to those of our previous molecular study of the disjunct ranges in the moth *Eversmannia exornata* (Eversmann, 1837) (Geometroidea: Uraniidae: Epipleminae) belonging to the same nemoral ecological complex as the three species of *Limenitis* with disjunct ranges (Solovyev et al., 2015a). In *E. exornata*, a similarly low variation in the *COI* and histone H1 gene was revealed (Solovyev et al., 2015a). The *COI* gene sequences were identical in Siberian and Far Eastern parts of the disjunct range of *E. exornata* while in the European range an additional allele differing in one synonymous substitution was also found. Two polymorphic sites were found in the histone H1 gene of this species: one synonymous substitution was found in all the three range parts while another one–in one specimen only. This low level of variation was compared to that of the difference observed in an amphi-Beringian generic disjunction in the genus *Oreta* from the sister superfamily Drepanoidea. Most (about 60) species of this genus inhabit eastern and south-eastern Asia and only one species, *Oreta rosea*, is Nearctic. The genetic distances between the Nearctic *O. rosea* and the closest Palaearctic species *O. paki* and *O. pulchripes* comprised 9–11% in the *COI* gene fragment and 5.6–5.7% in the histone H1 gene (Solovyev et al., 2015a). The amphi-Beringian disjunction of the genus *Oreta* could not take place after 4 mya, when the suitable nemoral vegetation disappeared from the banks of the Bering Strait (Fradkina, 1995; Dubatolov & Kosterin, 2000; Solovyev et al., 2015a), so both genes had enough time to diverge considerably. From this point of view, the lack of divergence of both *COI* and histone H1 genes in the isolated range parts of *E. exornata* suggests its very recent, most probably the Holocenian split.

The only known nemoral moth species with an amphi-Palaearctic range is *Lithosia quadra* (Linnaeus, 1758)

(Erebidae: Arctiinae), which inhabits Europe and Anterior and (scarcely) Central Asia in the west and Japan, Korea, NE China and the Far East of Russia from Transbaikalia to Sakhalin and the Kuriles in the east, and with just few records in between, from Saur Mts in E Kazakhstan and (dubious) Tomsk and Baikal (Dubatolov et al., 2016). The cited authors analysed the 5′-fragment of the mitochondrial *COI* in 32 specimens from different range parts including Spain, Lithuania, Bulgaria, Caucasus and Transcaucasia, Turkey, Iran, Uzbekistan, Russia from Transbaikalia to Sakhalin, Korea and Japan. The intra-specific variation for the studied fragment was found very scarce and many alleles were found in distant range parts so that specimens with remote provenances were interspersed in the phylogenetic tree without geographical regularity that was in line with our results on *Limenitis* spp., including the amphi-Palaearctic *L. camilla*.

Thus, nemoral Lepidoptera with disjunct Palearctic ranges representing three genera from different superfamilies, Papilionoidea and Geometroidea, exhibit no geographical variation in the nuclear histone H1 gene and the studied part of the *COI* gene and hence support the hypothesis by Dubatolov and Kosterin (2000) that their range disjunctions took place in the Holocene. This hypothesis can be extrapolated to other nemoral Lepidoptera species with disjunct ranges, e.g. *Apatura iris* (Linnaeus, 1758), *Apatura ilia* Denis & Schiffermüller, 1775, *Apatura metis* Freyer, 1829 and *Argynnis laodice* Pallas, 1771, which will be target species of further studies.

Similar results were obtained from the studies of the damselfly *Nehalennia speciosa* (Charpentier, 1840) (Odonata: Coenagrionidae) (Bernard et al., 2011; Suvorov, 2011). The genus *Nehalennia* is amphi-Beringian, with five species in the Americas (three in the Nearctic, one in Florida and one in Central and South America) and one, *N. speciosa*, in the boreal zone of the Palearctic. The latter probably has an amphi-Palearctic range in Eurasia; if so, it is the only such species among Odonata (Kosterin, 2005). Bernard et al. (2011) and Suvorov (2011) revealed very low divergence between the two parts of its amphilapearctic range: identical *ITS1* sequences, two polymorhic sites in the *COI* gene, 1 polymorphic site in the mitochondrial 16S rRNA-ND1 and 3 polymorphic sites in the COII gene (0.35% variable sites in the latter sequence). At the same time, the uncorrected *p*-distances in the *COII* gene between *N. speciosa* and the North American species *N. irene* and *N. gracilis* comprised 12.3% and 10.3%, respectively (Bernard et al., 2011). *N. speciosa* is most closely related to *N. gracilis* (Bernard et al., 2011). Most probably the ancestor of *N. speciosa* penetrated from North America to Eurasia rather recently, supposedly in the Late Pleistocene, spread throughout the Palaearctic and then underwent split of once continuous range into the Euro-West Siberian and Far Eastern parts (Bernard et al., 2011; Kosterin, 2002).

