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Taxonomic interpretation of chromosomal and mitochondrial DNA variability in the species complex close to *Polyommatus* (*Agrodiaetus*) *dama* (Lepidoptera, Lycaenidae)

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Abstract

In this paper, by using combination of molecular and chromosomal markers, populations of *Polyommatus* (*Agrodiaetus*) *karindus* (Riley, 1921) from north-west and central Iran are analyzed. It has been found that taxon usually identified as *P.* (*A.*) *karindus* is represented in Iran by two geographically separated groups of individuals, strongly differentiated by their karyotypes and mitochondrial haplotypes. It is demonstrated that populations from NW Iran have the haploid chromosome number n = 68, while the haploid chromosome number of *P.* (*A.*) *karindus* from central Iran is found to be n = 73. Phylogenetic analysis revealed that these groups also differ by at least eight nucleotide substitutions in a 690 bp fragment of the mitochondrial *COI* gene and form separated groups of clusters in Bayesian inference tree. Thus, population entities from central Iran are described here as a new subspecies *Polyommatus* (*Agrodiaetus*) *karindus saravandi* **ssp. n.** Strong chromosomal and molecular differentiation are confirmed between *P.* (*A.*) *karindus* and its sister species, *P.* (*A.*) *dama* (Staudinger, 1892).

Keywords

COI, Iran, karyotype, molecular marker, chromosome number

Introduction

Agrodiaetus Hübner, 1822 is the most species-rich subgenus within the genus *Polyommatus* Latreille, 1804 (Talavera et al. 2013a, Lukhtanov et al. 2015a). It consists of approximately 130 species distributed in the western Palearctic (Vila et al. 2010, Lukhtanov et al. 2008, 2014, Vershinina and Lukhtanov 2010, Przybyłowicz et al. 2014, Lukhtanov and Tikhonov 2015). Today *Agrodiaetus* has become a model group in studies of speciation (Lukhtanov et al. 2005, 2015b), intraspecific differentiation (Dincă et al. 2013, Przybyłowicz et al. 2014, Lukhtanov et al. 2015a), and rapid karyotype evolution (Lukhtanov et al., 2005, Kandul et al. 2007). From the point of view of taxonomy, *Agrodiaetus* is a very complicated group. Many *Agrodiaetus* taxa display extremely similar phenotype (Hesselbarth et al. 1995) and, in contrast to other Lepidoptera taxa, genitalia offer only few distinctive features. Furthermore, many taxa represent allopatric populations which differ only slightly in morphology, and a conclusion on their status as distinct species or subspecies is controversial and can be misleading (Wiemers 2003, Lukhtanov et al. 2015a). This resulted in description of numerous polytypic species based on geographic distribution and classic morphological characters (Forster 1956, 1960a, b, 1961).

In particular, *Polyommatus* (*Agrodiaetus*) *dama* (Staudinger, 1892) was traditionally regarded as a polytypic species that included two subspecies: *Polyommatus* (*Agrodiaetus*) *dama dama* (Staudinger, 1892) (orig. comb. *Lycaena Dama*) and *Polyommatus* (*Agrodiaetus*) *dama karindus* (Riley, 1921) (orig. comb. *Lycaena dama* subsp. *karinda*). *P.* (*A.*) *dama dama* has only been found in South Anatolia (a few localities in Malatya, Maraş, and Mardin provinces (Turkey), while *P.* (*A.*) *dama karindus* distribution range is restricted to Zagros Mountains in Iran.

The karyotype studies of de Lesse (1957, 1959a, b, c, d, 1960a, b, 1961, 1962a, b, 1963a, b, 1964, 1966, 1968) revealed that *Agrodiaetus* species exhibit a wide diversity of karyotypes. Karyotyping may provide necessary diagnostic character for many *Agrodiaetus* species, and therefore become an important requirement for describing new taxa (de Lesse 1960a, b, Lukhtanov and Dantchenko 2002, 2003, Lukhtanov et al. 2008). Karyological investigations showed strong chromosomal differentiation between Turkish and Iranian populations of *P.* (*A.*) *dama* s. l.. De Lesse (1959a) described karyotype of *P.* (*A.*) *dama dama* from Kahramanmaraş and Olivier et al. (1999) confirmed his results from the type locality Malatya. It has an asymmetric karyotype with n = 41 chromosomes, about eleven of them are large, gradually decreasing in size, the others medium–sized; whereas the karyotype of Iranian taxon was determined as n = 68 (Wiemers 2003). Thus, on the basis of karyotype studies, *P.* (*A.*) *dama* s. l. was split into two species, *P.* (*A.*) *dama* and *P.* (*A.*) *karindus*, that can be characterized by species-specific haploid chromosome numbers.

However, the chromosome number of *P.* (*A.*) *karindus* was determined only for one population from NW Iran (Saqqez, Kordestan Province) (Wiemers 2003). Further investigations showed that Iranian species *P.* (*A.*) *karindus* has complicated genetic and

phylogeographic structure (Lukhtanov et al. 2015b). Here a combination of molecular mitochondrial (*COI*) and nuclear chromosomal (karyotype) markers are used to analyze different Iranian populations of *P.* (*A.*) *karindus*. Our study demonstrates that butterflies from central Iran strongly differentiated by their karyotypes and mitochondrial haplotypes from NW Iranian populations. Thus, population entities from central Iran are described here as a separate subspecies *Polyommatus* (*Agrodiaetus*) *karindus saravandi* ssp. n.

Material and methods

Specimens sampling

The butterflies were collected in the period of 2007–2014 in Iran (list of collected specimens is given in Table 1). In north–west Iran we collected material in two localities: 1) in the mountain range between Saqqez and Baneh (30–40 km SW of Saqqez), and 2) in the vicinity of Dare Dozdan (30–40 km W of Divandarreh). In central Iran we collected butterflies in the vicinity of Vennai (18 km W of Borujerd), in the vicinity of Saravand (15 km SE of Dorud), in the vicinity of Nahavand and in the vicinity of Darreh Takht (35 km NE of Dorud) (information about sampling localities is given in Figure 1 and Table 1).

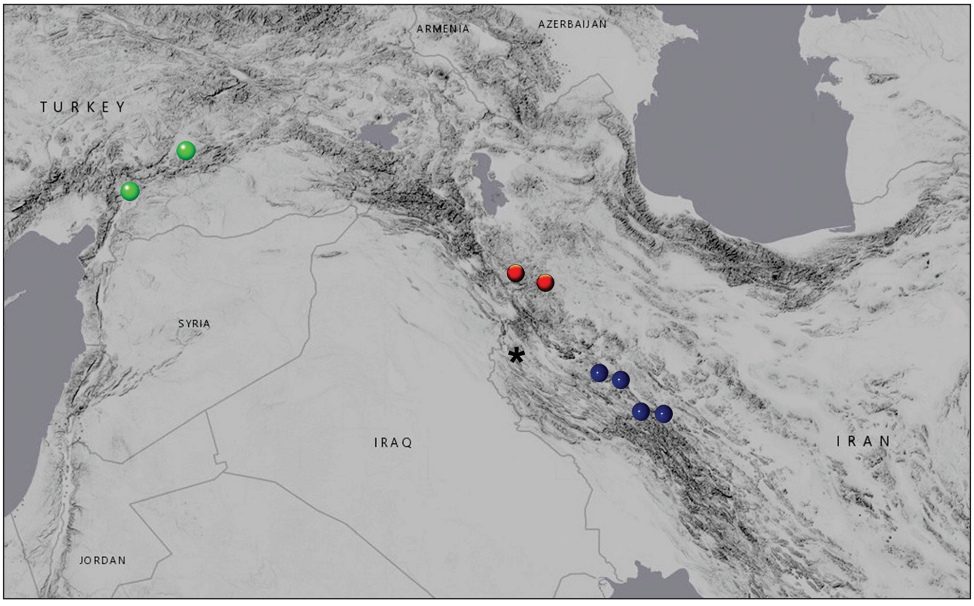

Figure 1. Distribution ranges of *P.* (*A.*) *dama* (green circles), *P.* (*A.*) *karindus karindus* (red circles) and *P.* (*A.*) *karindus saravandi* (blue circles). The asterisk indicates the type locality of *P.* (*A.*) *karindus karindus.*

Table 1. List of studied material (129 specimens) with information on karyotype (48 specimens) and *COI* sequences (54 specimens). Collectors: V. Lukhtanov (VL), N. Shapoval (NS) and A. Barabanov (AB).

Species	Sex	Sample ID	Chromosome number (n)	COI	GeneBank number	Province	Locality and coordinates	Altitude	Date	Collectors
karindus	M	E391	70			Kordestan	ca. 40 km SW Saqqez 36°06.18'N; 046°00.27'E	1725 m	29 July 2004	VL
karindus	M	E399	68			Kordestan	ca. 40 km SW Saqqez 36°06.18'N; 046°00.27'E	1725 m	29 July 2004	VL
karindus	M	E400	68			Kordestan	ca. 40 km SW Saqqez 36°06.18'N; 046°00.27'E	1725 m	29 July 2004	VL
karindus	M	E402	68			Kordestan	ca. 40 km SW Saqqez 36°06.18'N; 046°00.27'E	1725 m	29 July 2004	VL
karindus	M	Z726	68	h02/GH5	KT582701	Kordestan	ca. 40 km SW Saqqez 36°05.97'N; 045°59.63'E	1720m	30 July 2007	VL & NS
karindus	M	Z727	68	h03/GH5	KT582702	Kordestan	ca. 40 km SW Saqqez 36°05.97'N; 045°59.63'E	1720m	30 July 2007	VL & NS
karindus	M	Z729	68			Kordestan	ca. 40 km SW Saqqez 36°05.97'N; 045°59.63'E	1720m	30 July 2007	VL & NS
karindus	M	Z749		h01/GH5	KT582703	Kordestan	ca. 40 km SW Saqqez 36°04.82'N; 045°58.88'E	1880m	31 July 2007	VL & NS
karindus	M	Z750		h01/GH5	KT582704	Kordestan	ca. 40 km SW Saqqez 36°04.82'N; 045°58.88'E	1880m	31 July 2007	VL & NS
karindus	M	Z753	68	h01/GH5	KT582705	Kordestan	ca. 40 km SW Saqqez 36°04.82'N; 045°58.88'E	1880m	31 July 2007	VL & NS
karindus	M	Z800	68	h01/GH5	KT582706	Kordestan	ca. 40 km SW Saqqez 36°04.09'N; 045°58.82'E	2050m	31 July 2007	VL & NS
karindus	M	Z809	68	h01/GH5	KT582707	Kordestan	ca. 40 km SW Saqqez 36°04.09'N; 045°58.82'E	2050m	31 July 2007	VL & NS
karindus	M	Z820	68	h01/GH5	KT582708	Kordestan	ca. 40 km SW Saqqez 36°04.09'N; 045°58.82'E	2050m	31 July 2007	VL & NS
karindus	M	Z843	68	h01/GH5	KT582709	Kordestan	ca. 40 km SW Saqqez 36°04.64'N; 045°59.16'E	1920–1950m	1 August 2007	VL & NS
karindus	M	Z845	69	h01/GH5	KT582710	Kordestan	ca. 40 km SW Saqqez 36°04.64'N; 045°59.16'E	1920–1950m	1 August 2007	VL & NS
karindus	M	W253	68			Kordestan	ca. 40 km SW Saqqez 36°03.00'N; 045°58.54'E	2027m	29 July 2009	VL & NS
karindus	M	W254	68			Kordestan	ca. 40 km SW Saqqez 36°03.00'N; 045°58.54'E	2027m	29 July 2009	VL & NS
karindus	M	W259	68			Kordestan	ca. 40 km SW Saqqez 36°03.00'N; 045°58.54'E	2027m	29 July 2009	VL & NS
karindus	M	W271	68			Kordestan	ca. 40 km SW Saqqez 36°04.39'N; 045°59.06'E	1869m	29 July 2009	VL & NS
karindus	M	W272	68			Kordestan	ca. 40 km SW Saqqez 36°04.39'N; 045°59.06'E	1869m	29 July 2009	VL & NS
karindus	M	W273	68			Kordestan	ca. 40 km SW Saqqez 36°04.39'N; 045°59.06'E	1869m	29 July 2009	VL & NS
karindus	M	W274	68			Kordestan	ca. 40 km SW Saqqez 36°04.39'N; 045°59.06'E	1869m	29 July 2009	VL & NS
karindus	M	W275	68			Kordestan	ca. 40 km SW Saqqez 36°04.39'N; 045°59.06'E	1869m	29 July 2009	VL & NS
karindus	M	W276	68			Kordestan	ca. 40 km SW Saqqez 36°04.39'N; 045°59.06'E	1869m	29 July 2009	VL & NS
karindus	M	W277	68			Kordestan	ca. 40 km SW Saqqez 36°04.39'N; 045°59.06'E	1869m	29 July 2009	VL & NS
karindus	M	W278	68			Kordestan	ca. 40 km SW Saqqez 36°04.39'N; 045°59.06'E	1869m	29 July 2009	VL & NS
karindus	M	W279	68			Kordestan	ca. 40 km SW Saqqez 36°04.39'N; 045°59.06'E	1869m	29 July 2009	VL & NS

Species	Sex	Sample ID	Chromosome number (n)	COI	GeneBank number	Province	Locality and coordinates	Altitude	Date	Collectors
karindus	M	W280	68			Kordestan	ca. 40 km SW Saqqez 36°04.39'N; 045°59.06'E	1869m	29 July 2009	VL & NS
karindus	M	W281	68			Kordestan	ca. 40 km SW Saqqez 36°04.39'N; 045°59.06'E	1869m	29 July 2009	VL & NS
karindus	M	W282	68			Kordestan	ca. 40 km SW Saqqez 36°04.39'N; 045°59.06'E	1869m	29 July 2009	VL & NS
karindus	M	W283	68			Kordestan	ca. 40 km SW Saqqez 36°04.39'N; 045°59.06'E	1869m	29 July 2009	VL & NS
karindus	M	W340		h01/GH5	KT582732	Kordestan	Dare Dozdan 35°52.05'N; 046°33.03'E	2066m	30 July 2009	VL & NS
karindus	M	W341		h01/GH5	KT582733	Kordestan	Dare Dozdan 35°52.05'N; 046°33.03'E	2066m	30 July 2009	VL & NS
karindus	M	W342		h01/GH5	KT582734	Kordestan	Dare Dozdan 35°52.05'N; 046°33.03'E	2066m	30 July 2009	VL & NS
karindus	M	W354		h04/GH5	KT582737	Kordestan	Dare Dozdan 35°52.05'N; 046°33.03'E	2277m	31 July 2009	VL & NS
karindus	M	W355		h01/GH5	KT582735	Kordestan	Dare Dozdan 35°52.05'N; 046°33.03'E	2277m	31 July 2009	VL & NS
karindus	M	W361		h01/GH5	KT582736	Kordestan	Dare Dozdan 35°52.05'N; 046°33.03'E	2066m	31 July 2009	VL & NS
karindus	M	W366		h01/GH5	KT582738	Kordestan	Dare Dozdan 35°52.05'N; 046°33.03'E	2066m	31 July 2009	VL & NS
karindus	M	V069		h01/GH5	KT582739	Kordestan	Dare Dozdan 35°51.30'N; 046°42.60'E	2200m	27 July 2014	NS & AB
karindus	M	V070		h01/GH5	KT582740	Kordestan	Dare Dozdan 35°51.30'N; 046°42.60'E	2200m	27 July 2014	NS & AB
karindus	M	W370	73	h05/GH4	KT582722	Lorestan	Nahavand 34°02.57'N; 048°20.22'E	2173m	2 August 2009	VL & NS
karindus	M	W371	73	h05/GH4	KT582723	Lorestan	Nahavand 34°02.57'N; 048°20.22'E	2173m	2 August 2009	VL & NS
karindus	M	W372	73	h09/GH2	KT582724	Lorestan	Nahavand 34°02.57'N; 048°20.22'E	2173m	2 August 2009	VL & NS
karindus	M	W373	73	h05/GH4	KT582725	Lorestan	Nahavand 34°02.57'N; 048°20.22'E	2173m	2 August 2009	VL & NS
karindus	M	W374		h09/GH2	KT582726	Lorestan	Nahavand 34°02.57'N; 048°20.22'E	2173m	2 August 2009	VL & NS
karindus	M	W375		h09/GH2	KT582727	Lorestan	Nahavand 34°02.57'N; 048°20.22'E	2173m	2 August 2009	VL & NS
karindus	M	W376		h09/GH2	KT582728	Lorestan	Nahavand 34°02.57'N; 048°20.22'E	2173m	2 August 2009	VL & NS
karindus	M	W388		h08/GH2	KT582731	Lorestan	Nahavand 34°02.57'N; 048°20.22'E	1950–2173m	3 August 2009	VL & NS
karindus	M	W389		h05/GH4	KT582729	Lorestan	Nahavand 34°02.57'N; 048°20.22'E	1950–2173m	3 August 2009	VL & NS
karindus	M	W390		h09/GH2	KT582730	Lorestan	Nahavand 34°02.57'N; 048°20.22'E	1950–2173m	3 August 2009	VL & NS
karindus	M	W391				Lorestan	Nahavand 34°02.57'N; 048°20.22'E	1950–2173m	3 August 2009	VL & NS
karindus	M	W392				Lorestan	Nahavand 34°02.57'N; 048°20.22'E	1950–2173m	3 August 2009	VL & NS
karindus	M	U217				Lorestan	Nahavand, 34°02.92'N; 48°20.40'E	2161 m	19 July 2011	VL & NS
karindus	M	U218				Lorestan	Nahavand, 34°02.92'N; 48°20.40'E	2161 m	19 July 2011	VL & NS
karindus	M	U219				Lorestan	Nahavand, 34°02.92'N; 48°20.40'E	2161 m	19 July 2011	VL & NS
karindus	M	U220				Lorestan	Nahavand, 34°02.92'N; 48°20.40'E	2161 m	19 July 2011	VL & NS
karindus	M	U223				Lorestan	Nahavand, 34°02.92'N; 48°20.40'E	2161 m	19 July 2011	VL & NS

Species	Sex	Sample ID	Chromosome number (n)	COI	GeneBank number	Province	Locality and coordinates	Altitude	Date	Collectors
karindus	M	U228				Lorestan	Nahavand, 34°02.91'N; 48°21.08'E	2020 m	20 July 2011	VL & NS
karindus	M	U229				Lorestan	Nahavand, 34°02.91'N; 48°21.08'E	2020 m	20 July 2011	VL & NS
karindus	M	U230				Lorestan	Nahavand, 34°02.91'N; 48°21.08'E	2020 m	20 July 2011	VL & NS
karindus	M	U231				Lorestan	Nahavand, 34°02.91'N; 48°21.08'E	2020 m	20 July 2011	VL & NS
karindus	M	U232				Lorestan	Nahavand, 34°02.91'N; 48°21.08'E	2020 m	20 July 2011	VL & NS
karindus	M	U233				Lorestan	Nahavand, 34°02.91'N; 48°21.08'E	2020 m	20 July 2011	VL & NS
karindus	M	U234				Lorestan	Nahavand, 34°02.91'N; 48°21.08'E	2020 m	20 July 2011	VL & NS
karindus	M	U235				Lorestan	Nahavand, 34°02.91'N; 48°21.08'E	2020 m	20 July 2011	VL & NS
karindus	M	U236				Lorestan	Nahavand, 34°02.91'N; 48°21.08'E	2020 m	20 July 2011	VL & NS
karindus	M	U237				Lorestan	Nahavand, 34°02.91'N; 48°21.08'E	2020 m	20 July 2011	VL & NS
karindus	M	U238				Lorestan	Nahavand, 34°02.91'N; 48°21.08'E	2020 m	20 July 2011	VL & NS
karindus	M	U239				Lorestan	Nahavand, 34°02.91'N; 48°21.08'E	2020 m	20 July 2011	VL & NS
karindus	M	U240				Lorestan	Nahavand, 34°02.91'N; 48°21.08'E	2020 m	20 July 2011	VL & NS
karindus	M	U256				Lorestan	Nahavand, 34°02.91'N; 48°21.08'E	2020 m	20 July 2011	VL & NS
karindus	M	U257				Lorestan	Nahavand, 34°02.91'N; 48°21.08'E	2020 m	20 July 2011	VL & NS
karindus	M	U262				Lorestan	Nahavand, 34°02.91'N; 48°21.08'E	2020 m	20 July 2011	VL & NS
karindus	M	U263				Lorestan	Nahavand, 34°02.91'N; 48°21.08'E	2020 m	20 July 2011	VL & NS
karindus	M	U264				Lorestan	Nahavand, 34°02.91'N; 48°21.08'E	2020 m	20 July 2011	VL & NS
karindus	M	U265				Lorestan	Nahavand, 34°02.91'N; 48°21.08'E	2020 m	20 July 2011	VL & NS
karindus	M	U266				Lorestan	Nahavand, 34°02.91'N; 48°21.08'E	2020 m	20 July 2011	VL & NS
karindus	M	U267				Lorestan	Nahavand, 34°02.91'N; 48°21.08'E	2020 m	20 July 2011	VL & NS
karindus	M	U278				Lorestan	Nahavand, 34°02.91'N; 48°21.08'E	2020 m	20 July 2011	VL & NS
karindus	M	U279				Lorestan	Nahavand, 34°02.91'N; 48°21.08'E	2020 m	20 July 2011	VL & NS
karindus	M	U280				Lorestan	Nahavand, 34°02.91'N; 48°21.08'E	2020 m	20 July 2011	VL & NS
karindus	M	U281				Lorestan	Nahavand, 34°02.91'N; 48°21.08'E	2020 m	20 July 2011	VL & NS
karindus	M	Z381	73	h09/GH2	KT582691	Lorestan	W of Borujerd, Kuh-e Garin mount.Vennnai, 33°53.89'N; 48°34,03'E	2150m	21 July 2007	VL & NS
karindus	M	Z382	73	h09/GH2	KT582692	Lorestan	W of Borujerd, Kuh-e Garin mount.Vennnai, 33°53.89'N; 48°34,03'E	2150m	21 July 2007	VL & NS
karindus	M	Z396	73	h09/GH2	KT582693	Lorestan	W of Borujerd, Kuh-e Garin mount.Vennnai, 33°53.89'N; 48°34,03'E	2150m	21 July 2007	VL & NS

Species	Sex	Sample ID	Chromosome number (n)	COI	GeneBank number	Province	Locality and coordinates	Altitude	Date	Collectors
karindus	M	Z397	73	h10/GH2	KT582694	Lorestan	W of Borujerd, Kuh-e Garin mount.Vennnai, 33°53.89'N; 48°34,03'E	2150m	21 July 2007	VL & NS
karindus	M	Z398	73	h09/GH2	KT582695	Lorestan	W of Borujerd, Kuh-e Garin mount.Vennnai, 33°53.89'N; 48°34,03'E	2150m	21 July 2007	VL & NS
karindus	M	Z399	73	h11/GH1	KT582696	Lorestan	W of Borujerd, Kuh-e Garin mount.Vennnai, 33°53.89'N; 48°34,03'E	2150m	21 July 2007	VL & NS
karindus	M	Z400	73	h10/GH2	KT582697	Lorestan	W of Borujerd, Kuh-e Garin mount.Vennnai, 33°53.89'N; 48°34,03'E	2150m	21 July 2007	VL & NS
karindus	M	Z408	73	h10/GH2	KT582698	Lorestan	W of Borujerd, Kuh-e Garin mount.Vennnai, 33°53.89'N; 48°34,03'E	2150m	21 July 2007	VL & NS
karindus	M	Z412	73	h11/GH1	KT582700	Lorestan	W of Borujerd, Kuh-e Garin mount.Vennnai, 33°53.89'N; 48°34,03'E	2150m	22 July 2007	VL & NS
karindus	M	Z413				Lorestan	W of Borujerd, Kuh-e Garin mount.Vennnai, 33°53.89'N; 48°34,03'E	2150m	22 July 2007	VL & NS
karindus	M	Z416		h09/GH2	KT582699	Lorestan	W of Borujerd, Kuh-e Garin mount.Vennnai, 33°53.89'N; 48°34,03'E	2150m	22 July 2007	VL & NS
karindus	M	V331				Lorestan	W of Borujerd, Kuh-e Garin mount.Vennnai, 33°53.89'N; 48°34,03'E	2150m	2 August 2014	NS & AB
karindus	M	V335				Lorestan	W of Borujerd, Kuh-e Garin mount.Vennnai, 33°53.89'N; 48°34,03'E	2150m	2 August 2014	NS & AB
karindus	M	V336				Lorestan	W of Borujerd, Kuh-e Garin mount.Vennnai, 33°53.89'N; 48°34,03'E	2150m	2 August 2014	NS & AB
karindus	M	W061	73,74,75	h12/GH1	KT582711	Lorestan	Saravand, 33°22.39'N; 49°10.25'E	2070m	21 July 2009	VL & NS
karindus	M	W062	ca73			Lorestan	Saravand, 33°22.39'N; 49°10.25'E	2070m	22 July 2009	VL & NS
karindus	M	W063	71	h12/GH1	KT582712	Lorestan	Saravand, 33°22.39'N; 49°10.25'E	2070m	22 July 2009	VL & NS
karindus	M	W064	73	h12/GH1	KT582713	Lorestan	Saravand, 33°22.39'N; 49°10.25'E	2070m	22 July 2009	VL & NS
karindus	M	W065	ca73	h12/GH1	KT582714	Lorestan	Saravand, 33°22.39'N; 49°10.25'E	2070m	22 July 2009	VL & NS
karindus	M	W072	ca73	h12/GH1	KT582715	Lorestan	Saravand, 33°22.39'N; 49°10.25'E	2070m	22 July 2009	VL & NS
karindus	M	W073				Lorestan	Saravand, 33°22.39'N; 49°10.25'E	2070m	22 July 2009	VL & NS
karindus	M	W074				Lorestan	Saravand, 33°22.39'N; 49°10.25'E	2070m	22 July 2009	VL & NS
karindus	M	W075				Lorestan	Saravand, 33°22.39'N; 49°10.25'E	2070m	22 July 2009	VL & NS

Species	Sex	Sample ID	Chromosome number (n)	COI	GeneBank number	Province	Locality and coordinates	Altitude	Date	Collectors
karindus	M	W081				Lorestan	Saravand, 33°22.39'N; 49°10.25'E	2070m	22 July 2009	VL & NS
karindus	M	W082				Lorestan	Saravand, 33°22.39'N; 49°10.25'E	2070m	22 July 2009	VL & NS
karindus	M	W083				Lorestan	Saravand, 33°22.39'N; 49°10.25'E	2070m	22 July 2009	VL & NS
karindus	M	W084				Lorestan	Saravand, 33°22.39'N; 49°10.25'E	2070m	22 July 2009	VL & NS
karindus	M	W085				Lorestan	Saravand, 33°22.39'N; 49°10.25'E	2070m	22 July 2009	VL & NS
karindus	M	W086				Lorestan	Saravand, 33°22.39'N; 49°10.25'E	2070m	22 July 2009	VL & NS
karindus	M	W087				Lorestan	Saravand, 33°22.39'N; 49°10.25'E	2070m	22 July 2009	VL & NS
karindus	M	W093				Lorestan	Saravand, 33°22.39'N; 49°10.25'E	2070m	23 July 2009	VL & NS
karindus	M	W094				Lorestan	Saravand, 33°22.39'N; 49°10.25'E	2070m	23 July 2009	VL & NS
karindus	M	W095				Lorestan	Saravand, 33°22.39'N; 49°10.25'E	2070m	23 July 2009	VL & NS
karindus	M	W096				Lorestan	Saravand, 33°22.39'N; 49°10.25'E	2070m	23 July 2009	VL & NS
karindus	M	W377		h12/GH1	KT582716	Lorestan	Saravand, 33°22.39'N; 49°10.25'E	2100–2250m	3 August 2009	VL & NS
karindus	M	W378		h12/GH1	KT582717	Lorestan	Saravand, 33°22.39'N; 49°10.25'E	2100–2250m	3 August 2009	VL & NS
karindus	M	W379		h06/GH3	KT582718	Lorestan	Saravand, 33°22.39'N; 49°10.25'E	2100–2250m	3 August 2009	VL & NS
karindus	M	W380		h12/GH1	KT582719	Lorestan	Saravand, 33°22.39'N; 49°10.25'E	2100–2250m	3 August 2009	VL & NS
karindus	M	W381		h12/GH1	KT582720	Lorestan	Saravand, 33°22.39'N; 49°10.25'E	2100–2250m	3 August 2009	VL & NS
karindus	M	W382		h07/GH3	KT582721	Lorestan	Saravand, 33°22.39'N; 49°10.25'E	2100–2250m	3 August 2009	VL & NS
karindus	M	W383				Lorestan	Saravand, 33°22.39'N; 49°10.25'E	2100–2250m	3 August 2009	VL & NS
karindus	M	W386				Lorestan	Saravand, 33°22.39'N; 49°10.25'E	2100–2250m	3 August 2009	VL & NS
karindus	M	W387				Lorestan	Saravand, 33°22.39'N; 49°10.25'E	2100–2250m	3 August 2009	VL & NS
karindus	M	U168		h12/GH1	KT582741	Lorestan	Darreh Takht, 33°21.19'N; 49°22.34'E	2000–2100 m	18 July 2011	VL & NS
karindus	M	U178		h12/GH1	KT582742	Lorestan	Darreh Takht, 33°21.19'N; 49°22.34'E	2000–2100 m	18 July 2011	VL & NS
karindus	M	U179		h12/GH1	KT582743	Lorestan	Darreh Takht, 33°21.19'N; 49°22.34'E	2000–2100 m	18 July 2011	VL & NS
karindus	F	U169		h06/GH3	KT582744	Lorestan	Darreh Takht, 33°21.19'N; 49°22.34'E	2000–2100 m	18 July 2011	VL & NS

In addition, we used the following sequences from GenBank:

COI: AY557145 *P.* (*A.*) *karindus* (h01/GH5); AY557007 *P.* (*A.*) *dama*; AY556887 *P.* (*A.*) *birunii.*

Fresh (not worn) adult males were used to investigate the karyotypes. After capturing a butterfly in the field, it was placed in a glassine envelope for 1-2 hours to keep it alive until processed. Butterflies were killed by pressing the thorax. Testes for karyotype analysis were removed from the abdomen and placed into a 0.5 mL vial with a freshly prepared fixative (ethanol and glacial acetic acid 3:1). Then each wing was carefully removed from the body using forceps and placed into glassine envelope. The wingless body was placed into a plastic, 2 mL vial with pure 100% ethanol (for DNA analysis). Each vial with ethanol has already been numbered. This ID number was also used to label a vial with the fixative and a glassine envelope, in which the wings are preserved. Thus, each specimen was individually fixed. All collected specimens are kept in the Zoological Institute of the Russian Academy of Science (St. Petersburg) (ZIN RAS). All the testes are kept in the Department of Karyosystematics (ZIN RAS).

Chromosome preparation and karyotyping

Testes were stored in the fixative for 1–12 months at 4 °C. Then the gonads were stained in 2% acetic orcein for 30–60 days at 18–20 °C. Chromosome preparations were obtained as previously described (Talavera et al. 2013b). Different stages of male meiosis were examined by using a light microscope (Amplival, Carl Zeiss). An original two-phase method of chromosome analysis was used (Lukhtanov et al. 2006).

DNA Extraction and Sequencing

A fragment of the mitochondrial cytochrome *c* oxidase subunit I gene (first 690 positions) served as a mitochondrial molecular marker. Thoracic muscles and first abdominal segments were used for DNA extraction. The segments were homogenized in CTAB buffer and digested with proteinase K (10 mg/mL) for three hours at 60 °C. DNA was purified through successive ethanol precipitations and stored in dd H_2O at -20 °C.

For DNA amplification of *COI* we used primers K698 and Nancy (Caterino and Sperling 1999). PCR reactions (50 μl) contained 10 pmol each of forward and reverse primer, 1 mM dNTPs, 10x PCR Buffer (0.01 mM Tris-HCl, 0.05 M KCl, 0.1% Triton X–100: pH 9.0), 1 unit Taq DNA Polymerase (Fermentas), 5 mM $MgCl_2$ and were conducted using the following profile: initial 4 min denaturation at 94 °C and 30 cycles of 30 sec denaturation at 94 °C, 1 min annealing at 55 °C, 1 min extension at 72 °C and 5 min final elongation at 72 °C. PCR products were analyzed on 1.5% agarose gel, and purified using GeneJET PCR purification kit (Fermentas). Sequencing of double-stranded product was carried out at the Research Resource Center for Molecular and Cell Technologies (St. Petersburg State University).

Sequence alignments and phylogeny inference

The sequences were edited and aligned using CHROMAS 2.4.3 (http://www.technelysium.com.au/), Geneious 8.1.6 (Kearse et al. 2012), and BioEdit 7.0.3 (Hall 2011) software. The alignment was unambiguous, as all the sequences were of equal length and included no insertions/deletions. Primer sequences were cropped. This resulted in final alignment of 690 bp *COI* fragments. The analysis involved *COI* sequences inferred from 54 *P.* (*A.*) *karindus* specimens. Additional sequences of the *P.* (*A.*) *dama* (accession number AY557007) and *P.* (*A.*) *karindus* (accession number AY557145) were found in GenBank (Wiemers 2003) and were included into analysis, since these sequences completely overlapped with our fragment. We used sequence of *P.* (*A.*) *birunii* (Eckweiler and ten Hagen, 1998) (accession number AY556558) as an outgroup to root the phylogeny (according to available data, this species does not belong to the group closely related to *P.* (*A.*) *dama*). Thus, the final analysis included in total 57 *COI* sequences. A Bayesian approach for estimating phylogeny was used. Bayesian analyses were performed using the program MrBayes 3.2 (Ronquist et al. 2012), with the nucleotide substitution model GTR+G+I as suggested by jModelTest (Posada 2008). TRACER, v. 1.4 was used for summarizing the results of Bayesian phylogenetic analyses (http://beast.bio.ed.ac.uk/Tracer). A maximum–parsimony haplotype network was built using TCS v. 1.21, with a 99% parsimony connection limit (Clement et al. 2000).

Results

Analysis of karyotypes

Meiotic karyotypes were studied in 48 specimens of *P.* (*A.*) *karindus* from different Iranian localities. Depending on karyotypes and localities, 2 groups of individuals can be distinguished (Table 1 and see below).

Group I (*P.* (*A.*) *karindus* from NW Iran)

The haploid chromosome number n = 68 was found in meiotic metaphase I (MI) and meiotic metaphase II (MII) cells. The MI karyotype displayed 5 large bivalents in the center of metaphase plate and 63 smaller bivalents in the periphery (Fig. 2A).

Group II (will be described below as *P.* (*A.*) *karindus saravandi* from central Iran)

The haploid chromosome number n = 73 was found in meiotic MI and MII cells of studied individuals (Fig. 2B). The MI karyotype was strongly asymmetric with 5–6 larger bivalents in the center of the MI plate and 67–68 smaller bivalents in the periphery.

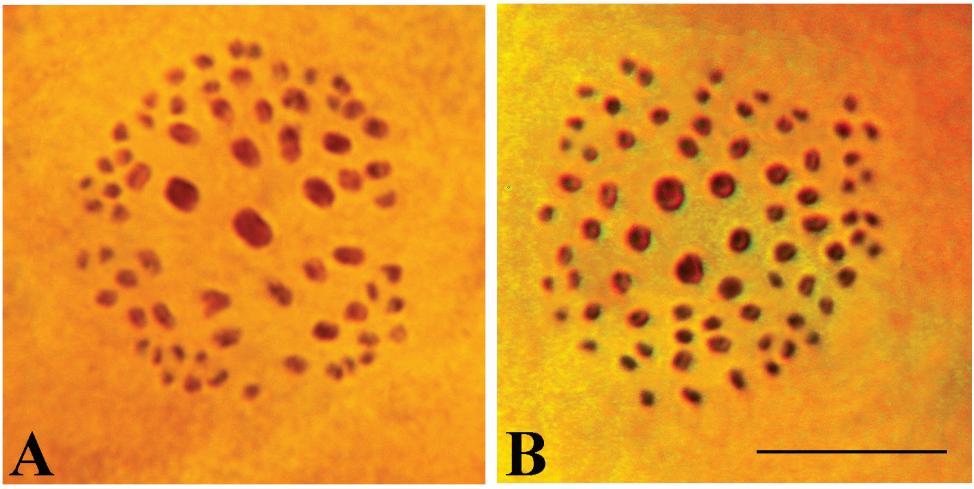

Figure 2. Male meiosis I karyotypes of: **A** *P.* (*A.*) *karindus karindus,* sample E399*,* Iran, Kordestan, 40 km SW Saqqez, 1800–1900 m, 2004.VII.29, V. Lukhtanov leg., n = 68 **B** *P.* (*A.*) *karindus saravandi*, sample W372, Iran, Nahavand 34°02.57'N; 048°20.22'E, 2173m, 2009.VIII.02, V. Lukhtanov & N. Shapoval leg., n = 73. Bar = 10 μm.

Phylogenetic analysis of molecular data

A Bayesian inference recovered *P.* (*A.*) *karindus* as a strongly supported monophyletic clade characterized by a specific set of fixed nucleotide substitutions (Fig. 3). Specimens of *P.* (*A.*) *karindus* were divided into several clusters: one cluster united specimens of *P.* (*A.*) *karindus* collected in north–west Iran (Fig. 3, GH5, highlighted in pink) and the others (Fig. 3, GH1–GH4, highlighted in blue) included specimens of central Iran populations (described here as a novel subspecies *P.* (*A.*) *karindus saravandi*). Most parsimonious *COI* haplotype network demonstrated similar pattern (Fig. 4). *P.* (*A.*) *dama* differs from *P.* (*A.*) *karindus* by at least 20 fixed nucleotide substitutions. Specimens of *P.* (*A.*) *karindus* form several haplotypes clustered in five different haplogroups. In general, composition of each haplogroup reflects geographical distribution of butterflies. Thus, majority of the specimens from easternmost (Saravand and Darreh Takht) and central west (Vennai, Nahavand) localities form two distinct haplogroups: GH1 and GH2. Nevertheless, two specimens from Vennai (approx. 80 km NW from Saravand) were found to have mitochondrial haplotype similar to that in easternmost populations, which has led to the suggestion that there is no complete isolation (reproductive or/and geographical) between population from Vennai and easternmost populations. The third haplogroup (GH3) consists of only three specimens, which were collected in Saravand and Darreh Takht. Interestingly, the third haplogroup differs drastically (by 10–12 fixed nucleotide substitutions) from the haplotypes, which comprise all other specimens from Saravand and Darreh Takht (group GH1). The fourth haplogroup (GH4) unites four specimens from Nahavand. Finally, all the haplotypes found in NW Iran constituted a subset of the distinct haplogroup (GH5). Thus, most parsimonious *COI* haplotype network reflects complex phylogeographic pattern of *P.* (*A.*) *karindus.*

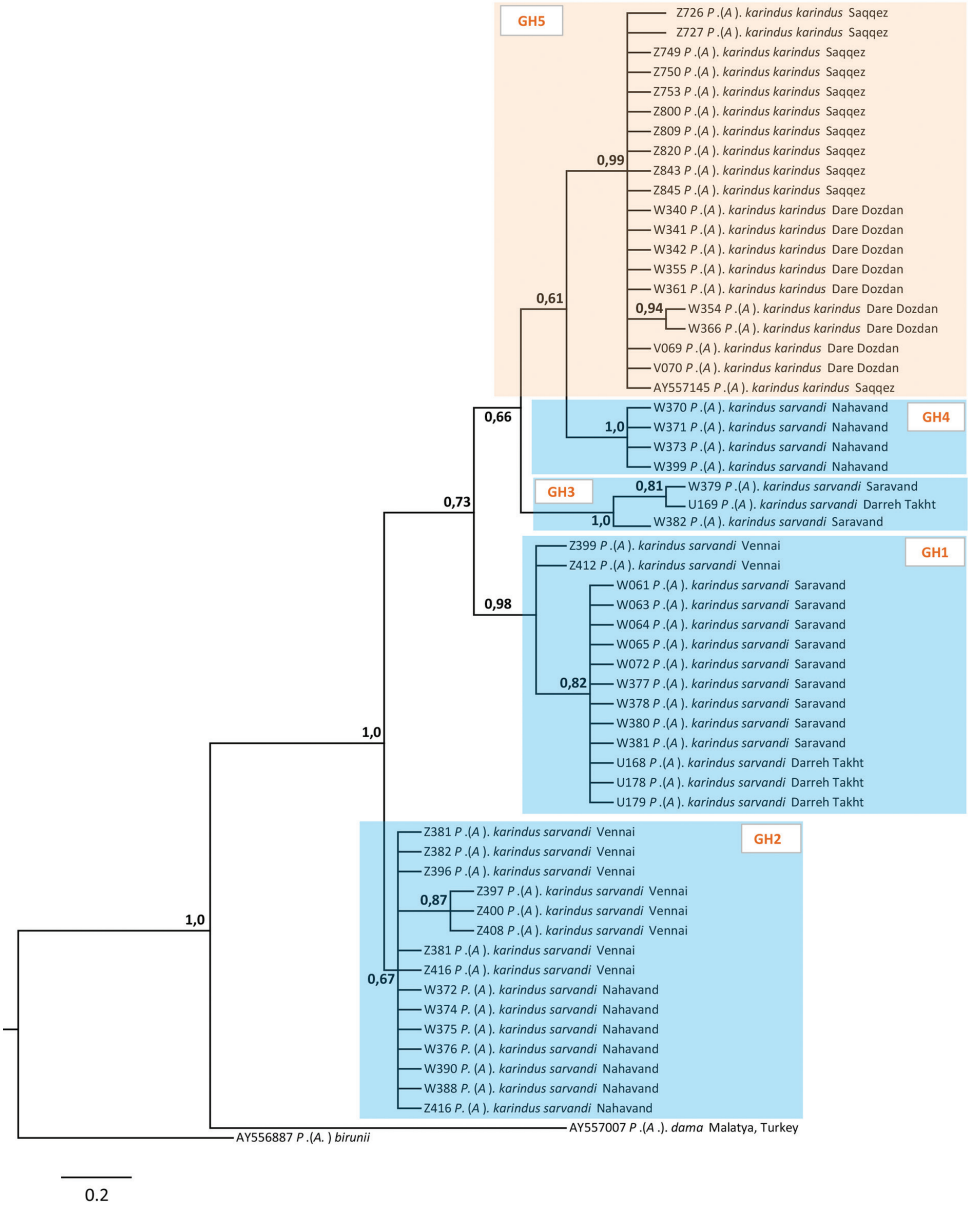

Figure 3. The Bayesian tree of *Polyommatus* (*Agrodiaetus*) *dama* and *Polyommatus* (*Agrodiaetus*) *karindus* based on analysis of the cytochrome c oxidase subunit I gene from 57 specimens. Numbers at nodes indicate Bayesian posterior probability. *Agrodiaetus karindus karindus* and *Agrodiaetus karindus saravandi* clusters highlighted in pink and blue respectively.

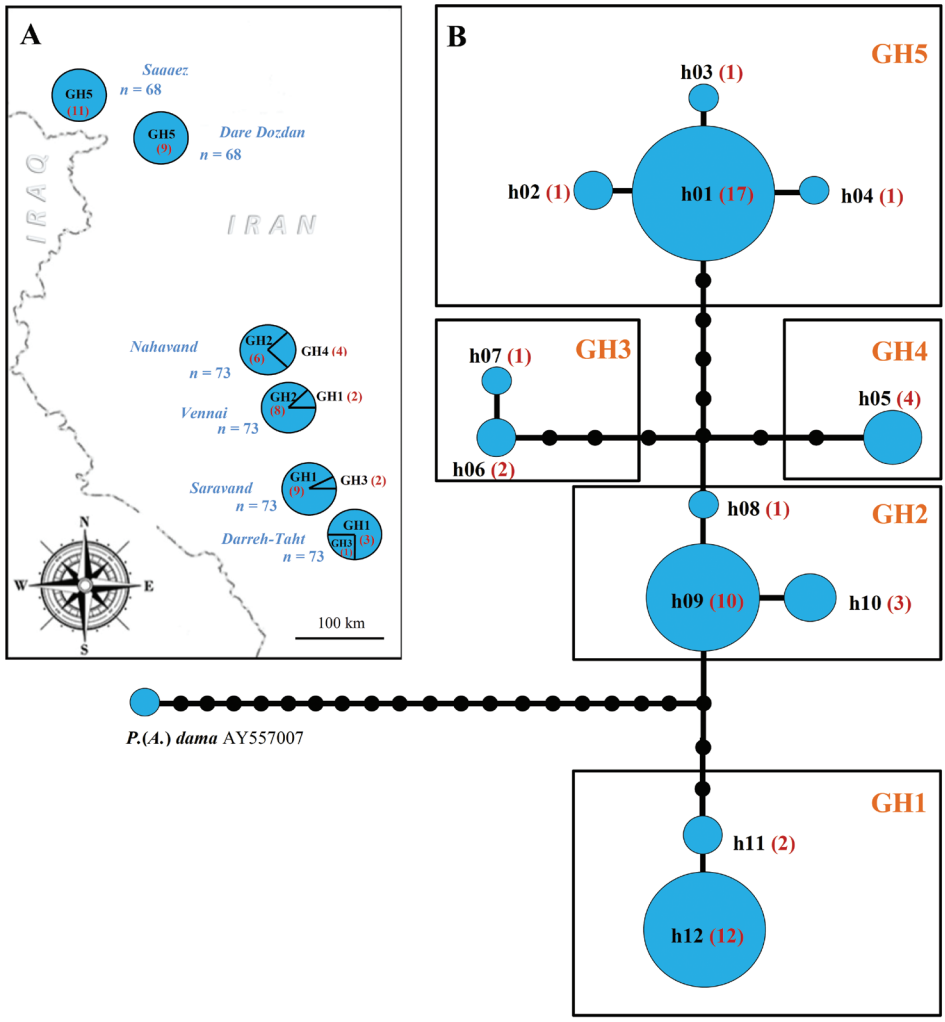

Figure 4. *COI* Haplotype analysis. **A** geographical distribution of haplogroups. Number of studied individuals sharing the same haplogroup is given in parentheses **B** most parsimonious *COI* haplotype network; h01–h12 are *COI* haplotypes; GH1–GH5 are *COI* haplogroups. Number of studied individuals sharing the same haplotype is given in parentheses.

Discussion

We have found that a taxon usually identified as *P.* (*A.*) *karindus* is represented in Iran by two geographically separated groups of individuals. The first group unites specimens collected in NW Iran, while the second group comprises specimens from central Iran. The representatives of these groups have different chromosome numbers, n = 68 and n = 73 respectively. They also have at least eight fixed nucleotide differences in 690 bp fragment of mitochondrial *COI* gene. The first group is monophyletic with respect

to both *COI* gene and karyotype (n = 68). The second group has complicated genetic structure, comprises several differentiated populations and is paraphyletic with respect to the *COI* gene. Despite this gene paraphyly, it appears as a clearly monophyletic group with respect to its karyotype (n = 73). Thus, the NW and central Iranian groups are differentiated by at least five fixed chromosome fusions/fissions. Fixed chromosome differences are often considered as characters associated with reproductive isolation (King 1993). From this point of view, the NW and central Iranian groups could be theoretically treated as a different species. However, our recent studies on *Agrodiaetus* demonstrated that multiple chromosome fusions and fissions did not block fertility in chromosomal hybrids (Lukhtanov et al. 2015b). In other words, differentiation by five fixed chromosome rearrangements would not guarantee impossibility of blending populations together when they occur in sympatry. Thus, NW and central Iranian groups of populations should be considered as a subspecies rather than separate species.

Since *Polyommatus* (*Agrodiaetus*) *karindus* (Riley, 1921) (orig. comb. *Lycaena dama* subsp. *karinda*) was described from NW Iran (type locality is "Harir, Karind, and Karind Gorge, N.W. Persia" according to original description, and "N.W. Persia, Karind Gorge, 6000 ft" according to lectotype designation made by Bálint (1999) (not from central Iran), the name *P. karindus karindus* should be attributed to the NW Iranian group of populations. The formal description and naming of the central Iranian group is provided below.

Description of the novel taxon

***Polyommatus (Agrodiaetus) karindus saravandi* ssp. n.**
http://zoobank.org/
Fig. 1 – map, Fig. 2B karyotype, Figs 3–4 phylogeny, Fig. 5 – Holotype of *P.* (*A.*) *karindus saravandi*, Fig. 6 A, B – Underside and upperside of the male and female wings

Holotype. ♂. Forewing length 34.0 mm. Iran, Lorestan province, Zagros Mt., vicinity of Saravand village, 33°22.39'N; 49°10.25'E, 2070 m, 22.07 2009. N. Shapoval and V. Lukhtanov leg. In the Zoological Institute of the Russian Academy of Sciences (St. Petersburg). Specimen field code W064, GenBank code for mitochondrial cytochrome *c* oxidase subunit I (*COI*) gene (partial cds) is KT582713.

Paratypes. 87 ♂♂, field codes W061, W062, W063, 21.07.2009; W065, W072, W073, W074, W075, W081, W082, W083, W084, W085, W086, W087, the same locality, date and collectors as the holotype. Field codes W093, W094, W095, W096 23.07.2009, the same locality and collectors as the holotype. Field codes W377, W378, W379, W380, W381, W382, W383, W386, W387 03.08.2009, the same locality and collectors as the holotype. Field codes W370, W371, W372, W373, W374, W375, W376, Iran, Lorestan province, Zagros Mt., vicinity of Nahavand village, 34°02.57'N; 048°20.22'E, 2170 m, 02.08.2009, the same collectors as the holotype. Field codes W388, W389, W390, W391, W392, Iran, Lorestan province, Zagros Mt., vicinity

Figure 5. Holotype of *P.* (*A.*) *karindus saravandi,* sample W064. Upperside (left) and underside (right) of the male wings.