The Holocene started ca 10 tya and its climatic optimum, the Atlantic time, took place ca 8 tya; then, our results fit the hypothesis that formation of continuous trans-Eurasian or Siberian-Far Eastern ranges of nemoral butterflies and their subsequent split was quite fast, taking no more than 5 thousand years. This is not surprising for such large flying insects. There is evidence that such events can in fact be even much faster. It was shown that for twentieth century the northern range limits in Europe of 21 of 25 investigated butterfly species have shifted to the north for 35–240 km, 7 species moved to the north also their southern limits; this move obviously reflected the northward shift of isotherms for ca 120 km (Parmesan et al., 1999). Similar northward range shifts were registered also in dragonflies (Paulson, 2001; Hassal & Thompson, 2008), so that some Mediterranean species successfully colonised Central and even North Europe (Ott, 2001, 2007). The opposite, southward shift of the range borders has been registered only in two butterfly species (Parmesan et al., 1999). It is noteworthy that one of them, *Apatura ilia*, has an amphi-Palearctic range resembling that of *L. camilla*. This large and conspicuous butterfly attracts attention of both professional and numerous amateur lepidopterologists, so its distribution was quite well documented.

The climate warming drives range shifts in butterflies not only to the north but also to higher altitudes. Rödder et al. (2021) documented range shifts to higher altitudes in the butterflies of the eastern Alps during the last 50 years. Sistri et al. (2021) estimated the rate of the uphill shift of the habitats of *Erebia pandrose* Borkhausen, 1788 in the Alps and Apennines as more than 3 m per year since the end of the nineteenth century and more than 22 m per year since 1995.

In 2018, a male of *L. camilla* was found by A.P. Sitnikov (pers. comm.) in the western West Siberian Lowland, 1 km N Yurty-Iska village (N 57.31° E 65.60°) near Tyumen City, which might indicate a recent eastward expansion of the European part of the range of this species, studied in this paper. Currently we evidence a fast shrinkage of the range gap of another butterfly with an amphi-Palearctic disjunctive range from the same family as *Limenitis*, namely *Apatura iris*. Until the end of twentieth century, it was considered to range in Europe, east to Ural, and then in the Pacific regions, not westerly of Transbaikalia (Dubatolov & Kosterin, 2000). However, in recent decades, this species expanded from Europe to West Siberia, as found since 1991 in Tyumen' Province (Sitnikov, 1992), since 1997 in Omsk Province (Knyazev & Kosterin, 2003), since 2006 in Tomsk Province at the longitude of Novosibirsk (Kosterin et al., 2007), since 2010 at Novosibirsk (Ivonin et al., 2013), since 2010 in Altaiskiy Kray, since 2012 in Kemerovo Province (Yakovlev & Kostyunin, 2015), and since 2017 in Khakasia (89°40′30.0″E) (Dragan, 2018). By reaching Altaiskiy Kray, the species completed occupation of suitable latitudes of West Siberia and by reaching Khakasia it entered Central Siberia. Hence, its eastward expanding for 2,000 km required ca 30 years that means a speed of 70 km/year. At the same

time the opposite westward expansion of the species from the Far East to Siberia was also observed. Korshunov (1970) did not report *Apatura iris* for Siberia at all but in 2009 it reached Lake Baikal (106°97′E) (Berlov & Berlov, 2021). By doubling the above estimate of the expansion speed, we may estimate that the rest 1,200 km of the range gap is shrinking with a speed of 140 km/year and it would take 9 years for the species to fill it. Hence, the restoration of the continuous Eurasian range of *A. iris* from an amphi-Palearctic range with a gap from Ural to Transbaikalia, driven by the current global warming, will take half a century. A similar estimation of 50 km/year was obtained for the rate of the eastward expansion of *Maniola jurtina* (Nymphalidae, Satyrinae) from Europe and Ural to West Siberia (Ivonin et al., 2016, 2019); for the time being the species reached Kemerovo Province (86° 22′E) (Kostyunin & Klyueva, 2020).

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Author contribution All authors contributed to the study conception and design. Specimens were collected by Oleg E. Kosterin and Vladimir V. Dubatolov. Vladimir I. Solovyev and Oleg E. Kosterin designed the experiments, performed phylogenetic analyses, Vladimir I. Solovyev performed molecular analysis, and Valeriya Y. Vavilova cloned histone H1 genes. Analyses of results were performed by Vladimir I. Solovyev, Oleg E. Kosterin, and Vladimir V. Dubatolov. The first draft of the manuscript was written by Vladimir I. Solovyev, Oleg E. Kosterin and Vladimir V. Dubatolov and all authors commented on subsequent versions of the manuscript. All authors read and approved the final manuscript.

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Availability of data and material The sequences are submitted to European Nucleotide Archive; the relevant accession numbers are provided in Table 1.

Declarations

Conflict of interest The authors declare no competing interests.

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