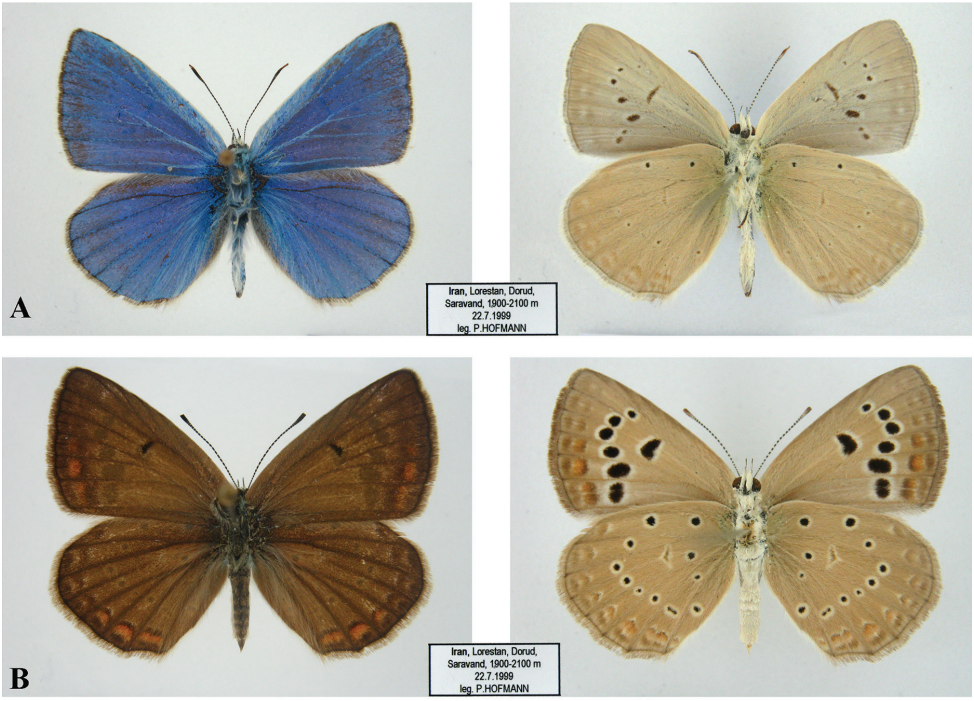

Figure 6. Underside and upperside of the *P.* (*A.*) *karindus saravandi* ssp. nov. wings. **A** upperside (left) and underside (right) of the male wings **B** upperside (left) and underside (right) of the female wings.

of Nahavand village, 34°02.57'N; 048°20.22'E, 2170 m, 02.08.2009, the same collectors as the holotype. Field codes U217, U218, U219, U220, U223, Iran, Lorestan province, Zagros Mt., vicinity of Nahavand village, 34°02.57'N; 048°20.22'E, 2170 m, 19.07.2011, the same collectors as the holotype. Field codes U228, U229, U230, U231, U232, U233, U234, U235, U236, U237, U238, U239, U240, U256, U257, U262, U263, U264, U265, U266, U267, U278, U279, U280, U281, Iran, Lorestan province, Zagros Mt., vicinity of Nahavand village, 34° 02.92'N; 48° 20.40'E, 2160 m, 20.07.2011 the same collectors as the holotype. Field codes Z381, Z382, Z396, Z397, Z398, Z399, Z400, Z408, Iran, Lorestan province, Zagros Mt., W of Borujerd, Kuh-e Garin mount mount., Vennnai, 33°53.89'N; 48°34.03'E, 2150 m, 21.07.2007, the same collectors as the holotype. Field codes Z412, Z413, Z416, Iran, Lorestan province, Zagros Mt., W of Borujerd, Kuh-e Garin mount., Vennnai, 33°53.89'N; 48°34.03'E, 2150 m, 22.07.2007, the same collectors as the holotype. Field codes V331, 335, V336, Iran, Lorestan province, Zagros Mt., W of Borujerd, Kuh-e Garin mount., Vennnai, 33°53.89'N; 48°34.03'E, 2150 m, 02.08.2014, N. Shapoval and A. Barabanov leg. Field codes U169, U178, U179, Iran, Lorestan province, Zagros Mt., Darreh Takht, 33° 21.19'N; 49° 22.34'E, 2000–2100 m, 18.07.2011, the same collectors as the holotype. 1 ♀, field code U169 Iran, Lorestan province, Zagros Mt., Darreh Takht, 33° 21.19'N; 49° 22.34'E, 2000–2100 m, 18.07.2011, same collectors as the holotype. All paratypes are kept in the Zoological Institute of the Russian Academy of Sciences (St. Petersburg). Gene Bank accession numbers of the paratypes are presented in the Table 1.

Derivatio nominis. The new taxon is named after the village Saravand, one of the places where it was found.

Description. *Male upperside.* Forewing length 30–36 mm, ground colour bright blue with azure tint. Discoidal, submarginal and antemarginal marking absent on both fore- and hindwings. Black outer marginal line on forewings and hindwings very narrow; forewing hind margin with long white pubescence. Fringes of both wings dark grey; tips of hindwings veins indicated with fine black.

Male underside. Ground colour light grey, white streak on the hindwings absent. Basal black spots present only on hindwings. Discoidal series of spots present on fore- and hindwings, although the black spots composing it are minute. Postdiscal black marking very narrow, longitudinal, present only on forewings. Submarginal and marginal lunules only faintly indicated.

Female upperside. Ground colour brown with vastly darker veins. Discoidal black spots present on forewings. Submarginal markings dark brown with orange submarginal lunules well developed on forewing and hindwing. Fringe greyish-brown.

Female underside. General design as in males, but ground colour slightly darker.

Genitalia. The male genitalia have a structure typical for other species of the subgenus *Agrodiaetus* (Coutsis 1986). No specific characters in genitalia are found.

Diagnosis. Genetically *P.* (*A.*) *karindus saravandi* differs from all other taxa of *Agrodiaetus* by fixed substitutions in mitochondrial gene *COI*. Phenotypically the new taxon is extremely similar to *P.* (*A.*) *karindus karindus* from north-west Iran, but they have different chromosome numbers, n=73 and n = 68 respectively.

Distribution. Central part of Zagros Mountains, Iran.

Flight period. From July to August.

Ecology. Dry slopes, gorges and plateaus with xerophyte or steppe vegetation, sometimes wooded areas from 1800 up to 2800 m. Butterflies fly together with *P.* (*A.*) *alcestis* (Zerny, 1932), *P.* (*A.*) *cyaneus* (Staudinger, 1899), *P.* (*A.*) *hamadanensis* (de Lesse, 1959), *P.* (*A.*) *lorestanus* (Eckweiler, 1997) and *P.* (*A.*) *zarathustra* (Eckweiler, 1997).

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Interpretation of mitochondrial diversity in terms of taxonomy: a case study of *Hyponephele lycaon* species complex in Israel (Lepidoptera, Nymphalidae, Satyrinae)

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Abstract

It is difficult to interpret mitochondrial diversity in terms of taxonomy even in cases in which a concordance exists between mitochondrial, ecological and morphological markers. Here we demonstrate this difficulty through a study of Israeli *Hyponephele* butterflies. We show that samples commonly identified as *Hyponephele lycaon* are represented on Mount Hermon in Israel by two sympatric groups of individuals distinct both in mitochondrial DNA-barcodes (uncorrected *p*-distance = 3.5%) and hindwing underside pattern. These two groups were collected in different biotopes. They also tended to be different in length of brachia in male genitalia, although the latter character is variable. We reject the hypothesis that the discovered *COI* haplogroups are selectively neutral intraspecific characters. We hypothesize that they represent: either (1) two different biological species, or (2) a consequence of a strong positive selection acting at intraspecific level and resulting in two intraspecific clusters adapted to low and to high elevations. If we accept the first hypothesis, then provisionally these two haplogroups can be attributed to transpalearctic *H. lycaon* sensu stricto and to *H. lycaonoides*, previously known from Iran and East Turkey.

Keywords

adaptation to high/low elevation, biodiversity, *COI*, cryptic species, DNA barcoding, disruptive selection, habitat-related selection, molecular markers

Introduction

Hyponephele Muschamp, 1915 is a large and taxonomically diverse genus of satyrine butterflies. The genus contains 39 species (Eckweiler and Bozano 2011) distributed throughout the Palearctic region, with the highest species diversity found in Central Asia, Iran and Turkey. This group was taxonomically revised by Samodurov with co-authors (Samodurov et al. 1995, 1996, 1997, 1999a, b, 2000, 2001) and by Eckweiler and Bozano (2011).

Within the genus, *Hyponephele lycaon* (Rottenburg, [1775]) is the best known and the most common species broadly distributed in the temperate zone of the Palearctic from Portugal in the west to Far East Russia in the east (Samodurov et al. 2001, Eckweiler and Bozano 2011). In south Palearctic it is replaced by closely related allopatric taxa *H. maroccana* Blachier, 1908 (North Africa), *H. galtscha* (Grum-Grshimailo, 1893) (Tajikistan) and *H. sifanica* (Grum-Grshimailo, 1891) (China) (Eckweiler and Bozano 2011). One more species, *H. lycaonoides* D. Weiss, 1978 was described from Zagros Mountains in Iran. *Hyponephele lycaonoides* was shown to be sympatric with *H. lycaon* in Iran (Weiss 1978, Eckweiler and Bozano 2011, Tshikolovets et al. 2014). *Hyponephele lycanoides* was also reported for Turkey (Koçak 1989, Eckweiler and Bozano 2011), but the reports for Turkey were questioned in the comprehensive analysis of Turkish butterfly fauna made by Hesselbarth et al. (1995). Male genitalia structures are commonly used for distinguishing between *H. lycaon* and *H. lycaonoides*, and specimens with long brachia are attributed to *H. lycaon*, whereas specimens with short brachia are attributed to *H. lycaonoides* (Weiss 1978). However, male genitalia are variable in both *H. lycaon* and *H. lycaonoides,* and intermediate forms are reported to be common (Eckweiler and Bozano 2011). Moreover, Hesselbarth et al. (1995) considered these traits (the long and short brachia) as intraspecific variations, rather than species-specific characters. Unfortunately, until now nobody used molecular markers to test the non-conspecifity of *H. lycaon* and *H. lycaonoides.*

In our study we analysed mitochondrial DNA barcodes and morphological and ecological markers to show that butterflies commonly identified as *Hyponephele lycaon* are represented in Israel by two sympatric groups of individuals. We further discuss different possible evolutionary and taxonomic interpretations of the pattern discovered.

Materials and methods

In the course of our DNA barcode survey of Israeli butterflies (2012–2015) we found butterflies similar to *H. lycaon* on Mount Hermon in northern Israel. They were collected in a small area situated between 33°17'12"N, 35°45'49"E, at 1440 m and 33°18'38"N, 35°47'07"E, at 2050 m. The distance between these extreme points of the collecting was 3460 m (measured using Google Earth map). Some of the butterflies were collected in the forest zone at 1450-1600 m above sea level, other were collected

Table 1. List of *Hyponephele* samples sequenced in the present study.

Haplogroup or taxon	Country	Ecological zone	Pattern of the wing underside	BOLD Process ID	Field ID	GenBank accession #
I	Israel	forest	contrasting	BPAL2756-15	CCDB-17969_A01	KT864697
I	Israel	forest	contrasting	BPAL2757-15	CCDB-17969_A02	KT864698
I	Israel	forest	contrasting	BPAL2758-15	CCDB-17969_A03	KT864699
I	Israel	forest	contrasting	BPAL2760-15	CCDB-17969_A05	KT864700
I	Israel	forest	contrasting	BPAL2761-15	CCDB-17969_A06	KT864701
I	Israel	forest	contrasting	BPAL2765-15	CCDB-17969_A10	KT864702
II	Israel	forest	pale	BPAL2695-14	CCDB-17968_C11	KT864691
II	Israel	subalpine	pale	BPAL2705-14	CCDB-17968_D09	KT864692
II	Israel	subalpine	pale	BPAL2706-14	CCDB-17968_D10	KT864693
II	Israel	subalpine	pale	BPAL2733-14	CCDB-17968_G01	KT864690
II	Israel	subalpine	pale	BPAL2762-15	CCDB-17969_A07	KT864694
II	Israel	subalpine	pale	BPAL2763-15	CCDB-17969_A08	KT864695
II	Israel	subalpine	pale	BPAL2764-15	CCDB-17969_A09	KT864696
H. lupinus	Israel	n/a	n/a	BPAL2719-14	CCDB-17968_E11	KT864688
H. lupinus	Israel	n/a	n/a	BPAL2683-14	CCDB-17968_B11	KT864689
H. maroccana	Morocco	n/a	n/a	BPAL1378-12	CCDB-03030_D12	KT864703
H. maroccana	Morocco	n/a	n/a	BPAL1377-12	CCDB-03030_D11	KT864704
H. maroccana	Morocco	n/a	n/a	BPAL1376-12	CCDB-03030_D10	KT864705

in the subalpine zone with predominance of xerophytous vegetation at 1800-2050 m above sea level (Table 1).

DNA barcodes, 658 bp fragments within mitochondrial gene, *cytochrome oxidase subunit I* (*COI*), were sequenced at the Canadian Centre for DNA Barcoding (CCDB, Biodiversity Institute of Ontario, University of Guelph) using standard high-throughput protocol described in deWaard et al. (2008). DNA was extracted from a single leg removed from each voucher specimen employing a standard DNA barcode glass fibre protocol (Ivanova et al. 2006). All polymerase chain reactions (PCR) and DNA sequencing were carried out following standard DNA barcoding procedures for Lepidoptera as described previously (Hajibabaei et al. 2005). Photographs of specimens used in the analysis are available in the Barcode of Life Data System (BOLD) at http://www.barcodinglife.org/. All voucher specimens are deposited at the Zoological Institute of the Russian Academy of Sciences and could be identified by the corresponding unique BOLD Process IDs, that were automatically generated by BOLD, and by GenBank accession numbers (Table 1).

The procedure of phylogenetic inference was described previously (Vershinina and Lukhtanov 2010, Talavera et al. 2013, Lukhtanov et al. 2014, 2015a, b). Briefly, the sequences were aligned using BioEdit version 7.1.7 software (Hall 1999) and edited manually. Phylogenetic relationships were inferred using Bayesian Inference and

the program MrBayes 3.2.2 (Ronquist et al. 2012). A GTR substitution model with gamma distributed rate variation across sites and with proportion of invariable sites was specified before running the program as suggested by jModelTest (Posada 2008). Two runs of 10,000,000 generations with four chains (one cold and three heated) were performed. Chains were sampled every 10,000 generations, and burn-in was determined based on inspection of log likelihood over time plots using TRACER, version 1.4 (available at http://beast.bio.ed.ac.uk/Tracer). For comparison we used additional *COI* barcodes of *Hyponephele* downloaded from GenBank (Lukhtanov et al. 2009, Dinca et al. 2011).

Butterfly photographs were taken with Nikon D810 digital camera equipped with a Nikon AF-S Micro Nikkor 105 mm lens. Genitalia photographs were taken with Leica M205C binocular microscope equipped with Leica DFC495 digital camera, and processed using the Leica Application Suite, version 4.5.0 software.

Results

During a 2012-2015 survey of Israeli fauna, *H. lycaon*-similar butterflies were found only on Mount Hermon in northern Israel. We never observed *H. lycaon*-similar butterflies in other parts of Israel, although the distantly related *H. lupinus* (Costa, 1836) was found not only in the northern, but also in central Israel. Thus, our observations support the finding that the geographic range of *H. lycaon* species complex is restricted in Israel to the northernmost part of the country (Benyamini 2002).

Molecular analysis of *H. lycaon*-similar samples (Table 1, Fig. 1) revealed two distinct mitochondrial haplogroups (I and II) that were strongly differentiated with respect to the *COI* gene. These two haplogroups differed from one another by 23 fixed nucleotide substitutions in the studied 658 bp fragment of the mitochondrial *COI* gene. When looking at the level of primary polypeptide structure, these differences translate to two fixed amino acid substitutions in the studied fragment. The minimal uncorrected *COI p*-distance between these two haplogroups was found to be as high as 3.5 %. *Hyponephele lupinus* from Israel was found to be closely related to *H. interposita* and distant from all the taxa of the *H. lycaon* complex.

With a single exception (female sample BPAL2695-14|CCDB-17968_C11, Fig. 1, Table 1), the representatives of these two *COI* haplogroups were collected in different biotopes (Fig. 2). The butterflies of haplogroup I were found on grassy slopes in the forest zone (1450-1600 m above see level) (Fig. 2a). The butterflies of haplogroup II were found in steppe lands of the subalpine zone (1800-2050 m alt.), where xerophytous thorny cushion vegetation formed by *Onobrychis cornuta* and *Astragalus* species (Fabaceae) was predominant (Fig. 2b).

Standard χ2-test was used to distinguish between random *vs*. non-random distribution haplogroups I and II in the low (forest) and high (subalpine) zones. Empirical and expected frequencies of *COI* haplogroups I and II in low and high altitude belts were compared (Table 2). The calculated χ^2 was larger than the tabular value (9.558 *vs*. 6.635,

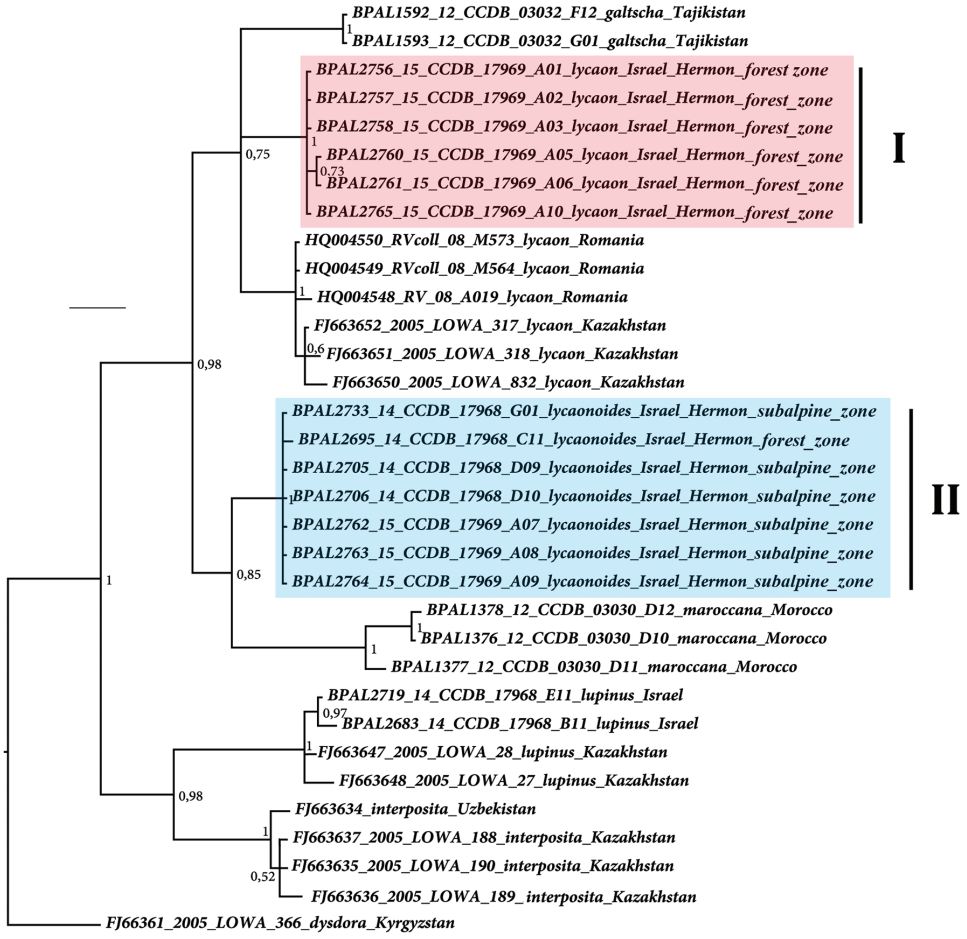

Figure 1. The Bayesian tree of the *Hyponephele lycaon* species complex based on analysis of *COI* DNA barcodes. Numbers at nodes indicate Bayesian posterior probability values. Sympatric haplogroups I and II from Israel are highlighted. Scale bar = 0.2 substitutions per position.

Table 2. Primary data (number of samples) for $\chi 2$-analysis of random *vs*. non-random distribution of the *COI* I and II haplogroups in the low (forest) and high (subalpine) zones.

	empirical values		expected values (in case of random distribution)	
	low altitude	high altitude	low altitude	high altitude
COI haplogroup I	6	0	3.234	2.772
COI haplogroup II	1	6	3.766	3.228

df = 1, 0.01 level of significance). Therefore, we reject the H_0 hypothesis and conclude that haplogroup I butterflies are significantly more frequent in the lower zone, whereas haplogroup II butterflies are significantly more frequent in the higher zone.

Figure 2. Biotopes on Mount Hermon, Israel where *COI* haplogroups I (**a**) and II (**b**) were collected.

The representatives of these two clusters were also different in the pattern on the hindwing underside (Figs 3 and 4). In haplogroup I this pattern had more contrast with clearly visible medial band, whereas in haplogroup II the hindwing underside was paler and had less contrast.

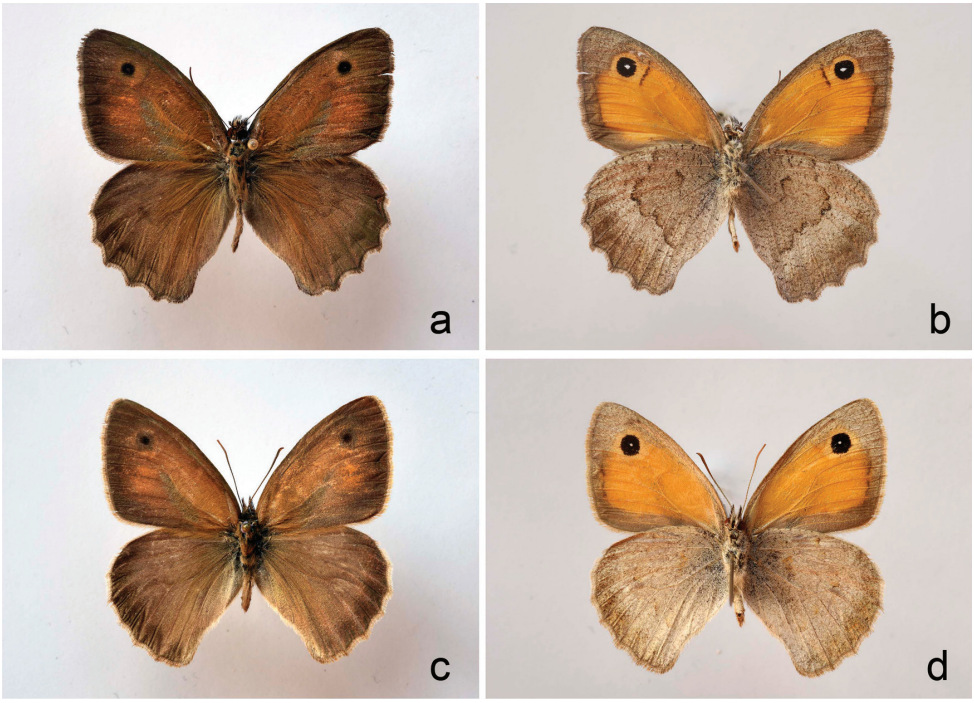

Figure 3. Wing pattern in haplogroup I and II samples from Mt Hermon, Israel. The pictures were taken using diffused daylight **a** sample CCDB-17969_A02, upperside **b** sample CCDB-17969_A02, underside **c** sample CCDB-17969_A09, upperside **d** sample CCDB-17969_A09, underside.

Table 3. Primary data (number of samples) for χ2-analysis of random *vs.* non-random association between the haplogroup I and II and the hindwing underside pattern.

	empirical values		**expected values (in case of random distribution)**	
	contrast pattern	**pale**	**contrast pattern**	**pale**
COI haplogroup I	6	0	2.772	3.234
COI haplogroup II	0	7	3.228	3.766

A standard χ2-test was used to distinguish between random *vs*. non-random association between haplogroups I and II and hindwing underside pattern (Table 3). The calculated χ^2 of 12.860 was larger than the tabular value (12.860 *vs.* 10.83, df = 1, 0.001 level of significance). Therefore, we reject the H_0 hypothesis and conclude that *COI* haplogroup I is significantly associated with contrast pattern of the hindwing underside, whereas *COI* haplogroup II is significantly associated with pale pattern of the hindwing underside.

The representatives of these two *COI* haplogroups also tended to be different in the length of the brachia in male genitalia (Fig. 5), although the latter character had high variability. Males of haplogroup I often had long brachia (Fig. 5a, b), whereas males of haplogroup II were mainly characterized by reduced brachia (Fig. 5c, d).

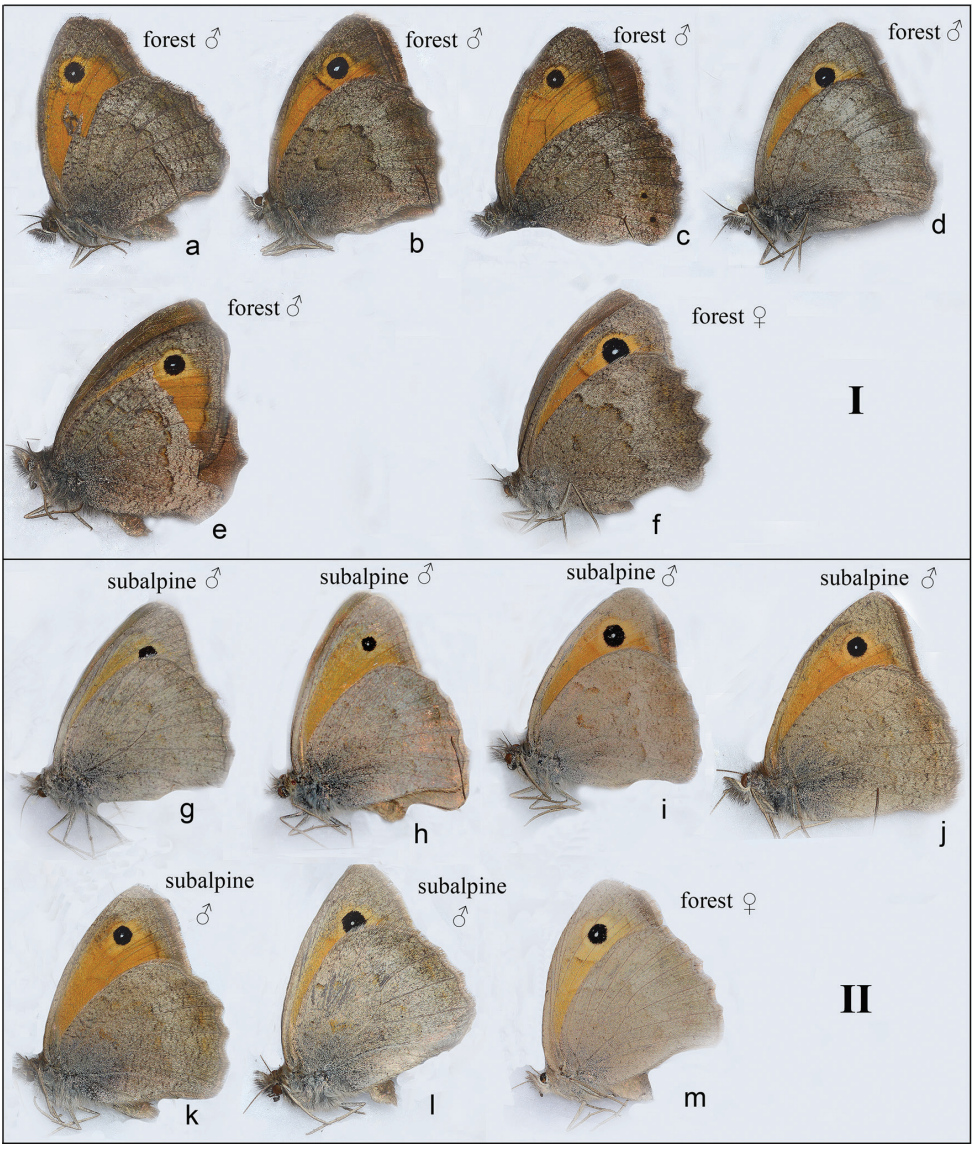

Figure 4. Pattern of the wing underside in haplogroups I and II samples. The pictures were taken using a flash **a** CCDB-17969_A01 **b** CCDB-17969_A02 **c** CCDB-17969_A03 **d** CCDB-17969_A06 **e** CCDB-17969_A10 **f** CCDB-17969_A05 **g** CCDB-17968_C11 **h** CCDB-17968_D09 **i** CCDB-17968_G01 **j** CCDB-17969_A07 **k** CCDB-17969_A08 **l** CCDB-17969_A09 **m** CCDB-17968_C11.

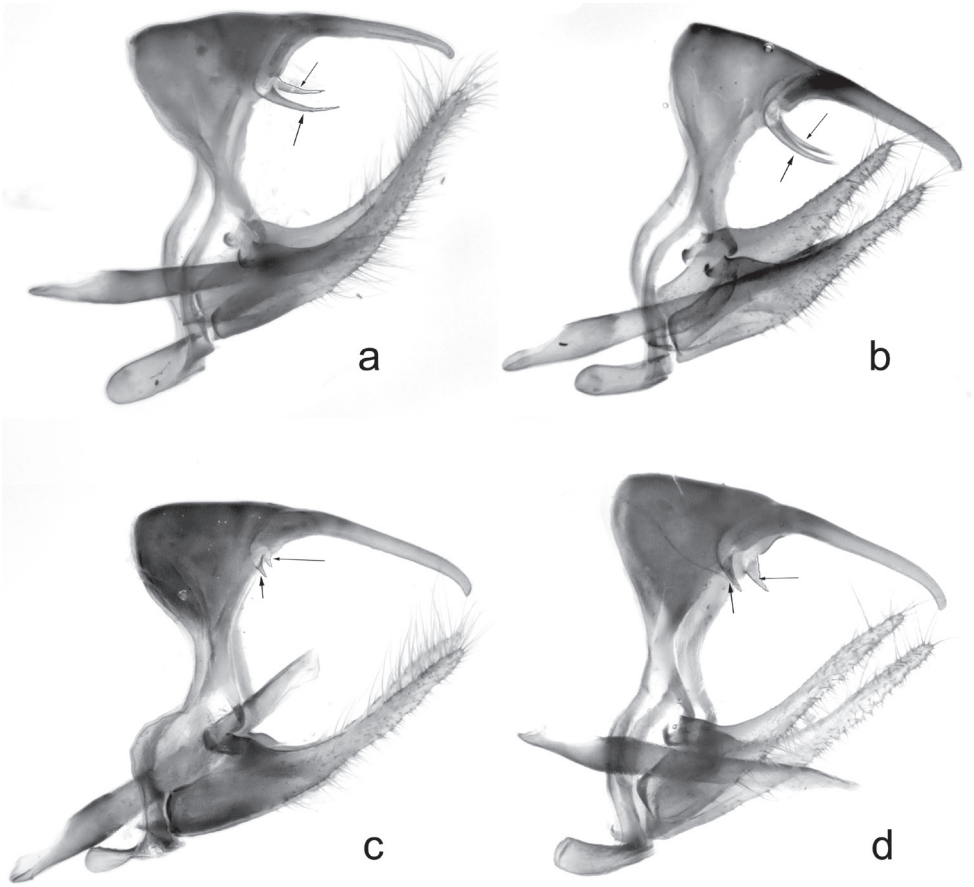

Figure 5. Typical male genitalia in haplogroups I (**a**, **b**) and II (**c**, **d**) from Mt Hermon, Israel. Lateral view. Brachia are indicated by arrow **a** specimen 17969_A10 **b** specimen 17969_A01 **c** specimen 17968_D10 **d** specimen 17968_D09.

Discussion

Evolutionary interpretation of the discovered pattern

The *COI* genetic distance between haplogroups I and II (3.5 %) is higher than the 'standard' 2.7–3.0% DNA-barcoding threshold commonly used as a tentative indicator for species distinctness of the taxa compared (Lambert et al. 2005, Lukhtanov et al. 2015a). It is known that *COI* barcodes alone are not sufficient for making any taxonomic decisions, since trees inferred from single markers sometimes display relationships that reflect the evolutionary histories of individual genes rather than the species being studied (Nichols 2001). Mitochondrial introgression (Zakharov et al. 2009) and *Wolbachia* infection (Ritter et al. 2013) can lead to additional bias when inferring taxonomic conclusions based on mitochondrial genes. Typically, multiple molecular markers or a combination of morphological and molecular markers are required for inferring taxonomic hypotheses. In our

research such additional information is represented by ecological characteristics (altitude belts). Less attention was attributed to the wing pattern, since we were not sure that it was an independent character. As the wing pattern strongly correlated with the ecology (low versus high elevation), one could hypothesize that the morphological difference is a consequence of phenotypic plasticity, i.e. ability of the same genotype to result in different phenotypes in response to changes in the environment (Price et al. 2003).

Three alternative explanations can account for bimodal sympatric distribution of mitochondrial markers. First, the diverged *COI* sequences may be selectively neutral intraspecific characters. Both preservation of a variety of ancestral haplotypes and mitochondrial introgression due to complex phylogeographic history could be responsible for such a neutral polymorphism (Avise 2000). Second, bimodal sympatric distribution of mitochondrial markers may be a result of a strong positive habitat-related selection working at intraspecific level and resulting in two *COI* clusters associated with different altitude belts (Cheviron and Brumfield 2009). Third, bearers of two diverged haplogroups may represent two different biological species (Avise 2000).

In our case the first hypothesis (neutral polymorphism) can be easily rejected. It predicts that the *COI* haplogroups I and II should be stochastically (i.e. randomly) distributed within high and low altitude belts. This prediction is not supported by $\chi 2$-test that demonstrated significantly non-random distribution of the *COI* haplogroups.

The second hypothesis (strong intraspecific positive selection) offers a more exotic, but not improbable, explanation. As *COI* sequence can be translated into a subunit of cytochrome c oxidase, a functional protein in mitochondria involved in energy metabolism (Kirk and Freedland 2011), this gene should be under natural selection (Castoe et al. 2008). Different haplotypes at this locus (or other linked mitochondrial genes) may be favoured in different environments. This could trigger a rapid sweep to fixation of a novel haplotype. This may result in sympatric clusters that differ in mitochondrial genes while exchanging alleles freely throughout the rest of the genome. Interestingly, such groups maintained by habitat-related selection could be considered species according to the genotypic cluster species concept (Coyne and Orr 2004, p. 448–449). The positive habitat-related selection of mitochondrial genome, despite its theoretical plausibility, has so far relatively low empirical support, although there are some data confirming mitochondrial evolution along temperature and altitude gradients (Cheviron and Brumfield 2009, Quintela et al. 2014).

The third hypothesis (two different species) seems to be a more likely explanation in the case of haplogroups I and II, especially if one takes into account the high level of genetic divergences between the haplogroups and concordance between molecular (Fig. 1), ecological (Fig. 2, Table 2) and morphological (Figs 3 and 4, Table 3) characters. More samples, especially from the intermediate elevation (1600-1800 m), and analysis of additional nuclear molecular markers across altitudinal transect will be required in future research to support or to reject the second (positive selection) and the third (two species) hypotheses and to reveal potential nuclear gene flow between haplogroups I and II.

Taxonomic interpretation of the discovered pattern

The presence of two sympatric, ecologically differentiated groups within *H. lycaon* complex in the Middle East is not a completely novel issue. A similar situation is known to exist in Iran and East Turkey (Weiss 1978, Eckweiler and Bozano 2011, Tshikolovets et al. 2014). It is accepted by *Hyponephele* genus experts (Eckweiler and Bozano 2011, Tshikolovets et al. 2014) that in Iran and Turkey these two groups represent two different species: *H. lycaon* and *H. lycaonoides* (but see the alternative opinion: Hesselbarth et al. 1995). Although we understand that this point of view requires an additional justification, we may accept it as a working hypothesis until further investigations and taxonomic revisions justify or falsify it.

If the species status of the discovered haplogroups will be confirmed in further studies, we suggest that, following Weiss (1978), the name *H. lycaon* (Rottenburg, [1775]) can be used for the Israeli taxon characterized by the contrast pattern on the hindwing underside and the predominance of longer brachia in male genitalia. Correspondingly, the name *H. lycaonoides* D. Weiss, 1978 can be used for the Israeli taxon characterized by the less contrasted pattern of the hindwing underside and the predominance of reduced brachia in male genitalia. However, this nomenclatural decision should be considered as a tentative one. First, despite recent revisions of the genus (Samodurov et al. 1995, 1996, 1997, 1999a, b, 2000, 2001, Eckweiler and Bozano 2011), no one studied type-specimens of numerous taxa that were described as subspecies and variations of *H. lycaon.* We cannot exclude that the name *lycaonoides* is a synonym of one of the previously described taxa, e.g. of *libanotica* (Staudinger, 1901). Second, molecular markers have never been used for analysis of taxonomic structure of *H. lycaon* species complex in its whole distribution range. Therefore, we will not be surprised if the true genetic and taxonomic structure of this group will be revealed as much more complex than a simple combination of two sympatric clusters as discovered in Iran, Turkey and Israel.

Acknowledgements

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DNA barcoding reveals twelve lineages with properties of phylogenetic and biological species within *Melitaea didyma* sensu lato (Lepidoptera, Nymphalidae)

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Abstract

The complex of butterfly taxa close to *Melitaea didyma* includes the traditionally recognized species *M. didyma*, *M. didymoides* and *M. sutschana*, the taxa that were recognized as species only relatively recently (*M. latonigena*, *M. interrupta*, *M. chitralensis* and *M. mixta*) as well as numerous described subspecies and forms with unclear taxonomic status. Here analysis of mitochondrial DNA barcodes is used to demonstrate that this complex is monophyletic group consisting of at least 12 major haplogroups strongly differentiated with respect to the gene *COI*. Six of these haplogroups are shown to correspond to six of the above-mentioned species (*M. didymoides*, *M. sutschana*, *M. latonigena*, *M. interrupta*, *M. chitralensis* and *M. mixta*). It is hypothesized that each of the remaining six haplogroups also represents a distinct species (*M. mauretanica*, *M. occidentalis*, *M. didyma*, *M. neera*, *M. liliputana* and *M. turkestanica*), since merging these haplogroups would result in a polyphyletic assemblage and the genetic distances between them are comparable with those found between the other six previously recognized species.

Keywords

Biodiversity, butterflies, *COI*, cryptic species, mitochondrial DNA, Nymphalidae, phylogeography, taxonomy

Introduction

The complex of butterfly taxa close to *Melitaea didyma* (Esper, 1779) is widely distributed in the Palaearctic region. This complex includes the traditionally recognized species *M. didyma*, *M. didymoides* Eversmann, 1847 and *M. sutschana* Staudinger, 1892 , the taxa that were recognized as species only recently (*M. latonigena* Eversmann, 1847, *M. interrupta* Colenati, 1846 , *M. chitralensis* Moore, 1901 and *M. mixta* Evans, 1912) as well as numerous described subspecies and forms with unclear taxonomic status (Higgins 1941, 1955, Hesselbarth et al. 1995, Kolesnichenko 1999, Kolesnichenko et al. 2011). All these taxa are similar in male and female wing pattern and genitalia structure (Higgins 1941). In our opinion, this complex does not include the species *M. deserticola* Oberthür, 1909, *M. ala* Staudinger, 1881, *M. enarea* Frühstorfer, 1917 and *M. persea* Kollar, 1849 which are similar to *M. didyma* in wing color and pattern but were shown to be distinctly different with respect to genitalia structure (Higgins 1941). The first significant review of this complex was published by Higgins (1941, 1955) in frame of a complete revision of the genus *Melitaea*. Recently the genus *Melitaea* was revised by Oorschot and Coutsis (2014). The taxa within the *M. didyma* complex have a strong morphological variation between individuals of different generations and indistinct clinal variability in wing size and color from north to south (Lvovsky and Morgun 2007). Available cytogenetic (Lukhtanov and Kuznetsova 1989), morphological (Lvovsky and Morgun 2007, Kolesnichenko et al. 2011, Oorschot and Coutsis 2014) and molecular (Wahlberg and Zimmermann 2000, Lukhtanov et al. 2009, Dincă et al. 2015) data show that the *M. didyma* species complex requires a more detailed taxonomic revision.

Here analysis of mitochondrial DNA barcodes is used to demonstrate that this complex is a natural (monophyletic) group consisting of at least 12 major haplogroups strongly differentiated with respect to the gene *COI*. Then the taxonomy of the *M. didyma* species complex is discussed.

Material and methods

Standard *COI* barcodes (658-bp 5' segment of mitochondrial *cytochrome oxidase subunit I*) were studied. *COI* sequences were obtained from 85 specimens collected in Afghanistan, Armenia, Austria, Bulgaria, China, Israel, Kazakhstan, Kyrgyzstan, Mongolia, Morocco, Russia, Syria, Tajikistan, Turkey and Uzbekistan. Collection data of these samples are presented in the Suppl. material 1.

Legs from 24 specimens (KT792884 - KT792908, see the Suppl. material 2) were processed at the Department of Karyosystematics of the Zoological Institute of the Russian Academy of Sciences. The set of voucher specimens of these butterflies is kept in the Zoological Institute of the Russian Academy of Science (St. Petersburg). DNA was extracted from a single leg removed from each voucher specimen. For DNA extraction we used the GeneJet Genomic DNA Purification Kit (Fermentas)

in accordance with the manufacturer's instructions. Extracted DNA samples were stored at -20 °C.

For DNA amplification we used primers LepF 5'- ATTCAACCAATCATAAAGATATTGG-3' and LepR (5'-TAAACTTCTGGATGTCCAAAAAATCA-3' (deWaard et al. 2008). The polymerase chain reaction (PCR) was carried out in 25-mL reactions using a DNA Engine thermal cycler (Eppendorf Mastercycler personal), and typically contained 0.5 mM of each primer, 0.8 mM dNTPs, Fermentas PCR buffer with additional MgCl2 to a final concentration of 2 mM and 1.25 units Fermentas Taq DNA polymerase. All reactions were initially denatured at 94 °C for 2 min, and then subjected to 30 cycles of 60 s at 94 °C denaturation, 60 s at 47 °C and 90 s at 72 °C extension. After amplification, double-stranded DNA was purified using GeneJet PCR Purification Kit (Fermentas). Sequencing of double-stranded product was carried out at the Research Resource Center for Molecular and Cell Technologies.

Legs from 61 specimens of *Melitaea* (HM404715 - HM404718, KT874693 - KT874751, see the Suppl. material 2) were processed at the Canadian Centre for DNA Barcoding (CCDB, Biodiversity Institute of Ontario, University of Guelph) using standard high-throughput protocol described in deWaard et al. (2008). The set of voucher specimens of these butterflies is kept at the McGuire Center for Lepidoptera and Biodiversity (University of Florida), at the Zoological Institute of the Russian Academy of Science (St. Petersburg) and in Museum for Insects, Pyatigorsk, Russia (Suppl. material 1).

The analysis involved 148 *COI* sequences (including outgroup). Among them there were 63 published sequences (Wahlberg and Zimmermann 2000, Vila and Bjorklund 2004, Leneveu et al. 2009, Lukhtanov et al. 2009, Dincă et al. 2011, 2015, Hausmann et al. 2011, Ashfaq et al. 2013) collected from GenBank (Suppl. material 2). Sequences were aligned using BioEdit software (Hall 1999) and edited manually. Phylogenetic hypotheses were inferred using Bayesian inference (BI), maximum-likelihood (ML) and maximum-parsimony (MP) analyses as described previously (Vershinina and Lukhtanov 2010, Talavera et al. 2013a). Briefly, Bayesian analyses were performed using the program MrBayes 3.1.2 (Huelsenbeck and Ronquist 2001) with default settings as suggested by Mesquite (Maddison and Maddison 2015): burn-in=0.25, nst=6 (GTR + I + G). Two runs of 10,000,000 generations with four chains (one cold and three heated) were performed. Chains were sampled every 10,000 generations. The average value of the Potential Scale Reduction Factor (PSRF) was 1.002 and average standard deviation of split frequencies was 0.01492, to the end of the analysis indicating that convergence was achieved, and a good sample from the posterior probability distribution was obtained.

The ML trees were inferred by using MEGA6 (Tamura et al. 2013) with the nucleotide substitution model T92 (Tamura 1992) as suggested by jModelTest (Posada 2008).

MP analysis was performed using a heuristic search as implemented in MEGA6 (Tamura et al. 2013). A heuristic search was carried out using the close-neighbour-interchange algorithm with search level 3 (Nei and Kumar 2000) in which the initial trees were obtained with the random addition of sequences (100 replicates). We used

non-parametric bootstrap values (Felsenstein 1985) to estimate branch support on the reconstructed ML and MP tree. Branch support was assessed using 1000 bootstrap replicates.

Results and discussion

This analysis recovered the *M. didyma* group as a strongly supported monophyletic clade (Fig. 1). Within this group many clades were well supported, whereas some of the relationships were not fully resolved (Figs 2 and 3). Within the complex we identified 12 differentiated major *COI* haplogroups. All of them showed a strict attachment to the localities (Fig. 4). Therefore in order to designate these haplogroups, we chose the oldest available name that was described from the area of each haplogroup: *M. mauretanica* Oberthür, 1909 , *M. occidentalis* Staudinger, 1861, *M. didyma* Esper, 1779, *M. neera* Fischer de Waldheim, 1840, *M. interrupta* Colenati, 1846, *M. liliputana* Oberthür,

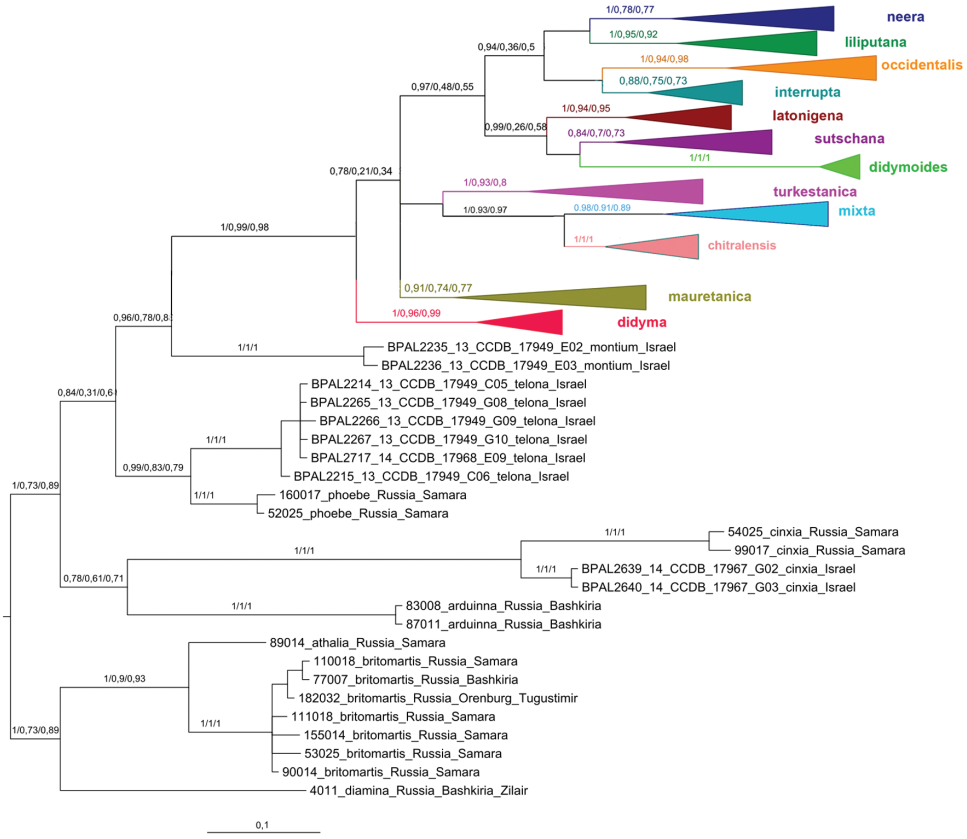

Figure 1. The Bayesian tree of *Melitaea* based on analysis of *the cytochrome oxidase subunit I* (*COI*) gene. Numbers at nodes indicate Bayesian posterior probability/ML bootstrap/MP bootstrap values. Scale bar = 0.1 substitutions per position.

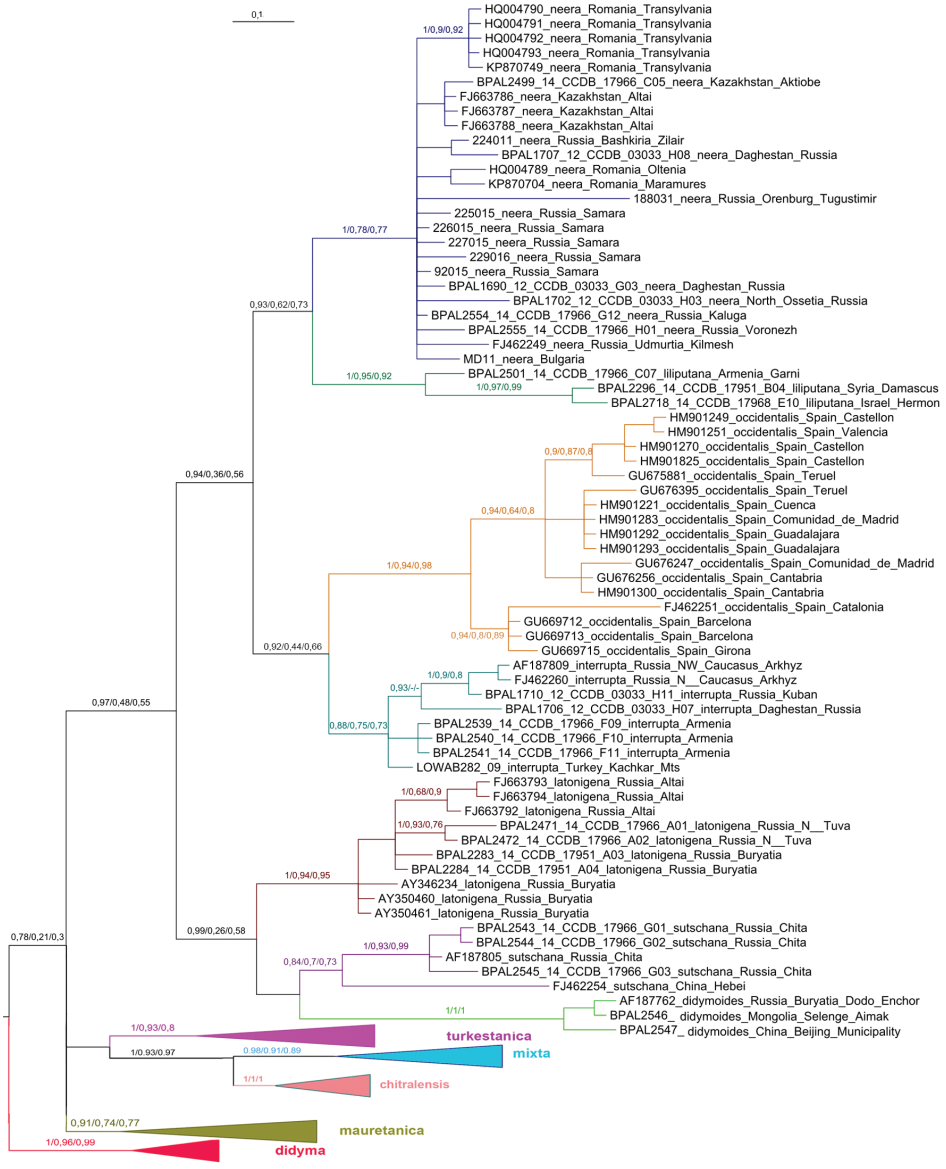

Figure 2. Fragment of the Bayesian tree of *Melitaea didyma* complex (haplogroups *neera, liliputana, occidentalis, interrupta, latonigena, sutschana* and *didymoides*) based on analysis of the *cytochrome oxidase subunit I* (*COI*) gene. Numbers at nodes indicate Bayesian posterior probability/ML bootstrap/MP bootstrap values, with nonmatching clades using different analyses indicated by '-'. Scale bar = 0.1 substitutions per position.

1909, *M. turkestanica* Sheljuzhko, 1929, *M. mixta* Evans, 1912, *M. chitralensis* Moore, 1901, *M. latonigena* Eversmann, 1847, *M. didymoides* Eversmann, 1847 and *M. sutschana* Staudinger, 1892 (Figs 2 and 3). The name *M. liliputana* was selected for the Middle

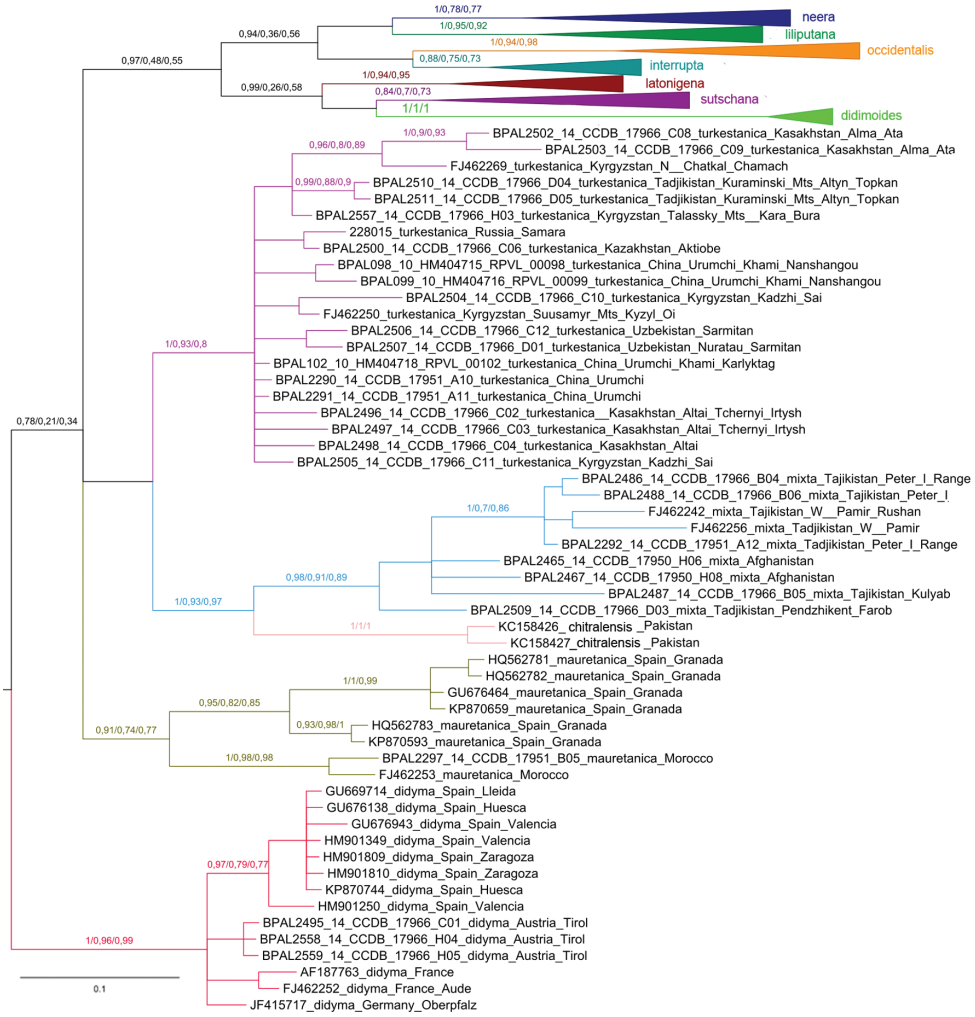

Figure 3. Fragment of the Bayesian tree of *Melitaea didyma* complex (haplogroups *turkestanica, mixta, chitralensis, mauretanica* and *didyma*) based on analysis of the *cytochrome oxidase subunit I* (*COI*) gene. Numbers at nodes indicate Bayesian posterior probability/ML bootstrap/MP bootstrap values, with non-matching clades using different analyses indicated by '-'. Scale bar = 0.1 substitutions per position.

East populations of the *M. didyma* complex. These populations have been known under the name *libanotica* Belter, 1934 in the literature (Larsen 1974, Benyamini 2002, Tshikolovets 2011). However, the name *liliputana* was preferred since ICZN states priority of the oldest available name (article 23, Principle of Priority).

The discovered haplogroups correspond to two traditionally recognized species (*M. didymoides* and *M. sutschana*) (Higgins 1941), to four taxa that were recognized as species relatively recently (*M. latonigena*, *M. interrupta, M. chitralensis* and *M. mixta*) (Lukhtanov and Kuznetsova 1989, Hesselbarth et al. 1995, Kolesnichenko 1999, Kole-

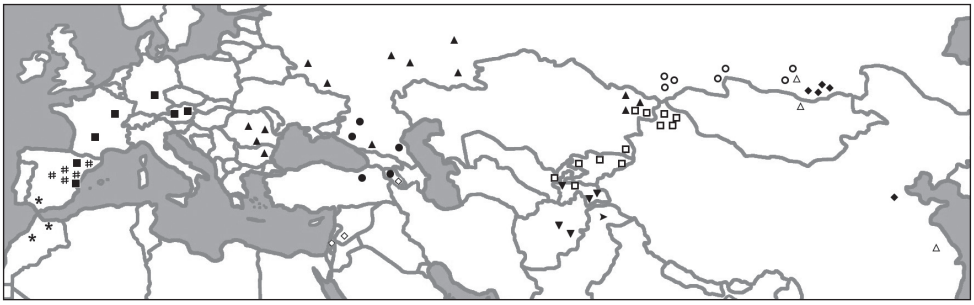

Figure 4. Distribution ranges of haplogroups *didyma* (■), *didymoides* (Δ), *interrupta* (●), *latonigena* (○), *liliputana* (◊), *mauretanica* (*), *mixta* (▼), *neera* (▲), *occidentalis* (#), *sutschana* (♦), *turkestanica* (□) and *chitralensis* (►).

Table 1. Minimal uncorrected *COI* p-distances between 12 major haplogroups of the *M. didyma* species complex (%).

	1.	2.	3.	4.	5.	6.	7.	8.	9.	10.	11.
1. *neera*											
2. *liliputana*	1.3										
3. *occidentalis*	2.7	3.9									
4. *interrupta*	1.8	3	1.9								
5. *latonigena*	1.9	3.2	3.6	3.26							
6. *sutschana*	2.2	3.6	3	3.28	1.89						
7. *didymoides*	3.8	4.8	4.4	3	3.6	3.29					
8. *turkestanica*	1.6	2.7	2.4	2.43	2.16	2.73	3.89				
9. *mixta*	2.7	3.6	3	3.2	3.86	3.87	4.77	1.89			
10. *chitralensis*	4.3	4.7	4.6	4.1	4.3	4.3	5.2	3,2	2.4		
11. *mauretanica*	1.6	2.9	2.16	1.9	2.16	3	3.88	1.6	2.18	3.8	
12. *didyma*	1.9	3	2.73	2.4	2.44	3	4.48	1.6	3	3.3	1.61

Sympatry (or at least parapatry) (shown by green color) was demonstrated for the following taxa pairs: *mixta* and *turkestanica* (Kolesnichenko et al. 2011), *mixta* and *chitralensis* (Higgins 1941), *didymoides* and *sutschana* (Gorbunov 2001), *didymoides* and *latonigena* (Gorbunov 2001), *sutschana* and *latonigena* (Gorbunov 2001), *latonigena* and *neera* (Lukhtanov et al. 2007), *interrupta* and *neera* (parapatry in the North Caucasus, Tuzov and Churkin 2000) and *interrupta* and *liliputana* (parapatry in Armenia and Turkey, Hesselbarth et al. 1995).

Here we also report an observation of parapatry between *neera* and *turkestanica* in South Altai and Zaisan valley in East Kazakhstan (shown by green color). In this area the distribution ranges of these taxa overlap, however, the taxa are separated ecologically: *M. neera* is associated with the steppe biotopes and *M. turkestanica* is associated with deserts.

Sympatry was also found between haplogroups *occidentalis* and *didyma* sensu stricto in Spain (shown by yellow, Dincă et al. 2015). However, morphology and ecology of the bearers of these haplogroups were not analyzed in the contact zone. Therefore, evolutionary and taxonomic interpretation of this case of sympatry is difficult. It may represent sympatric distribution of two different species or may be a consequence of mitochondrial introgression between the allopatric pair *occidentalis-didyma*.

snichenko et al. 2011), to five recognized subspecies (*M. didyma occidentalis*, *M. didyma didyma*, *M. didyma neera*, *M. didyma liliputana* and *M. didyma turkestanica*) (Higgins 1941, Larsen 1974, Benyamini 2002, Tshikolovets 2011) and to one form (*M. mauretanica*) whose status (subspecies or individual variations) is unclear (Higgins 1941).

There is good evidence based on analysis of morphology and observations of taxa in sympatry that *M. didymoides*, *M. sutschana*, *M. latonigena*, *M. interrupta, M. chitralensis* and *M. mixta* represent true biological species (Higgins 1941, Lukhtanov and Kuznetsova 1989, Hesselbarth et al. 1995, Kolesnichenko 1999, Kolesnichenko et al. 2011). Theoretically, the remainder of the *M. didym*a complex can be interpreted as a single species *M. didyma*. However, such an interpretation meets two difficulties. Firstly, such a lumping would result in a polyphyletic assemblage. Monophyly is the basic principle of phylogenetics and taxonomy. The majority of taxonomists currently believe that monophyly, in the narrow sense used by Hennig (Hennig 1966, Envall 2008, Hörandl and Stuessy 2010) is mandatory. Thus avoiding non-monophyletic groups and focusing on monophyletic entities is the preferable option in practical terms (Talavera et al. 2013b). The *COI* barcodes alone can provide weak evidence for monophyly of taxa since trees inferred from single markers sometimes display relationships that reflect the evolutionary histories of individual genes rather than the species being studied. Mitochondrial introgression (Zakharov et al. 2009) and *Wolbachia* infection (Ritter et al. 2013) can lead to additional bias in inferring phylogenetic relationships. Despite these limitations, we argue that, until not falsified, clusters based on DNA barcode monophyly represent preferable primary taxonomic hypotheses than the clusters based on para- or polyphyletic DNA barcode assemblages.

Secondly, the uncorrected p-distances between these taxa are high (from 1.3% between *neera* and *liliputana* to 3.9% between *liliputana* and *occidentalis*). Although some of them are lower than the 'standard' 2.7–3.0% DNA-barcoding threshold usually used for allopatric taxa as an indicator for their species distinctness (Lambert et al. 2005, Lukhtanov et al. 2015), even the lowest distances are comparable with those found between other six well recognized species. For example, distances between *interrrupta*, *latonigena* and *mixta* and their sympatric/parapatric non-conspecifics are 1.6-1.9% (Table 1).

Finally, five of the six remaining haplogroups (*occidentalis*, *didyma* sensu stricto, *neera*, *liliputana* and *turkestanica*) are morphologically distinct and have been considered as separate taxonomic entities (subspecies) (Higgins 1941, Larsen 1974, Benyamini 2002, Tshikolovets 2011). Their monophyly with respect to the *COI* gene reinforces the conclusion that they represent independent lineages of evolution.

Therefore, we hypothesize that the *M. didyma* complex is represented by the following 12 species that can be recognized by a phylogenetic species concept (Cracraft 1989, Coyne and Orr 2004) (taxa 1–5) and by both phylogenetic and biological species concepts (taxa 6–12):

1) *M. liliputana* Oberthür, 1909 (Armenia, Turkey, Syria, Israel)
2) *M. occidentalis* Staudinger, 1961 (Spain)

3) *M. didyma* Esper, 1779 (west Europe)
4) *M. neera* Fischer de Waldheim, 1840 (east Europe, north Caucasus, west Siberia, north Kazakhstan)
5) *M. mauretanica* Oberthür, 1909 (north Africa, south Spain)
6) *M. interrupta* Colenati, 1846 (Caucasus, Turkey, Iran)
7) *M. turkestanica* Sheljuzhko, 1929 (Kazakhstan, Kyrgyzstan, Uzbekistan, Tajikistan, west China)
8) *M. mixta* Evans, 1912 (Tajikistan, Pakistan, Afghanistan)
9) *M. chitralensis* Moore, 1901 (north Pakistan)
10) *M. latonigena* Eversmann, 1847 (Asian Russia, north-east Kazakhstan, Mongolia, north-west China)
11) *M. didymoides* Eversmann, 1847 (Asian Russia, Mongolia, North China)
12) *M. sutschana* Staudinger, 1892 (Far East Russia, Korea, North-East China)

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Supplementary material 1

Table S1

Authors: Elena A. Pazhenkova, Evgeny V. Zakharov, Vladimir A. Lukhtanov

Data type: Excel table.

Explanation note: Collection data of the samples sequenced in this study.

Supplementary material 2

Table S2

Authors: Elena A. Pazhenkova, Evgeny V. Zakharov, Vladimir A. Lukhtanov

Data type: Excel table.

Explanation note: List of the samples used in this study

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Chromosome numbers in antlions (Myrmeleontidae) and owlflies (Ascalaphidae) (Insecta, Neuroptera)

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Abstract

A short review of main cytogenetic features of insects belonging to the sister neuropteran families Myrmeleontidae (antlions) and Ascalaphidae (owlflies) is presented, with a particular focus on their chromosome numbers and sex chromosome systems. Diploid male chromosome numbers are listed for 37 species, 21 genera from 9 subfamilies of the antlions as well as for seven species and five genera of the owlfly subfamily Ascalaphinae. The list includes data on five species whose karyotypes were studied in the present work. It is shown here that antlions and owlflies share a simple sex chromosome system XY/XX; a similar range of chromosome numbers, 2n = 14-26 and 2n = 18-22 respectively; and a peculiar distant pairing of sex chromosomes in male meiosis. Usually the karyotype is particularly stable within a genus but there are some exceptions in both families (in the genera *Palpares* and *Libelloides* respectively). The Myrmeleontidae and Ascalaphidae differ in their modal chromosome numbers. Most antlions exhibit 2n = 14 and 16, and Palparinae are the only subfamily characterized by higher numbers, 2n = 22, 24, and 26. The higher numbers, 2n = 20 and 22, are also found in owlflies. Since the Palparinae represent a basal phylogenetic lineage of the Myrmeleontidae, it is hypothesized that higher chromosome numbers are ancestral for antlions and were inherited from the common ancestor of Myrmeleontidae + Ascalaphidae. They were preserved in the Palparinae (Myrmeleontidae), but changed via chromosomal fusions toward lower numbers in other subfamilies.

Keywords

Male chromosome numbers, sex chromosomes, distant pairing of sex chromosomes, lacewings, Myrmeleontoidea

Introduction

Within the holometabolous (= Endopterygota) insect order Neuroptera (lacewings) including a total of 17 or 18 currently recognized families (Aspöck et al. 2012), the Myrmeleontidae (antlions) comprise the most species-rich and most widespread family, with over 1500 valid extant species in 191 genera (Stange 2004). The closely related Ascalaphidae (owlflies) are a moderately speciose neuropteran family encompassing approximately 400 valid extant species assigned to about 65 genera, with wide distributional range in tropical and temperate areas of the world (Sekimoto and Yoshizawa 2007).

The Myrmeleontidae and Ascalaphidae belong to the superfamily Myrmeleontoidea (suborder Myrmeleontiformia), together with another four extant families, Nemopteridae, Crocidae, Psychopsidae, and Nymphidae. Despite the controversial hypotheses on the interfamilial phylogenetic relationships within this group, different phylogenetic analyses based on morphological and genetic data provide almost universal support for the monophyly of Myrmeleontoidea and the sister relationship between Myrmeleontidae and Ascalaphidae (Stange 1994, Aspöck 2002, Haring and Aspöck 2004, Winterton et al. 2010, Aspöck et al. 2012). However, molecular analyses are not always concordant with the monophyly of these families (Winterton et al. 2010).

Within Myrmeleontidae, the higher-level classification is controversial (reviewed in Mansell 1999), with several authors proposing various taxonomic divisions at the subfamily, tribe and subtribe levels (e.g. Banks 1899, 1927, New 1985a, b, c, Stange 1994, 2004, Krivokhatsky 2011). In his recent monography on the world fauna of Myrmeleontidae, Stange (2004) recognized three subfamilies, Stilbopteryginae, Palparinae, and Myrmeleontinae, with 14 tribes and 191 genera. Myrmeleontidae were further classified by Krivokhatsky (2011) who subdivided the family into 12 subfamilies (Palparinae, Pseudimarinae, Stilbopteryginae, Dimarinae, Echthromyrmicinae**,** Dendroleontinae, Nemoleontinae, Glenurinae, Myrmecaelurinae, Acanthaclisinae, Brachynemurinae, and Myrmeleontinae), with 23 tribes.

The Ascalaphidae are poorly-understood and taxonomically weakly-elaborated family. It was extensively revised only by van der Weele (1908) and now it comprises at least three subfamilies, Schizophthalminae (now Ascalaphinae), Holophthalminae (now Haplogleniinae), and Albardiinae, with a total of 15 tribes. Two-thirds of the species are placed in the first subfamily, and the remaining species (approximately 90) are placed in the second one, whereas the third subfamily contains only one species (van der Weele 1908, Sekimoto and Yoshizawa 2007). To date, no wide-ranging modern phylogenetic analyses of higher ascalaphid relationships have been published (Fischer et al. 2006).

Mansell (1999: p. 3) pointed out that the antlions, "apart from their obvious biological significance, are ideal subjects for the study of insect behavior, physiology, biogeography and evolution, and consequently a group urgently warrants study and con-

servation". Although chromosomal investigations have a long history in systematics and evolutionary biology (White 1973, King 1993), and a large body of data has been accumulated for insects (e.g., butterflies: Lukhtanov 2014; beetles: Angus et al. 2013, Blackmon and Demuth 2014, 2015; true bugs: Papeschi and Bressa 2006, Kuznetsova et al. 2011; aphids: Gavrilov-Zimin et al. 2015; coccids: Gavrilov 2007; cicadas: Kuznetsova and Aguin-Pombo 2015; grasshoppers: Warchałowska-Śliwa et al. 2005, parasitic wasps: Gokhman 2009), both antlions and owlflies were largely ignored in this respect. Our present knowledge of their karyotypes is scarce and fragmentary, being completely confined to the number of chromosomes and, additionally, to the meiotic behavior of the sex chromosomes that is of a very peculiar type in many neuropteran groups (Naville and de Beaumont 1932, 1933, Hughes-Schrader 1969, 1975a, b, 1979, Nokkala 1986) including the Myrmeleontidae (Naville and de Beaumont 1932, 1933, Hughes-Schrader 1983). In the Myrmeleontidae and Ascalaphidae, chromosomal studies were initiated in the 1930s with the pioneering works of Oguma and Asana (1932), Naville and de Beaumont (1932, 1933, 1936), Ikeda and Kichijo (1935), Asana and Kichijo (1936), and Katayama (1939). Since that time only scarce chromosome studies were performed on the Myrmeleontidae (Hirai 1955a, b, Highes-Schrader 1983, Klok and Chown 1993) while no further work on the Ascalaphidae appeared except for the re-investigation of *Ascalohybris subjacens* (Walker, 1853) karyotype (Hirai 1955a, b: as *Hybris* Lefèbvre, 1842) earlier studied by Katayama (1939: as *Hybris*).

Thus, cytogenetic studies on the families Myrmeleontidae and Ascalaphidae virtually ceased a few decades ago. The latest checklist of chromosome numbers in antlions published by Klok and Chown (1993) suffers from many shortcomings including imperfect references, erroneous identifications, outdated species names and synonymy. In order to fill this gap, an updated and comprehensive checklist of chromosome numbers of antlions and owlflies is provided here by integrating the published data together with our latest unpublished results.

Material and methods

Insects

Four antlion species (only males), namely *Palpares libelluloides*, *Distoleon tetragrammicus*, *Macronemurus bilineatus*, *Myrmecaelurus trigrammus*, and male owlfly *Bubopsis hamatus*, were used in the present study. The specimens were collected from May to October 2013 in the Republic of Dagestan (North-East Caucasus, Russia). The material was collected by G. Khabiev. Collection sites, sampling dates, and the numbers of studied males are given in Table 1. In the field, adult individuals were fixed in a solution of 96% alcohol and glacial acetic acid (3:1) and then stored at 4°C until required.

Table 1. Material used.

Taxon	Sampling locality and date of collection	No. of studied males
Myrmeleontidae		
Palparinae		
Palpares libelluloides (Linnaeus, 1764)	Russia, Dagestan, near Makhachkala 43°00'00"N, 47°13'33"E; V.2013	2
Nemoleontinae		
Distoleon tetragrammicus (Fabricius, 1798)	Russia, Dagestan, near Makhachkala 43°00'29"N, 47°14'51"E VII.2013	1
Macronemurus bilineatus Brauer, 1868	Russia, Dagestan, near Makhachkala 42°59'58"N 47°13'30"E; VI.2013	7
Myrmecaelurinae		
Myrmecaelurus trigrammus (Pallas, 1771)	Russia, Dagestan, near Makhachkala 43°01'26"N, 47°15'12"E; 42°57'19"N, 47°28'51"E; 42°58'07.2"N, 47°20'03"E; VI-VII.2013	23
Ascalaphidae		
Bubopsis hamatus (Klug in Ehrenberg, 1834)	Dagestan, Gumbetovsky district, near Chirkata village; 42°47'53"N, 46°41'14"E; VII.2013	2

Chromosome preparation

Air-dried preparations were made by macerating testicular follicles in a drop of 45% acetic acid on a glass microscope slide and squashing under a cover slip. The preparations were frozen using dry ice, the cover slips were removed with a razor blade, and the preparations were dehydrated in fresh fixative (3:1) for 20 min and air dried. Slides were first examined under a phase-contrast microscope to check for the availability of meiotic divisions and quality of chromosome spreads. Counts were based on samples of one to 23 individuals. The preparations and remains of the specimens are stored at the Department of Karyosystematics, Zoological Institute, RAS.

Chromosome staining

Meiotic chromosomes were stained using the Feulgen-Giemsa method developed by Grozeva and Nokkala (1996).

Microscopy and imaging

Chromosome preparations were analyzed under a Leica DM 4000B microscope with a 100x objective. Images were taken with a Leica DFC 345 FX camera using Leica Application Suite 3.7 software with an Image Overlay module.

Results

Only meiotic divisions in adult males were available for analysis during the present study. In five examined species belonging to the families Myrmeleontidae (four species) and Ascalaphidae (one species) (Table 1), as many as three different chromosome numbers were found. Males of *P. libelluloides* showed 12 autosomal bivalents and univalent X and Y chromosomes at spermatocyte metaphases I (MI) suggesting the diploid karyotype formula of this species is 2n = 26(24A + XY). Unfortunately, our method proved to be inappropriate for effective and reliable detection of the centromere positions in chromosomes and, hence, analysis of their morphology. Nonetheless, most autosomes were suggested to be one-armed, with at least one clear exception of a large pair of bi-armed submetacentric chromosomes (Fig. 1). Males of *Macronemurus bilineatus* and *Myrmecaelurus trigrammus* showed 7 autosomal bivalents and univalent X and Y chromosomes at spermatocyte MI suggesting the diploid karyotype formula is 2n = 16(14A + XY). Males of *D. tetragrammicus* and *B. hamatus* showed 8 autosomal bivalents and univalent X and Y chromosomes at spermatocyte MI suggesting the diploid karyotype formula is 2n = 18(16A + XY). In the four low-numbered species, the chromosomes seemed to be essentially bi-armed (Figs 2–5).

A peculiar feature of all the species was that at metaphase I, the univalent X and Y chromosomes were disposed on the opposite sides of the division spindle whereas autosomal bivalents showed a typical metaphase location on the equator of the nucleus (Figs 1–5). In each species, the behavior of sex chromosomes was traced in the meiotic nuclei throughout all stages and these data will be presented elsewhere.

The new findings and references to previous reports of chromosome numbers in Myrmeleontidae and Ascalaphidae are given in Table 2. The subfamilial and tribal classification of the Myrmeleontidae used in this paper follows Krivokhatsky (2011) and that of the Ascalaphidae follows van der Weele (1908).

Discussion

Chromosome numbers

In the Myrmeleontidae, with the original data presented here, karyotype data have been made available for 37 species and 21 genera in 9 out of 12 subfamilies accepted by Krivokhatsky (2011). Having regard to 1500 valid species and 191 valid genera in this family (Stange 2004), the proportion of the studied species and genera is approximately 2.5% and 11% respectively. The karyotypes (chromosome numbers and sex chromosome systems) are presently known for the subfamilies Palparinae (3 species/2 genera), Pseudimarinae (1/1), Dendroleontinae (2/2), Nemoleontinae (6/4), Glenurinae (2/2), Myrmeleontinae (11/3), Brachynemurinae (7/3), Myrmecaelurinae (2/1), and Acanthaclisinae (3/3). The family demonstrates a relatively high diversity of karyotypes, with diploid chromosome numbers (2n) of 37 studied species ranging

Table 2. Data on karyotypes in the Myrmeleontidae and the Ascalaphidae (Neuroptera: Myrmeleontoidea).

No	Taxon	2n (karyotype formula) ♂	Sampling locality	Reference
	Family Myrmeleontidae Latreille, 1802			
	Subfamily Palparinae Banks, 1911			
1	*Indopalpares pardus* (Rambur, 1842)	24(22+XY)	East India: Ahmedabad	Oguma and Asana 1932 (as *Palpares* sp.)[1]
2	*Palpares libelluloides* (Linnaeus, 1764)	26(24+XY) 26(24+XY)	Switzerland: Geneve, France: Banyuls-sur-Mer Russia: Dagestan	Naville and De Beaumont 1936 Present data
3	*Palpares sobrinus* Péringuey, 1911	22(20+XY)	South Africa: Transvaal	Klok and Chown 1993
	Subfamily Pseudimarinae Markl, 1954			
	Tribe Palparidiini Markl, 1954			
4	*Palparidius concinnus* Péringuey, 1910	18(16+XY)	South Africa: Transvaal	Klok and Chown 1993
	Subfamily Dendroleontinae Banks, 1899			
	Tribe Dendroleontini Banks, 1899			
5	*Epacanthaclisis moiwanus* (Okamoto, 1906)	16(14+XX) (♀)	Japan	Hirai 1955a, b
6	*Dendroleon jezoensis* Okamoto, 1910	16(14+XY)	Japan	Hirai 1955 a, b
	Subfamily Nemoleontinae Banks, 1911			
	Tribe Distoleontini Tillyard, 1916			
7	*Distoleon tetragrammicus* (Fabricius, 1798)	18(16+XY)	Russia: Dagestan	Present data
	Tribe Neuroleontini Banks, 1911			
8	*Neuroleon* sp.[2]	16(14+XY)	Western India: Bombay [Mumbai]	Asana and Kichijo 1936
	Tribe Macronemurini Esben-Petersen, 1919			
9	*Macronemurus appendiculatus* (Latreille, 1807)	16(14+XY)	France: Banyuls-sur-Mer	Naville and De Beaumont 1933
10	*Macronemurus bilineatus* Brauer, 1868	16(14+XY)	Russia: Dagestan	Present data
11	*Macronemurus* sp.	16(14+XY)	Western India: Bombay [Mumbai]	Asana and Kichijo 1936 (as *Macronemurus* sp.?)
	Tribe Creoleontini Markl, 1954			
12	*Creoleon lugdunensis* (Villers, 1789)	18(16+XY)	France: Banyuls-sur-Mer	Naville and De Beaumont 1936 (as *Creagris plumbea* Navás, 1928)[3]
	Subfamily Glenurinae Banks, 1927			
	Tribe Glenurini Banks, 1927			
13	*Euptilon arizonensis* (Banks, 1935)	16(14+XY)	USA	Hughes-Schrader 1983 (as *Psammoleon arizonensis* Banks, 1935)
14	*Paraglenurus japonicus* (MacLachlan, 1867)	16(14+XY)	Japan	Hirai 1955a, b (as *Glenuroides japonicus* MacLachlan, 1867)

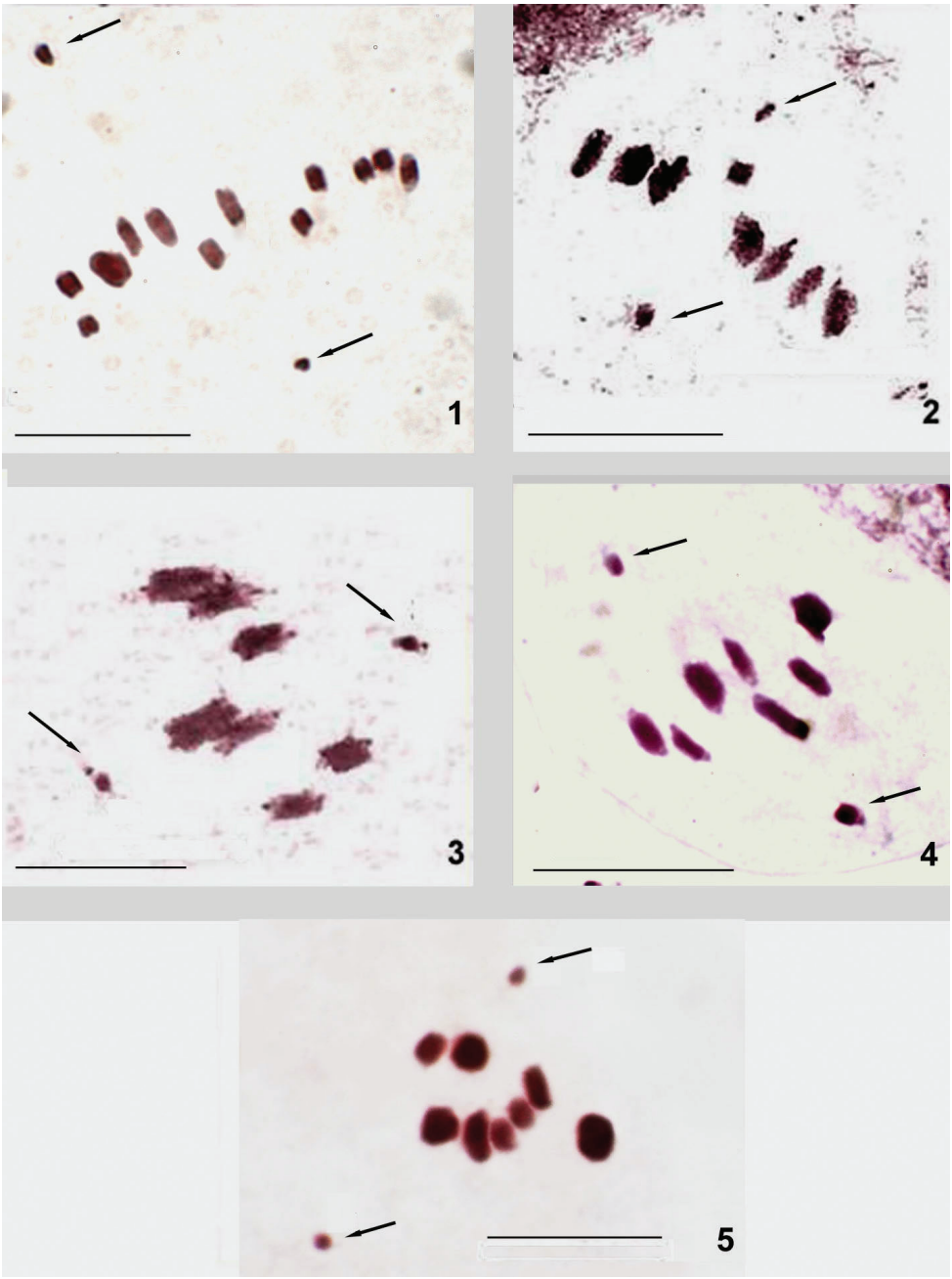
No	Taxon	2n (karyotype formula) ♂	Sampling locality	Reference
	Subfamily Myrmeleontinae Latreille, 1802			
	Tribe Myrmeleontini Latreille, 1802			
15	*Baliga micans* (McLachlan, 1875)	14(12+XY)	Japan	Hirai 1955a, b (as *Hagenomyia micans* McLachlan, 1875)
16	*Baliga sagax* (Walker, 1853)	14(12+XY)	Western India: Bombay [Mumbai]	Asana and Kichijo 1936 (as *Myrmeleon* sp., probably *M. sagax* Walker, 1853)
17	*Euroleon nostras* (Fourcroy, 1785)	14(12+XY) 14(12+XX) (♀)	Switzerland, Geneva	Naville and De Beaumont 1932, 1933 (as *Myrmeleon europaeus* McLachlan, 1873)
18	*Myrmeleon alcestris* Banks, 1911	14(12+XY)	South Africa: Transvaal	Klok and Chown 1993
19	*Myrmeleon californicus* Banks, 1943	14(12+XY)	USA	Hughes-Schrader 1983
20	*Myrmeleon exitialis* Walker, 1853	14(12+XY)	USA	Hughes-Schrader 1983
21	*Myrmeleon formicarius* Linnaeus, 1767[2]	14(12+XY) 14(12+XY)	Western India: Bombay [Mumbai] Japan	Ikeda and Kichijo 1935, Hirai 1955a, b
22	*Myrmeleon hyalinus* Olivier, 1811	14(12+XY)	France: Corse	Naville and De Beaumont 1936 (as *Morter hyalinus* (Olivier, 1811))
23	*Myrmeleon immaculatus* DeGeer, 1773	14(12+XY)	USA	Hughes-Schrader 1983
24	*Myrmeleon mexicanus* Banks, 1903	14(12+XY)	USA	Hughes-Schrader 1983
25	*Myrmeleon obscurus* Rambur, 1842	14(12+XY)	South Africa: Transvaal	Klok and Chown 1993
	Subfamily Brachynemurinae Banks, 1927			
	Tribe Brachynemurini Banks, 1927			
26	*Brachynemurus hubbardi* Currie, 1898	14(12+XY)	USA	Hughes-Schrader 1983
27	*Brachynemurus mexicanus* Banks, 1895	14(12+XY)	USA	Hughes-Schrader 1983
28	*Clathroneuria coquilletti* (Currie, 1898)	14(12+XY)	USA	Hughes-Schrader 1983 (as *Brachynemurus coquilletti* Currie, 1898)
29	*Clathroneuria schwarzi* (Currie, 1903)	14(12+XY)	USA	Hughes-Schrader, 1983 (as *Brachynemurus schwarzi* Currie, 1903)
30	*Scotoleon dissimilis* (Banks, 1903)	16(14+XY)	USA	Hughes-Schrader 1983 (as *Brachynemurus dissimilis* Banks, 1903)
31	*Scotoleon niger* (Currie, 1898)	16(14+XY)	USA	Hughes-Schrader 1983 (as *Brachynemurus niger* Currie, 1898)
32	*Scotoleon nigrilabris* (Hagen, 1888)	16(14+XY)	USA	Hughes-Schrader 1983 (as *Brachynemurus nigrilabris* Hagen, 1888)

No	Taxon	2n (karyotype formula) ♂	Sampling locality	Reference
	Subfamily Myrmecaelurinae Esben-Petersen, 1919			
	Tribe Myrmecaelurini Esben-Petersen, 1919			
33	*Myrmecaelurus* sp.[2]	14(12+XY)	Western India: Bombay [Mumbai]	Asana and Kichijo 1936 (as *Myrmecaelurus* sp , probably *M. acerbus* (Walker, 1853))
34	*Myrmecaelurus trigrammus* (Pallas, 1771)	16(14+XY)	Russia: Dagestan	Present data
	Subfamily Acanthaclisinae Navás, 1912			
35	*Synclisis japonica* (McLachlan, 1875)	14(12+XY) 14(12+XY)	Western India: Bombay [Mumbai] Japan	Ikeda and Kichijo 1935, Hirai 1955a, b (as *Acanthaclisis japonica* Hagen, 1866)
36	*Centroclisis brachygaster* (Rambur, 1842)	14(12+XY)	South Africa: Transvaal	Klok and Chown 1993
37	*Vella fallax* (Rambur, 1842)	14(12+XY)	USA	Hughes-Schrader 1983
	Family Ascalaphidae Rambur, 1842			
	Subfamily Ascalaphinae Rambur, 1842			
	Tribe Hybrisini Lefèbvre, 1842			
38	*Ascalohybris subjacens* (Walker, 1853)	22(20+XY) 22(20+XX) (♀)	Japan Japan	Katayama 1939 (as *Hybris subjacens* (Walker, 1853)), Hirai 1955a, b (as *Hybris subjacens*)
39	*G1yptobasis dentifera* (Westwood, 1847)	22(20+XY)	Western India: Bombay [Mumbai]	Asana and Kichijo 1936
	Tribe Ascalaphini Rambur, 1842			
40	*Libelloides corsicus* Rambur, 1842)	20	France: Corse	Naville and De Beaumont 1936 (as *Ascalaphus ictericus corsicus* Rambur, 1842)
41	*Libelloides coccajus* (Denis & Schiffermüller, 1775)	22(20+XY) 22(20+XX) (♀)	Switzerland: Geneva, Valais	Naville and De Beaumont 1933, 1936 (as *Ascalaphus libelluloides* Schäffer, 1763)
42	*Libelloides longicornis* (Linnaeus, 1764)	22(20+XY)	Switzerland: Valais	Naville and De Beaumont 1936 (as *Ascalaphus longicornis* (Linnaeus, 1764))
	Tribe Encyoposini McLachlan, 1871			
43	*Bubopsis hamatus* (Klug in Ehrenberg, 1834)	18(16+XY)	Russia: Dagestan	Present data
44	*Ogcogaster segmentator* (Westwood, 1847)	22(20+XY)	Western India: Bombay [Mumbai]	Asana and Kichijo 1936

[1] Later described as *Palpares pardus asanai* Kuwayama, 1933 (Oguma and Asana 1932, Kuwayama, 1933)

[2] Presence of these taxa in Bombay [Mumbai] is doubtful

[3] Wrong identifications: all records of *Creoleon plumbeus* from West Europe actually belong to *C. lugdunensis* (Hölzel 1976, Krivokhatsky 2011)



Figures 1–5. Meiotic (MI) karyotypes of antlions (**1–4**) and owlflies (**5**). **1** *Palpares libelluloides*, n = 12AA+XY (2n = 26, XY) **2** *Distoleon tetragrammicus*, n = 8AA+XY (2n = 18, XY) **3** *Myrmecaelurus trigrammus*, n = 7AA+XY (2n = 16, XY) **4** *Macronemurus bilineatus*, n = 7AA+XY (2n = 16, XY), **5** *Bubopsis hamatus*, n = 8AA+XY (2n = 18, XY). Arrows point to X and Y sex chromosomes. Scale bars = 10 μm

from 14 to 26 including four intermediate counts, i.e. 16, 18, 22 and 24. The highest numbers, 26, 24 and 22, occur only in the subfamily Palparinae, in three species of the genera *Palpares* Rambur, 1842 and *Indopalpares* Insom & Carfi, 1988. Other numbers, 2n = 14, 16 and 18, are encountered in the remaining subfamilies. In the Pseudimarinae, the only studied species, *Palparidius concinnus*, exhibits the next highest number found in antlions, i.e. 2n = 18. In the Nemoleontinae, with the exception of *Distoleon tetragrammicus* and *Creoleon lugdunensis* displaying 2n = 18, all studied species, including three *Macronemurus* Costa, 1855 species, have karyotypes with 2n = 16. The latter value is also found in all studied Dendroleontinae and Glenurinae. The subfamilies Brachynemurinae, Myrmecaelurinae and Acanthaclisinae include species both with 2n = 16 and 2n = 14, whereas Myrmeleontinae show 2n = 14 in all the studied species. It is noteworthy that, with the exception of *Palpares* (but see below), all these genera do not show interspecific variation in the chromosome number. This is especially remarkable for those genera where several species have been studied, e.g. *Macronemurus* (Nemoleontinae) and *Scotoleon* Banks, 1913 (Brachynemurinae). In each of these genera, three studied species share 2n = 16. Moreover, in *Myrmeleon* Linnaeus, 1767 (Myrmeleontinae) all eight studied species have 2n = 14. It is noteworthy that closely related genera, *Baliga* Navás, 1912 and *Euroleon* Esben-Petersen, 1918 in the Myrmeleontini, show the same karyotype with 2n = 14. It is unclear at present whether the chromosome number varies within the genus *Palpares*. The highest chromosome number, 2n = 26, is found in *P. libelluloides*, the type species of the genus. *Palpares pardus asanai* Kuwayama, 1933 with 2n = 24 (Oguma and Asana 1932) is treated here as a member of *Indopalpares*. Additionally, there is a possibility that *Palpares sobrinus* with 2n = 22 (Klok and Chown 1993) represents in fact *Pseudopalpares sparsus* (McLachlan, 1867). Although few members of the Palparinae are studied at present, karyotypic differences between the genera of this subfamily probably occur.

Compared to the Myrmeleontidae, karyotypes of the Ascalaphidae are less studied. The chromosome numbers are currently known in only seven owlfly species from the genera *Ascalohybris* Sziraki, 1998, *Ogcogaster* Westwood, 1847, *Libelloides* Schaeffer, 1766, *Bubopsis* McLachlan, 1898, and *Glyptobasis* McLachlan, 1871, all presently classified within the subfamily Ascalaphinae. The species studied show relatively high chromosome numbers, i.e. 2n = 18 in *Bubopsis hamatus*, 20 in *Libelloides corsicus*, and 22 in all the remaining species, including two other studied members of the genus *Libelloides*.

Although Myrmeleontidae and Ascalaphidae show a similar range of chromosome numbers (2n = 14 - 26 in the former and 18 - 22 in the latter), these families differ in the modal numbers. Of 37 species studied in the Myrmeleontidae, 19 species display 2n = 14, and 12 species have 2n = 16. On the other hand, five of seven species studied in the Ascalaphidae display 2n = 22. Other chromosome numbers occur only occasionally within the families except for high numbers characteristic of the antlion subfamily Palparinae.

In different eukaryotic organisms, evolutionary changes in the chromosome number happen via polyploidy, aneuploidy or fusion/fission events. In animals polyploidy is known to be rare, whereas chromosomal fusions and fissions are common. As stated

above, most Myrmeleontidae possess lower chromosome numbers, 2n = 14 and 2n = 16, which are encountered in all subfamilies, with the only exception of the Palparinae. The latter is the only subfamily characterized by higher numbers, 2n = 26, 24, and 22, and the higher number, 2n = 18, is also found in the only studied species of the related subfamily Pseudimarinae. The higher numbers, 2n = 22, 20 and 18, are also characteristic of the sister family Ascalaphidae. Since Palparinae represent a basal phylogenetic lineage of the Myrmeleontidae (Krivokhatsky 2011), it is hypothesized that higher chromosome numbers are ancestral for antlions. Most likely, the higher chromosome numbers were inherited from the common ancestor of Myrmeleontidae + Ascalaphidae. It was preserved in the subfamily Palparinae (Myrmeleontidae) but changed via chromosomal fusions toward lower numbers, 2n = 18, 16 and 14, in other subfamilies.

Knowledge of the chromosome morphology in the low-numbered and high-numbered chromosome complements would help in understanding the karyotype evolution in the Myrmeleontidae and Ascalaphidae and testing the above hypothesis. Unfortunately, despite several efforts to identify chromosomal morphology within particular karyotypes (e.g. Asana and Kichijo 1936, Hughes-Schrader 1983, present study), this important question remains unresolved. Special staining methods, e.g. C-banding, are therefore needed to identify the centromeric position in the chromosomes and thus their morphology. However, these techniques have never been used in neuropteran cytogenetics, and therefore this is the most serious objective in the chromosome research of antlions and owlflies.

Sex chromosome system

All Myrmeleontidae and Ascalaphidae species, including those studied here, exhibit a simple sex chromosome system XY/XX, which is characteristic of the whole order Neuroptera (White 1973, Blackman 1995). Both antlions and owlflies demonstrate a very peculiar behavior of sex chromosomes in males (Naville and de Beaumont 1933, 1936, Asana and Kichijo 1936, Hughes-Schrader 1983, Klok and Chown 1993, present paper). In spermatocyte meiosis of those insects, sex chromosomes take up positions at opposite halves of the meiotic spindle at metaphase I before segregating into the daughter spermatocytes. It means that the X and Y chromosomes get segregated to opposite poles of the spindle long before the autosomal half-bivalents disjoin at anaphase I and move to the poles. The same pattern, the so-called "distance pairing" of sex chromosomes first discovered by Naville and de Beaumont (1933) in antlions, is known to be characteristic of the related neuropteran families Chrysopidae, Mantispidae, Sisyridae, Osmylidae, and Hemerobiidae (Naville and de Beaumont 1936, Hughes-Schrader 1969, 1975b, 1980, Nokkala 1986) and probably of the order Neuroptera in general. The biological role of this unusual behavior of sex chromosomes is unclear. In any case, this mechanism observed in brown lacewings (Hemerobiidae) showed no significance for the regular segregation of the sex chromosomes in meiosis (Nokkala 1986).

The order Neuroptera belongs to the superorder Neuropterida, which comprises another two orders, namely, Raphidioptera with two extant families, Raphidiidae and Inocelliidae, and Megaloptera with two extant families, Corydalidae and Sialidae (Aspöck and Aspöck 2007). Interestingly, the Neuroptera share the "distance pairing" of sex chromosomes with Raphidioptera (Naville and de Beaumont 1936, Hughes-Schrader 1975a) but not with Megaloptera. In the latter group, all hitherto studied species, which belong to the single family Corydalidae, show another very specific "parachute-like" sex bivalent in spermatocyte meiosis (Hughes-Schrader 1980, Takeuchi et al. 2002). In this case, the X and Y chromosomes form a pseudo-bivalent that is situated together with the autosomes on the equator of the spindle and segregates synchronously with them at the first meiotic anaphase. This unique meiotic sex chromosome configuration called Xy_p (Smith 1950) is the well-known characteristic feature of the related order Coleoptera, and is encountered in almost all coleopteran families. Therefore Xy_p is considered ancestral for beetles (Smith 1950), at least for the suborder Polyphaga (Petitpierre 1987).

The variety and distribution of sex chromosome systems in different orders of the class Insecta have been comprehensively reviewed by Blackman (1995). The X(0) system was shown to predominate in the lower orders and is considered as ancestral condition for several major groups and for Insecta as a whole. The XY systems when occur are all derived from an X(0) one. The sex chromosome systems seem to provide useful phylogenetic evidence. Within Holometabola orders, besides simple X(0) and XY, there are some peculiar systems, e.g., those involving female heterogamety (XY/XX or ZW/ZZ) shared by Lepidoptera and Trichoptera, haplodiploid sex determination characteristic of Hymenoptera, and some others. Of these, distance pairing of the X and Y chromosomes in spermatocyte meiosis and the parachute Xy_p system are hypothesized to be synapomorphies respectively of the clade Neuroptera + Raphidioptera and of the clade Megaloptera + Coleoptera (Blackman 1995, Takeuchi et al. 2002).

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Comparative cytogenetics of Auchenorrhyncha (Hemiptera, Homoptera): a review

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Abstract

A comprehensive review of cytogenetic features is provided for the large hemipteran suborder Auchenorrhyncha, which currently contains approximately 42,000 valid species. This review is based on the analysis of 819 species, 483 genera, and 31 families representing all presently recognized Auchenorrhyncha superfamilies, e.i. Cicadoidea (cicadas), Cercopoidea (spittle bugs), Membracoidea (leafhoppers and treehoppers), Myerslopioidea (ground-dwelling leafhoppers), and Fulgoroidea (planthoppers). History and present status of chromosome studies are described, as well as the structure of chromosomes, chromosome counts, trends and mechanisms of evolution of karyotypes and sex determining systems, their variation at different taxonomic levels and most characteristic (modal) states, occurrence of parthenogenesis, polyploidy, B-chromosomes and chromosome rearrangements, and methods used for cytogenetic analysis of Auchenorrhyncha.

Keywords

chromosome structure, chromosome numbers, sex chromosome systems, B-chromosomes, polyploidy, polymorphism, meiosis, chromosome evolution, Cicadoidea, Cercopoidea, Membracoidea, Myerslopioidea, Fulgoroidea, Cicadomorpha, Fulgoromorpha

Introduction

The hemipteran (homopteran) suborder Auchenorrhyncha is divided into two major lineages: the infraorder Cicadomorpha with superfamilies Cicadoidea (cicadas), Cercopoidea (spittle bugs), Membracoidea (leafhoppers and treehoppers), and Myerslopioidea (ground-dwelling leafhoppers), and the infraorder Fulgoromorpha with the single superfamily Fulgoroidea (planthoppers) (Szwedo et al. 2004, Aguin-Pombo and Bourgoin 2012). More than 42,000 valid species of Auchenorrhyncha have been reported worldwide (Deitz 2008), which, depending on the classification followed, can be grouped roughly into 30 to 40 families.

Olli Halkka (Halkka 1959), one of the earliest and most well-known researchers of chromosomes in Auchenorrhyncha, concluded that they "are a group well suited for comparative karyological work. Technically, this group presents no special difficulties. The numbers of the chromosomes are relatively low and the chromosomes themselves are fairly large". The first cytogenetic studies on Auchenorrhyncha provided data for the cicada species *Diceroprocta tibicen* Linnaeus (Cicadidae) (Wilcox 1895) and the spittlebug species *Lepyronia quadrangularis* Say (Aphrophoridae) (Stevens 1906). Shortly afterwards Boring (1907) initiated research on the comparative karyology of Auchenorrhyncha with a study of 22 species belonging to five families. Documented lists of Auchenorrhyncha chromosome numbers have been published by several authors. Those of Halkka (1959) and Kirillova (1986, 1987) cover the complete suborder Auchenorrhyncha. The first author discussed different aspects of auchenorrhynchan cytogenetics, while the second reported only chromosome numbers and sex determining systems. Later, lists for particular fulgoromorphan families were published by Kuznetsova et al. (1998) for Cixiidae, Meenoplidae, Derbidae, Achilidae, Nogodinidae, Tropiduchidae, and Flatidae; by Maryańska-Nadachowska et al. (2006) for Issidae, Caliscelidae, and Acanaloniidae; by Kuznetsova et al. (2009a) for Dictyopharidae and Fulgoridae; and then, Tian et al. (2004) added data for 19 species of the families Cixiidae, Delphacidae, Fulgoridae, Ricaniidae, Issidae, Flatidae, and Achilidae, while Kuznetsova et al. (2010) for 14 species of the issid tribe Issini. In contrast to Fulgoromorpha, the data on cicadomorphan families have never been tabulated after the comprehensive reviews by Halkka (1959) and Kirillova (1986, 1987). Quite recently, chromosome numbers were reported for 91 species of Cicadellidae (Wei 2010, Juan 2011) and 25 species of Membracidae (Tian and Yuan 1997) from China. Several additional species were also karyotyped within the families Cicadidae, Cercopidae, Aphrophoridae, Cicadellidae, and Myerslopiidae (Marin-Morales et al. 2002, Perepelov et al. 2002, Kuznetsova et al. 2003, 2013, 2015a, Aguin-Pombo et al. 2006, 2007, Maryańska-Nadachowska et al. 2008, 2012, Castanhole et al. 2010, de Bigliardo et al. 2011, Golub et al. 2014).

At the present time, approximately 819 auchenorrhynchan species (nearly 2% of the total number of species described) are known from a cytogenetic viewpoint (V. Kuznetsova, unpublished checklist). These species represent 483 genera and 31 families from all the superfamilies of Auchenorrhyncha. Of these taxa, 511 species,

335 genera and 11 families belong to Cicadomorpha, while 308 species, 148 genera, and 20 families belong to Fulgoromorpha (Figs 1-8). The available data were chiefly obtained using conventional cytogenetic techniques and concerned, almost entirely, chromosome numbers, sex determining systems, and, in outline, the behaviour of chromosomes during meiosis. A few recent studies have used modern cytogenetic techniques to identify the individual chromosomes in karyotypes and specific regions in chromosomes of auchenorrhynchan species (Kuznetsova et al. 2003, 2009b, 2010, 2015a, Maryańska-Nadachowska et al. 2008, 2012, 2013, Golub et al. 2014). The application of new techniques, primarily fluorescence *in situ* hybridization (FISH), opened a promising area of research, which yields more detailed karyotype information (Maryańska-Nadachowska et al. 2013, Golub et al. 2014, Kuznetsova et al. 2015a).

Since the Halkka's (1959) excellent review, the comparative cytogenetics of Auchenorrhyncha has never been rigorously addressed. The only exception is the two-part paper of Emeljanov and Kirillova (1990, 1992), which presents a comprehensive analysis of chromosome numbers and their variation at different taxonomic levels within every auchenorrhynchan family explored at that time. Thus, nearly fifty five years after Halkka's and twenty five years after Emeljanov and Kirillova's publications, we discuss here different aspects of cytogenetics of Auchenorrhyncha and summarize progress and problems in the field.

Chromosome structure

The overwhelming majority of eukaryotic organisms have monocentric chromosomes. These chromosomes possess the localized centromere, a region where two chromatids join and where spindle fibers attach during mitosis and meiosis. Like all Hemiptera, Auchenorrhyncha have holokinetic (holocentric) chromosomes. In contrast to monocentric chromosomes, holokinetic chromosomes have no localized centromere. The latter is considered to be diffuse and is formed by a large kinetochore plate (a circular plaque structure on the centromere by which the chromosomes are attached to spindle polar fibers) extending along all or most of the length of the holokinetic chromosome (Schrader 1947, Wolf 1996). Holokinetic chromosomes are sometimes designated as holocentric despite the fact that they lack a proper centromere. These chromosomes occur in certain scattered groups of plants and animals, being particularly widespread in insects, including Odonata (Palaeoptera), Dermaptera (Polyneoptera), Psocoptera, Phthiraptera, Hemiptera (Paraneoptera), Lepidoptera, Trichoptera (Oligoneoptera) (White 1973), and the enigmatic Zoraptera (Kuznetsova et al. 2002). Thus, holokinetic chromosomes occur in every major phylogenetic lineage (cohort) of Pterygota suggesting that they are likely to have evolved at least four times independently in insect evolution.

In theory, the large kinetochore plate facilitates rapid karyotype evolution via occasional fusion/fission events. Firstly, fusion of holokinetic chromosomes would not create the problems characteristic of a dicentric chromosome in monocentric organ-

isms (i.e. displaying chromosomes with localized centromeres). Secondly, fission of a holokinetic chromosome should create chromosome fragments that exhibit a part of the kinetochore plate and can attach themselves to the spindle fibers at cell divisions. As a result, chromosome fragments that would be acentric (lacking a centromere) and hence lost in organisms with monocentric chromosomes may be inherited in holokinetic organisms. The gametes harboring chromosome fragments are consequently expected to be viable (Hipp et al. 2010). Fusion/fission rearrangements are therefore conventionally accepted as the commonest mechanisms of chromosome evolution in holokinetic groups. This assumption seems to receive support from the fact that the greatest range of within-genus variation in chromosome number related to the fusion/fission rearrangements is described in organisms with holokinetic chromosomes (reviewed in Kuznetsova et al. 2011). The evidence for the unique potential of holokinetic chromosomes' fissions is provided by the blue butterfly *Polyommatus atlanticus* Elwes (Lycaenidae), 2n = ca 448-452, holding the record of the highest number of chromosomes in the non-polyploid eukaryotic organisms (Lukhtanov 2015).

Although variations in chromosome number of related species are probably due to both fissions and fusions, fusions are suggested to be more common in holokinetic groups (White 1973) including Auchenorrhyncha (Halkka 1959, 1964). The point is that a chromosome, whether holokinetic or monocentric, has to display two functional telomeres in order to survive a mitotic cycle. A chromosome resulting from a fusion event will always display two functional telomeres originated from the two ancestral chromosomes, whereas a chromosome from a fission event will have to be able to develop a functional telomere *de novo* (White 1973, Nokkala et al. 2007).

Chromosome numbers and possible trends of their evolution

Variation in chromosome number. The currently known diploid chromosome numbers in Auchenorrhyncha range between 8 and 38 (here and elsewhere chromosome numbers are provided for females), being the lowest in Cicadomorpha (Cicadellidae) and the highest in Fulgoromorpha (Delphacidae and Dictyopharidae). The infraorders differ in the limits of variation in chromosome number and in the modal numbers (sometimes referred to as the type numbers or basic numbers). Within each infraorder, many taxa have more than one modal number and these are characteristically lower in Cicadomorpha than in Fulgoromorpha. In Cicadomorpha, chromosome numbers vary from 2n = 8 (*Orosius* sp. from Cicadellidae) to 2n = 32 (*Peuceptyelus coriaceus* Fallén from Aphrophoridae). The numbers in most cicadomorphan species lie between 16 and 22, with rare exceptions above and below these limits. In Fulgoromorpha, chromosome numbers vary from 2n = 20 (*Pentastiridius hodgarti* Distant from Cixiidae) to 2n = 38 (*Scolops* spp. from Dictyopharidae and *Paraliburnia clypealis* Sahlberg from Delphacidae) with strongly marked modes at 28 (prevailing), 30 (second) and 26. The variation in chromosome number in various groups of Auchenorrhyncha is shown in Figs 1–8.

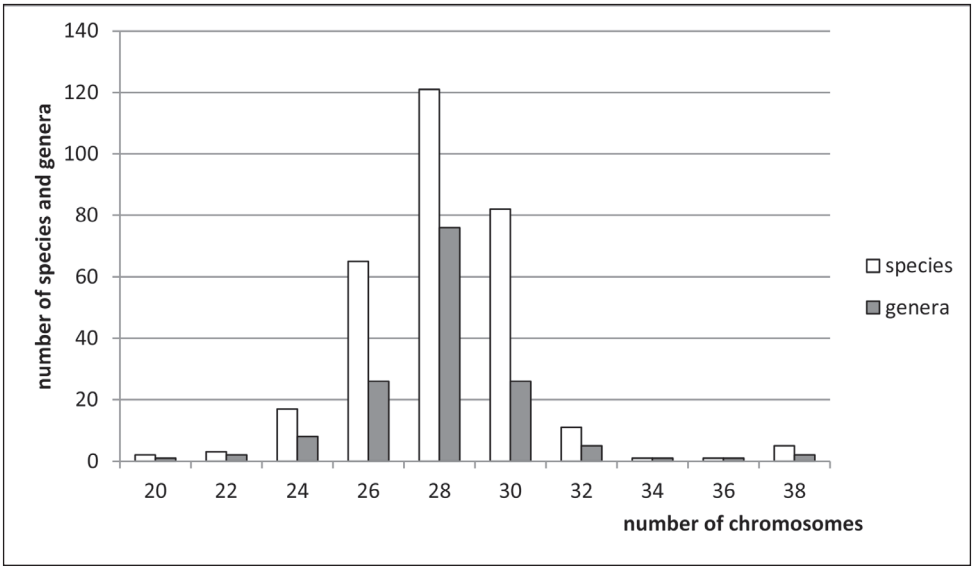

Figure 1. Histogram showing the distribution of female diploid chromosome numbers in Fulgoroidea at species and generic levels, based on analysis of 308 species and 148 genera of the families Tettigometridae, Delphacidae, Cixiidae, Kinnaridae, Meenoplidae, Derbidae, Achilidae, Achilixiidae, Dictyopharidae, Fulgoridae, Issidae, Caliscelidae, Acanaloniidae, Nogodinidae, Ricaniidae, Flatidae, Hypochthonellidae, Lophopidae, Eutybrachyidae, and Gengidae.

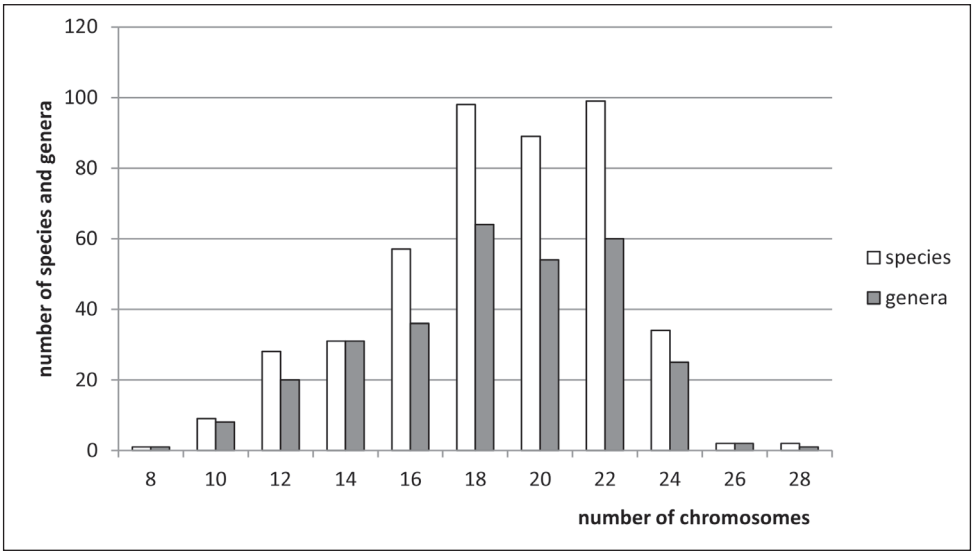

Figure 2. Histogram showing the distribution of female diploid chromosome numbers in Membracoidea at species and generic levels, based on analysis of 450 species and 302 genera of the families Cicadellidae, Membracidae, Ulopidae, Ledridae, and Aetalionidae.

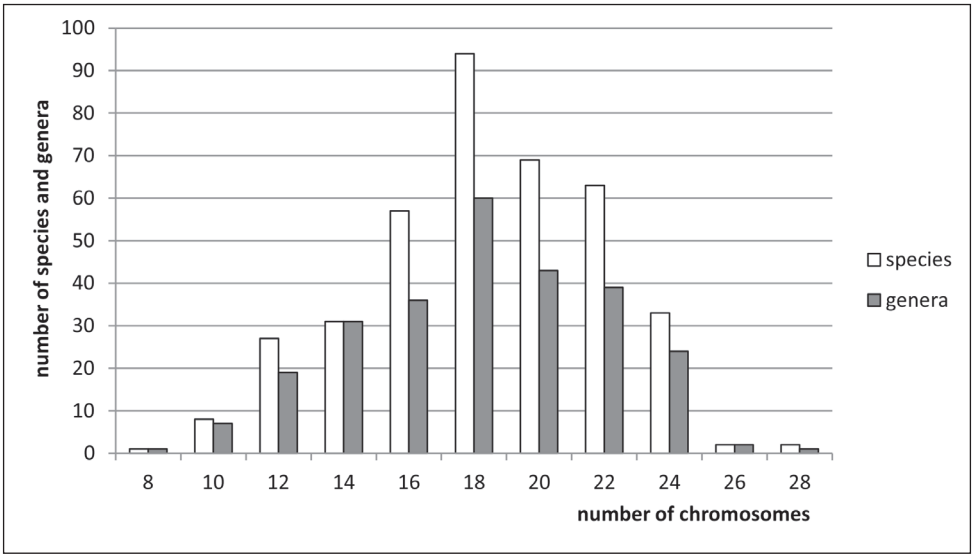

Figure 3. Histogram showing the distribution of female diploid chromosome numbers in Cicadellidae at species and generic levels, based on analysis of 387 species and 263 genera.

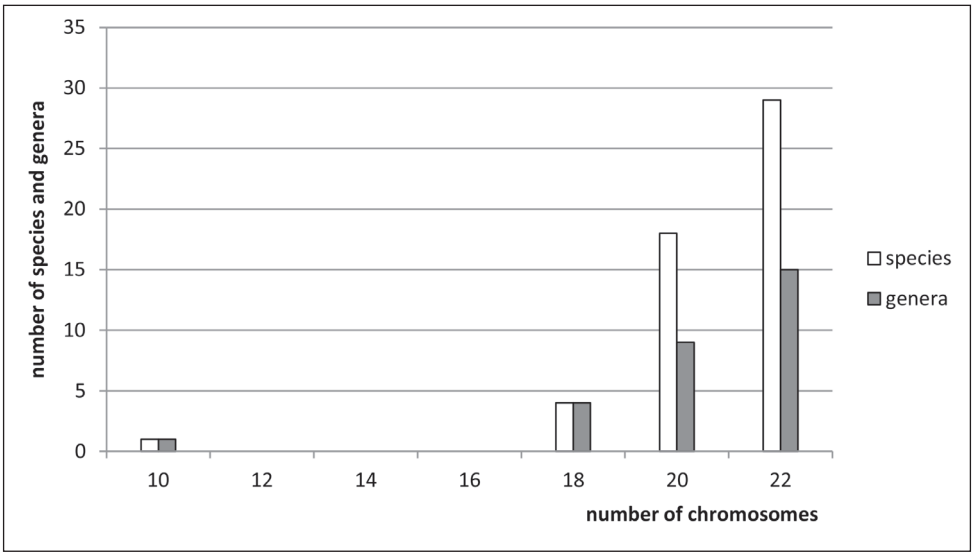

Figure 4. Histogram showing the distribution of female diploid chromosome numbers in Membracidae at species and generic levels, based on analysis of 52 species and 29 genera.

Despite the fact that all Auchenorrhyncha possess holokinetic chromosomes, many higher taxa of the suborder show stable or only slightly variable karyotypes. Quite often the chromosome number is constant within the same genus. Within Cicadellidae, the

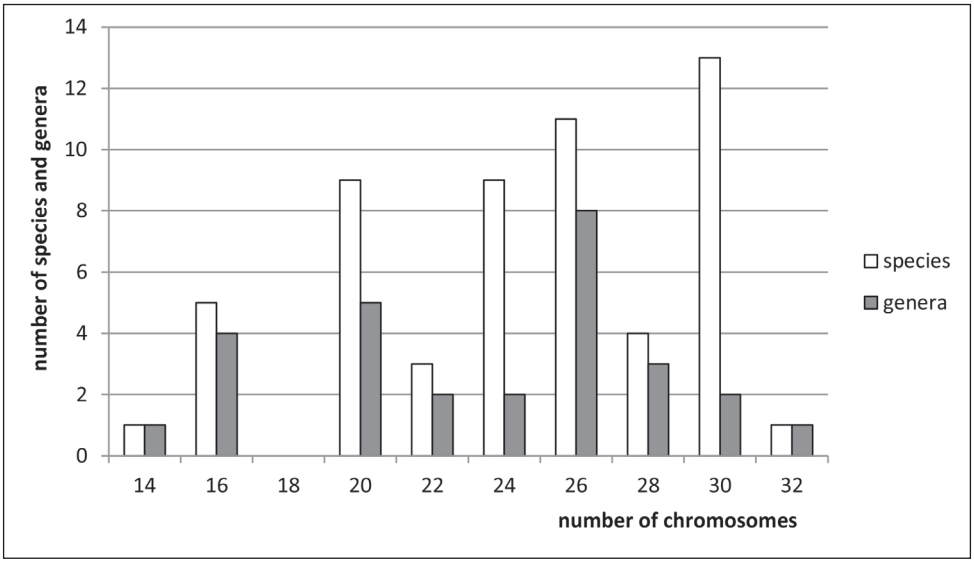

Figure 5. Histogram showing the distribution of female diploid chromosome numbers in Cercopoidea at species and generic levels, based on analysis of 50 species and 23 genera of the families Cercopidae, Aphrophoridae, Machaerotidae, and Clastopteridae.

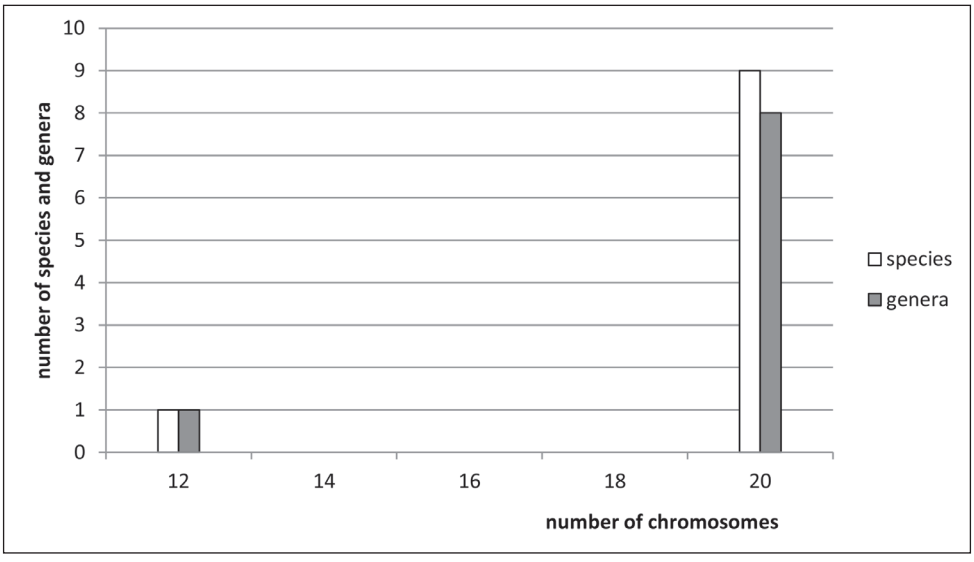

Figure 6. Histogram showing the distribution of female diploid chromosome numbers in Cicadoidea at species and generic levels, based on analysis of 10 species and 9 genera of the family Cicadidae.

genera *Eurymela* Le Peletier & Serville, *Eurymeloides* Ashmead, and *Cicadula* Zetterstedt are examples. In the first, all three studied species, *E. distincta* Signoret, *E. erythrocnemis* Burmeister, and *E. fenestrata* Peletier & Serville, share 2n = 22; in the second,

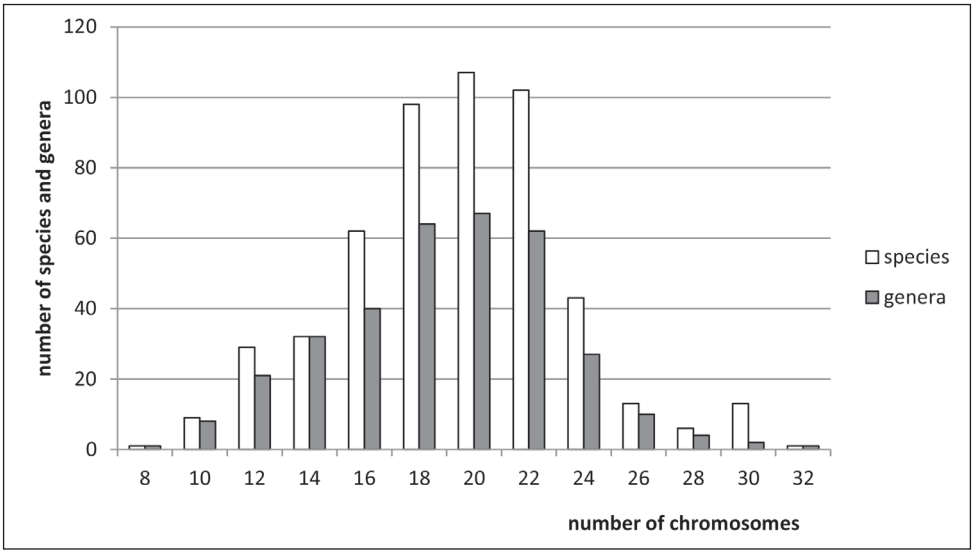

Figure 7. Histogram showing the distribution of female diploid chromosome numbers in Cicadomorpha at species and generic levels, based on analysis of 511 species and 335 genera of the families Cicadellidae, Membracidae, Ulopidae, Ledridae, Aetalionidae, Cercopidae, Aphrophoridae, Machaerotidae, Clastopteridae, Cicadidae, and Myerslopiidae.

all four studied species, *E. bicincta* Erichson, *E. perpusilla* Walker, *E. pulchra* Signoret, and *E. punctata* Signoret, possess likewise 2n = 22; in the third, four studied species, *C. intermedia* Boheman, *C. quadrinotata* Fabricius, *C. persimilis* Edwards, and *C. saturata* Edwards, possess 2n = 16 (de Lello et al. 1982, Kirillova 1987). In the family Aphrophoridae, the large genus *Aphrophora* Germar is characterized by 2n = 30, whereas in the Membracidae, the majority of species in the genera *Gargara* Amyot & Serville and *Leptocentrus* Stål shows 2n = 20 and 2n = 22, respectively (Kirillova 1987). The most impressive examples of chromosome stability come from groups which have been extensively studied. Thus, nearly all species and genera of the subfamily Eurymelinae (Cicadellidae) have 2n = 22 (Whitten 1965), while those (33 species) of the tribe Issini (Issidae) – 2n = 28 (Maryańska-Nadachowska et al. 2006, Kuznetsova et al. 2010). Similarly, within the family Dictyopharidae, almost all so far studied representatives of the tribe Dictyopharini (9 species) have 2n = 30; those of the tribe Ranissini (8 species) 2n = 28, while those of the tribe Almanini (16 species) – 2n = 26 (Kuznetsova 1986, Kuznetsova et al. 2009a). The conservative numbers suggest no evidence that fusions/fissions have played a role in speciation and evolution of these groups.

By contrast, there are some groups in which a wide variety of chromosome numbers occurs suggesting that both fusions and fissions have established themselves during their evolution. In Cicadellidae, within the genus *Eurhadina* Haupt, the 19 studied species vary broadly in chromosome number: 2n = 12, 14, 16, 18, and 20 (Halkka 1957, Juan 2011). The genus *Empoasca* Walsh is another group, which seems to show a striking range in chromosome number. In this genus, the twelve species examined so

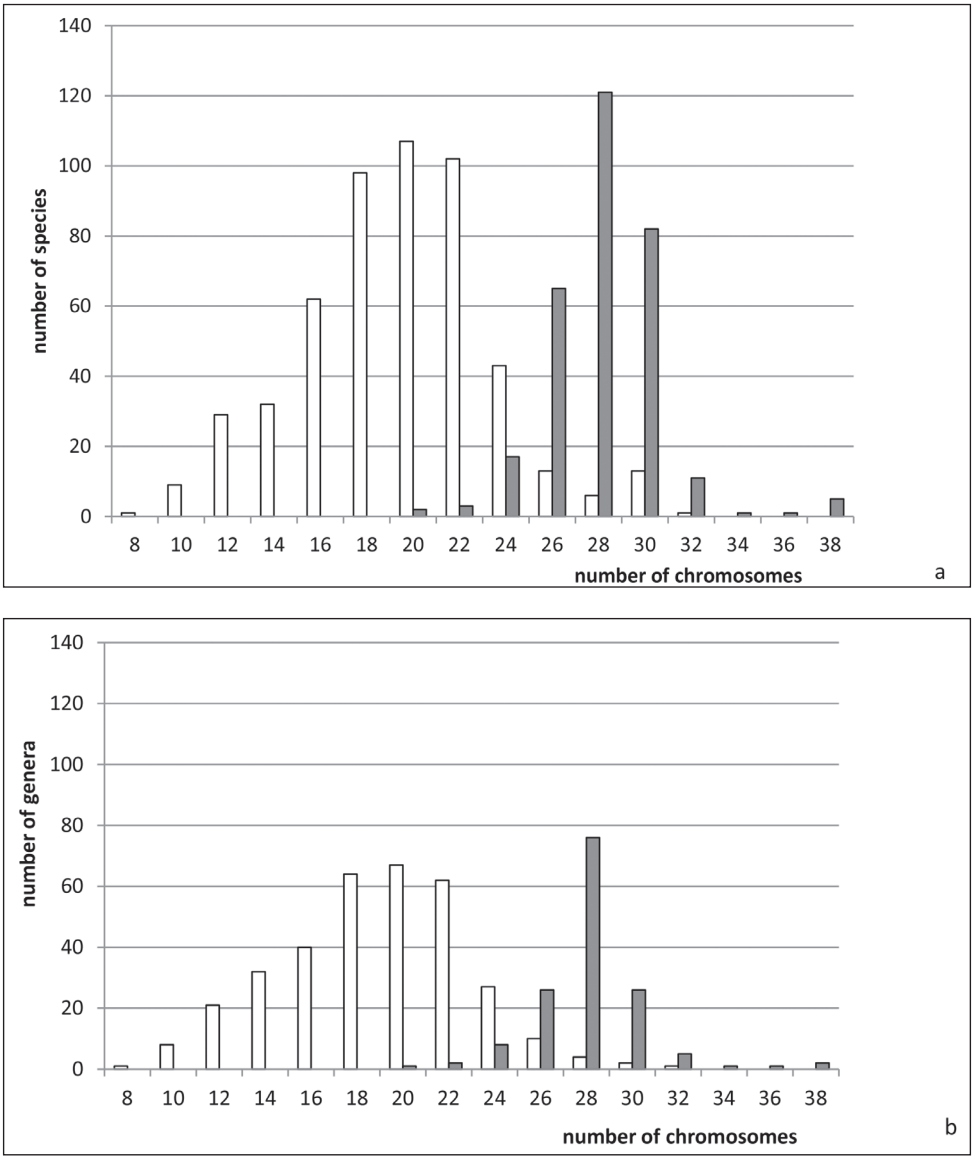

Figure 8. Histograms showing the distribution of female diploid chromosome numbers in Cicadomorpha (**a, b** white columns) and Fulgoromorpha (**a, b** black columns) at species (**a**) and generic (**b**) levels, based on analysis of 819 species and 483 genera.

far display 2n = 16 (4 species), 18 (2 species), 20 (4 species), and 22 (2 species) (Kirillova 1988, Aguin-Pombo et al. 2006, Juan 2011). This cosmopolitan genus with more than 1,000 described species is by far the most speciose genus in Cicadellidae. *Empoasca* is recognized as a genus requiring comprehensive revision (Southern and Dietrich 2010), and a cytogenetic approach might be useful to clarify the species-level systemat-

ics of this group. Likewise, the eight species recognized in the remarkably polymorphic spittlebug genus *Philaenus* Stål (Aphrophoridae) display three different chromosome numbers in males: 20, 23, and 24 (Maryańska-Nadachowska et al. 2012).

The modal and ancestral chromosome numbers. Emeljanov and Kirillova (1992) argued that the ancestral chromosome numbers were as follows: 2n = 30 in Fulgoroidea, 2n = 26-28 in Cercopoidea, 2n = 20 in Cicadoidea, and 2n = 22 in Membracoidea. Although 2n = 22, discovered later in the most primitive membracoid family Aetalionidae (Kuznetsova and Kirillova 1993), seemed to confirm the ancestrality of this number in Membracoidea, a more definite solution of this problem will be possible only after a more thorough investigation of every superfamily. For example, the currently available data on Fulgoroidea (308 species and 148 genera) are greatly skewed owing to the focus on the families Dictyopharidae and Delphacidae and on the tribe Issini (Issidae), in which altogether over 160 species in 122 genera have been karyotyped. In Cercopoidea, chromosome numbers are known for 50 species (23 genera), half of which belong to the family Aphrophoridae, while in Cicadoidea only ten species (nine genera) in the Cicadidae have been studied. Within Membracoidea, 450 species in 302 genera have been karyotyped, of which at least 65% of species and 53% of genera belong to the subfamilies Typhlocybinae and Delthocephalinae (Cicadellidae). It should be added here that *Mapuchea chilensis* Nielson, the recently studied first representative of the cicadomorphan superfamily Myerslopoidea (Myerslopiidae), was found to exhibit 2n = 18(16 + XY) (Golub et al. 2014). This chromosome number fits well into the range of most characteristic numbers in Cicadomorpha as a whole (16-22) being close to the numbers accepted by Emeljanov and Kirillova (1992) as putative ancestral ones for Cicadoidea (2n = 20) and Membracoidea (2n = 22) but not for Cercopoidea (2n = 26-28).

Opinions on the ancestral chromosome number in Auchenorrhyncha as a whole differ considerably (Halkka 1959, Kuznetsova et al. 1998, Emeljanov and Kirillova 1992). The solution of the problem very much depends on phylogeny accepted and method of ancestral number inference adopted. One approach to inferring the ancestral karyotype is mapping chromosome numbers typical for particular superfamilies on phylogenetic trees. Some researchers treat Fulgoroidea (Fulgoromorpha) as the most basal branch within Auchenorrhyncha (e.g. Shcherbakov 1984). This idea recently supported by molecular data (Cryan and Urban 2012) is tempting to speculate that the typical number for Fulgoromorpha, 2n = 30 (Halkka 1959) or 2n = 28 (Kuznetsova et al. 1998), is the ancestral state in Auchenorrhyncha. However, based on a large number of morphological characters, Emeljanov (1987) hypothesized that the common ancestor of the four (now five including Myerslopioidea) recent superfamilies differentiated first into cercopoid-cicadoid and fulgoroid-cicadelloid branches, thus becoming the clade Cercopoidea + Cicadoidea, the sister group of the clade Fulgoroidea + Membracoidea. Comparison of the putative ancestral numbers of the superfamilies (see above) with the Emeljanov's phylogenetic scheme of Cercopoidea + Cicadoidea suggests that the characteristic karyotype of Cercopoidea, 2n = 28 or 26, would be the most likely ancestral chromosome number in Auchenorrhyncha as a whole (Emeljanov and Kirillova 1992).

Another approach to inferring the ancestral karyotype is a comparison of typical chromosome numbers between the target group and outgroups. Emeljanov and Kirillova (1992) used this approach and compared chromosome numbers in Auchenorrhyncha with those in other hemipteran lineages – Aphidomorpha, Coccomorpha, Psyllomorpha, Aleyrodomorpha, and Heteroptera. This led to the conclusion that the common ancestor of all Auchenorrhyncha had a diploid set of 20-22 chromosomes.

Since Emeljanov and Kirillova's (1992) publication, a large body of new cytogenetic data on hemipterans has been obtained. There is a good reason to reconsider the problem based on the data available at present time. In Coccomorpha, diploid numbers vary from 4 to 192, with comparatively low numbers in "archaeococcoids" comprising the most basal families of scale insects – Ortheziidae (2n = 14, 16, 18), Margarodidae (modal number of 2n = 4), and Phenacoleachiidae (2n = 8 in the only studied species *Phenacoleachia zealandica* Cockerell) (Gavrilov 2007). In Aphidomorpha, chromosome numbers vary between 4 and 72, the ancestral number most likely being between 8 and 20 (Blackman 1980) though, in our opinion, more likely between 8 and 22. In the most primitive families, 2n = 22 (Adelgidae), 2n = 8 and 12 (Phylloxeridae) and 2n = 20 (Eriosomatidae = Pemphigidae) seem to be most characteristic (Kuznetsova and Shaposhnikov 1973, Blackman 1980, Gavrilov-Zimin et al. 2015), but the sampling is still inadequate, at least for Adelgidae and Phylloxeridae (Gavrilov-Zimin et al. 2015). Although it is not necessary for a modal number to be ancestral in a group, it seems reasonable to assume that in Psyllomorpha the karyotype of 2n = 26, which has been conserved in 72% of the species and in 50% of the genera studied, is their ancestral trait (Maryańska-Nadachowska 2002). In Aleyrodomorpha, chromosome numbers are known for only four species (see Blackman and Cahill 1998): *Trialeurodes vaporariorum* Westwood (2n = 22), *Aleurotulus nephrolepidis* Quaintance (2n = 26 and/or 28), *Aleurodes proletella* Linnaeus (2n = 26), and *Bemisia tabaci* Gennadius (2n = 20). In one of the most primitive true bug infraorder, the Dipsocoromorpha, chromosome numbers have been recorded for males of three representatives of the family Dipsocoridae: 2n = 20 + X(0) in *Cryptostemma rufescens* Sahlberg, 2n = 20 + XY_1Y_2 in *C. pussillimum* Sahlberg, 2n = 20 + XY in *C. hickmani* Hill, 2n = 20? + XY in *C. castaneovitreus* Linnavuori, and for males of two species of the family Schizopteridae: 2n = 32 + X(0) in *Pateena elimata* Hill and *Rectilamina australis* Hill (Grozeva and Nokkala 1996). For the two studied Coleorrhyncha species, *Xenophyes cascus* Bergroth and *Peloridium pomponorum* Shcherbakov, male karyotypes of 2n = 26 + X(0) and 2n = 30 + X(0) respectively were recorded (Grozeva at al. 2014, Kuznetsova et al. 2015b).

In Psocomorpha, a sister group to the rest of Paraneoptera, the modal karyotype of 2n = 18 is considered as the ancestral one, although there appears to be considerable variation in chromosome number within more primitive suborder Trogiomorpha: 2n = 18 and 22 in Trogiidae, 2n = 20 in Psoquillidae, and 2n = 30 in Psyllipsocidae (Golub and Nokkala 2009). Thus, the available data on the higher hemipteran groups appear insufficient and too heterogeneous to reconstruct the chromosome number ancestral for Auchenorrhyncha.

Sex determining systems

Genetic sex determination predominates in higher animals, including insects, and is often accompanied by the presence of a heteromorphic chromosome pair in one sex (White 1973). The Auchenorrhyncha, in common with most other insects, display male heterogamety. The XX/X(0) sex determination (where 0 denotes the absence of the Y chromosome) is of common occurrence and seems to be an ancestral trait in this group (Halkka 1959, Emeljanov and Kirillova 1990, 1992) and in Hemiptera as a whole (Blackman 1995). Thus, in females two Xs are present, while in males only one X is present. Despite evolutionary stability, in some cases the X(0) system has been replaced by an XY system in species within the same genus that is otherwise exclusively X(0). When such cases occur in Auchenorrhyncha, it is often clear that the Y is a neo-Y (Blackman 1995). Examples of this are found in the genera *Oncopsis* Burmeister from Cicadellidae (John and Claridge 1974) and *Philaenus* from Aphrophoridae (Maryańska-Nadachowska et al. 2012). A highly peculiar situation occurs in the cytologically well-studied tribe Almanini (Dictyopharidae), in which all species have a neo-XY system and the only exception is *Almana longipes* Dufour with an X(0) system in males. In contrast to Almanini, species from all other tribes of this family are characterized by an X(0) system (Kuznetsova 1986, Kuznetsova et al. 2009a).

In organisms with XY systems, recombination between X and Y chromosomes is usually suppressed (White 1973) except for the cases when the XY is a neo-system. This type of sex determination usually arises from the ancestral X(0) system as a result of fusion between the original X chromosome and an autosome, the homologue of this autosome becoming a neo-Y chromosome (White 1973, Blackman 1995). Clearly, the derived karyotype should have one pair of autosomes less than the ancestral one. For example, the species of the tribe Almanini (Dictyopharidae) have 2n = 26 + neo-XY, whereas those of the tribe Ranissini 2n = 28 + X(0). In a recently formed neo-XY system, the autosomally derived Y chromosome (a neo-Y) and the autosomal part of the neo-X chromosome are still homologous, and therefore synapse in prophase I of meiosis. At metaphase I, the neo-XY bivalent is usually large and clearly heteromorphic, indicating a recent fusion between the X and an autosome pair.

Once a neo-XY system has arisen, it can undergo a further transformation into a multiple X_1X_2Y system as a result of a translocation involving the Y chromosome and another pair of autosomes. This may have occurred in the evolution of the sex chromosome mechanism in *Philaenus italosignus* Drosopoulos & Remane, which has 2n = 20 + neo-X_1X_2Y against 2n = 22 + neo XY found in *P. signatus* Melichar, *P. tarifa* Remane & Drosopoulos, and *P. maghresignus* Drosopoulos & Remane (Maryańska-Nadachowska et al. 2012, 2013). Multiple sex chromosomes of the X_1X_2Y type, which form chiasmate trivalents in meiosis, have also been described in *Austragalloides* sp. (Cicadellidae), however, in this case the X_1X_2Y system represents an example of sex chromosome polymorphism (Whitten 1968).

A different, achiasmate XY system, with a fairly small Y chromosome, is found in the planthoppers *Limois emelianovi* Oshanin and *L. kikuchii* Kato (Fulgoridae)

(Kuznetsova 1986, Tian et al. 2004). The origin of the Y chromosome in these species is not entirely clear, but it seems likely that it has been derived from a mitotically stable B chromosome that has become a standard member of the karyotype, that is, the B chromosome transformed into the Y chromosome during evolution (discussed in section **"Polymorphism for B-chromosomes"**).

Polyploidy

Polyploidy, that is, multiplication of the chromosome set is well known to play a major role in speciation and evolution of plants, but is a fairly rare phenomenon in sexually reproducing animals (White 1973). Evolutionary polyploidy had occurred in a number of animal species that reproduce parthenogenically. In Auchenorrhyncha, all so far known cases of polyploidy are connected with parthenogenesis, either with gynogenesis, sometimes referred to "pseudogamy" (where the egg is activated by sperm borrowed from conspecific or closely related males, but without fusion of the egg and sperm nuclei), or with true parthenogenesis, more often referred to "thelytoky". Although it is not necessary for all parthenogenetic forms to be polyploids, these are universally triploids in both leafhoppers and planthoppers. Only two planthopper genera, *Muellerianella* Wagner and *Ribautodelphax* Wagner (Delphacidae), are known to comprise a number of gynogenetic triploid forms (Drosopoulos 1976, 1977, den Bieman and Eggers-Schumacher 1986). In contrast, leafhoppers of the genus *Empoasca* (Aguin-Pombo et al. 2006) and planthoppers of the genus *Delphacodes* Fieber (den Bieman and de Vrijer 1987) comprise triploid forms, which reproduce by true parthenogenesis. The genus *Empoasca* is a good case in point. In this diverse, complex and cosmopolitan genus, the bisexual species are diploid, with hitherto known chromosome numbers of 2n = 16, 18, 20 and 22. In Madeira Island, besides the bisexual species *E. decedens* Paoli (2n = 14 + XX), *E. alsiosa* Ribaut (2n = 16 + XX) and *E. fabalis* DeLong (2n = 20 + XX), three all-female morphotypes (A, B and C) were discovered. In these females, the chromosomal complements are triploid, consisting thus of two female genomes and one male genome: 2n = 3x = 28 + XXX in morphotype A, 24 + XXX in morphotype B, and 21 + XXX in morphotype C. The study revealed that their reproduction follows an apomictic type (Aguin-Pombo et al. 2006).

In apomictic parthenogenesis, meiosis is completely suppressed, and eggs pass through a mitosis-like cell division, i.e. without formation of bivalents and recombination, and genetic heterozygosity is thus preserved. The heterozygosity is expected to be perpetuated from generation to generation, increasing slightly through mutations. It is generally proposed that most polyploid animals are allopolyploids, tending to be of hybrid origin (White 1973, Bullini 1985). It is assumed that such is most likely the case of the triploid forms of *Muellerianella* (Drosopoulos 1976). In contrast, in some groups, triploids seem to have an autopolyploid origin and this is probably true for triploids found in the genus *Ribautodelphax* (den Bieman 1988). The origin of the above-listed *Empoasca* parthenoforms still remains unknown. Some of them seem to

be closely related to bisexual species which are yet extant. Several hypotheses, including that of their hybrid origin, were made in Aguin-Pombo et al. (2006) but still much more information is needed to decide between the hypotheses.

Polymorphism for B-chromosomes

B-chromosomes (also referred to as supernumerary, additional or accessory) are chromosomes found in addition to chromosomes of the standard complement (A chromosomes) and occur in approximately 15% of living species (Beukeboom 1994). Little consensus has been achieved in understanding their origin, role, transmission, inheritance and evolution. B-chromosomes appear only in some individuals of some populations of the same species. Their presence is considered to be beneficial, harmful or neutral, and several authors consider B-chromosomes as parasitic and selfish (reviewed in Camacho 2004).

Small chromosomes additional to the standard complements and interpreted as B-chromosomes have been found in the leafhoppers *Alebra albostriella* Fallen and *A. wahlbergi* Boheman (Kuznetsova et al. 2013) and in several species of planthoppers (Halkka 1959, Booij 1982, den Bieman 1988, Kirillova and Kuznetsova 1990). Males of *A. albostriella* and *A. wahlbergi* were collected from a range of food plants in different localities in Greece. B-chromosomes were found in 3 out of 6 populations of *A. albostriella* and in 2 out of 7 populations of *A. wahlbergi.* A single B chromosome or sometimes two B-chromosomes were present in males. As is often the case, B-chromosomes were significantly smaller than chromosomes of the standard complements and negatively heteropycnotic during meiotic prophases and metaphases. No correlation was found between the occurrence and frequencies of B-chromosomes in populations with habitat or altitude (Kuznetsova et al. 2013).

It is suggested that inter-population differences in B chromosome distribution depend on selective factors (Camacho 2004). It is interesting to note in this connection that in the planthopper species *Javesella pellucida* Fabricius, *Criomorphus borealis* Sahlberg, and *Saccharosydne procera* Matsui (Delphacidae), B-chromosomes were present only in populations inhabiting Northeast Siberia and Kamchatka, whereas individuals sampled from different populations in European Russia (e.g. those of *J. pellucida*) lacked B-chromosomes (Kirillova and Kuznetsova 1990). The point that should be mentioned is that both these planthopper species and aforesaid *Alebra* Fieber leafhoppers showed no more than 1 or 2 B-chromosomes per individual. One must suggest that this number is tolerable for B chromosome carriers and the natural selection operates to eliminate individuals with more than two B-chromosomes in all these populations.

The commonly accepted view is that B-chromosomes are derived from the standard complement of a species, including the X chromosome (Camacho 2004). On the other hand, the possibility of integrating a B chromosome into the standard chromosome complement has been suggested in a number of studies (reviewed in Nokkala et al. 2000). Recently, it has been claimed that the achiasmate Y chromosome in *Drosophila*

Fallén (Diptera) might have evolved from a B chromosome (Carvalho 2002). To explain the formation of the achiasmate Y chromosome in separate species of psyllids (a related group sharing predominant X(0) sex determining system with Auchenorrhyncha), Nokkala et al. (2000, 2003) suggested that the Y chromosome has evolved from a mitotically stable B chromosome that was integrated into an achiasmate segregation system with the X chromosome. Later, this chromosome would become fixed in the karyotype as the Y chromosome. In this connection, it should be noted that at metaphase I of some delphacid species, a B chromosome appeared closely associated with the univalent X chromosome forming a pseudo-bivalent XB (Kirillova and Kuznetsova 1990). The X and B-chromosomes tended to segregate (i.e. pass to opposite poles) at anaphase I suggesting thus an increasing transmission of B-chromosomes to sons while a decreased transmission to daughters. This means that the B-chromosomes are able to spread through the male line, whereas are removed from the female line. In this connection, it is interesting to note that a Y chromosome of unknown origin has been described in several species of the family Fulgoridae (Kuznetsova 1986, Kuznetsova et al. 2010, see also section "**Sex determining systems"**). In these species, the XY chromosome pair was located outside the autosomal bivalents in the place where a univalent X chromosome is usually located in the X(0) auchenorrhynchan species. The XY pair differed distinctly from a neo-XY bivalent (characteristic, for example, of the closely related to Fulgoridae family Dictyopharidae) by its morphology and location at metaphase I. On the other hand, this XY pair appears to be identical to the XB pair described by Kirillova and Kuznetsova (1990) in *J. pellucida*, *C. borealis*, and *S. procera*. According to Nokkala et al. (2003: 331), "the evolutionary dynamics of B-chromosomes, that is, the ability to transform into A chromosomes or vice versa, might have played a much more important role in the evolution of karyotypes than previously understood".

Noteworthy is the different behaviour of B chromosomes in the leafhoppers *Alebra albostriella* and *A. wahlbergi* (Kuznetsova et al. 2013). In these species, B-chromosome(s) did not connect to the univalent X chromosome at MI, and when there were two B chromosomes in a set, they did not pair and passed randomly through meiosis as univalents, being still maintained in the populations by unknown means.

Other cases of polymorphism

Fission and fusion of holokinetic chromosomes do not result in unbalanced meiotic products, and so these rearrangements may be preserved through generations and establish variations in chromosome number within populations. Yet, descriptions of chromosomal polymorphisms are quite rare in Auchenorrhyncha. One can anticipate that it is due to very few studies at the population level in this group. However some chromosomal polymorphisms (other than polymorphism for B-chromosomes) do occur in natural populations of leafhoppers and planthoppers.

Polymorphism for sex chromosomes. Some cases of sex chromosome polymorphism were discovered in the leafhoppers *Austragalloides* sp. (Whitten 1968), *Parabo-*

locratus albomaculatus Distant (Manna and Bhattacharya 1973), *Oncopsis tristis* Zetterstedt, and *O. flavicollis* (Linnaeus) (John and Claridge 1974), as well as in the planthoppers *Dicranotropis hamata* Boheman (Delphacidae) and *Repetekia orbicularis* Oshanin (Dictyopharidae) (Halkka 1959, Kuznetsova 1986). In *P. albomaculatus* from India, the males were found to be dimorphic in sex chromosome constitution. Out of 30 males studied, 16 were of an X(0) type (designated as Type A), while 14 were of an XY type (designated as Type B). Type A was characterized by 17 chromosomes in the spermatogonial complement, with a pair of conspicuously large autosomes and a single medium-sized X chromosome. Type B displayed 2n = 18 with X being the largest, Y relatively small, and the pair of large autosomes present in Type A was absent. The sex chromosomes in Type B were suggested to have originated as neo-X and neo-Y by the X-autosome translocation from Type A (Manna and Bhattacharya 1973).

A very interesting example of sex chromosome polymorphism was revealed by John and Claridge (1974) in British populations of *Oncopsis flavicollis*. In this species, mountain populations occurring on *Betula pubescens* Ehrhart were X(0)-monomorphic, whereas populations in lowland woodlands were polymorphic, containing a mixture of X(0) and neo-XY males in the same or different populations.

Halkka (1959) studied a Finnish population of *Dicranotropis hamata* and found that some males in this population displayed a Y chromosome, while others did not. He suggested that the absence of Y chromosome in these males was a result of its loss, the Y being still inherited in part of the population.

Polymorphism for autosomes. Some impressive cases of a fission/fusion polymorphism for autosomes have been described in the Australian leafhopper species *Deltocephalus longuinquus* Kirkaldy (Whitten 1965) and *Alodeltocephalus draba* Evans (Whitten and Taylor 1968), as well as in Greek populations of *Alebra albostriella* and *A. wahlbergi* (Kuznetsova et al. 2013). In eight studied populations of *Alodeltocephalus draba*, specimens appeared invariable in having one of the following four chromosome complements: (1) three bivalents + X, (2) four bivalents + X, (3) two bivalents + one trivalent + X, and (4) one bivalent + one tetravalent + X. The reduction in the number of chromosomes has reached different stages in different areas. At Lake Pedder, the chromosome number was almost fixed (2n = 7, three bivalents + X), while at Bruny Island, there occurred a cline in chromosome number decreasing from north to south. This cline was caused by differences in the frequency of chromosome fusions. *A. draba* was suggested to be under a process of speciation driven by the reorganization of chromosomes that is initiated in some local populations through the fixation of a particular chromosome rearrangement (Whitten and Taylor 1968).

The brown planthopper *Nilaparvata lugens* Stål (Delphacidae) is the only auchenorrhynchan species studied cytogenetically both from natural populations and laboratory cultures. It is notable that natural populations of this species across a wide geographic range revealed almost no instances of chromosomal polymorphism (den Hollander 1982), whereas males from the stock cultures showed a great amount of polymorphism (Liquido 1986, Goh et al. 1992). These differences in the level of chromosomal polymorphism between natural populations and laboratory cultures deserve further investigation.

Meiosis in males and females

Meiosis in normal spermatogenesis. Within Hemiptera, some very interesting and highly aberrant chromosome cycles and anomalous types of meiosis occur in aphids, scale insects, whiteflies, and true bugs, including moss bugs (Coleorrhyncha) (White 1973, Blackman and Hales, 1986, Normark 2003, Papeschi and Bressa 2006, Kuznetsova et al. 2011, 2015b). In contrast to all these insects, meiosis in Auchenorrhyncha is essentially simple and uniform in different species and follows the classical "pre-reductional" scheme: during first meiotic division homologous chromosomes undergo pairing, synapsis and recombination at prophase I and segregation at anaphase I. As with autosomes, sex chromosomes undergo pre-reductional meiosis. During second division, sister chromatids separate and migrate to opposite poles at anaphase II creating then haploid daughter cells.

The number of chiasmata in bivalents. It is common knowledge that in meiosis, chiasmata (presumed to be the points of genetic crossing-over) are formed uniting homologous chromosomes together until their separation in the reductional division. In most organisms there are one to three chiasmata per bivalent, although in some organisms the number of chiasmata in a bivalent (i.e. the chiasma frequency) varies considerably being typically higher in plants than in animals (White 1973). Halkka (1964) analyzed the number of chiasmata in males and females of species belonging to six families of Auchenorrhyncha, namely, Cicadellidae, Cixiidae, Delphacidae, Issidae, Cercopidae, and Membracidae. In all species, bivalents were found to display one or occasionally two chiasmata, and no great differences in chiasma frequencies were detected between males and females, as well as between leafhoppers and planthoppers.

Similarly, the low number of chiasmata (estimated to be 1-2 from cytogenetic analyses) is a rule in psyllids (Maryańska-Nadachowska 2002) and true bugs (Kuznetsova et al. 2011) and is suggested to represent one of the peculiar features of holokinetic bivalents as such (Nokkala et al. 2004). Based on a detailed analysis of meiosis in a psyllid species, *Beopelma foersteri* Flor, Nokkala et al. (2004) concluded that the cells carrying more than two chiasmata were inevitably eliminated creating thus a strong selection against the formation of multiple chiasmata in holokinetic bivalents. The main cause of this pattern was suggested to be a specific condensation process inherent to holokinetic chromosomes. It is worth noting that bivalents with multiple chiasmata have been observed occasionally in holokinetic groups, including Auchenorrhyncha (see for references Kuznetsova et al. 2009b); however, these observations never advanced beyond metaphase I of spermatogenesis, and therefore, the further fate of the cells with multichiasmate bivalents remained unknown.

Meiosis in normal oogenesis. In comparison to the rather abundant data available on male meiosis in Auchenorrhyncha, there have been no comprehensive investigations of chromosome behaviour in female meiosis. The only exceptions are the few descriptions of meiosis in parthenogenetic forms (see section "**Polyploidy"**) and the studies done by Halkka (1959, 1964) on female meiosis of several bisexual species. First, Halkka (1964) provided evidence for the low chiasma frequencies in females.

Second, he revealed that females of the leafhopper species *Athysanus argentarius* Metcalf displayed pre-reductional meiosis both for autosomes and sex chromosomes and one chiasma per bivalent (in contrast to two chiasmata in males) (Halkka 1959).

Meiotic abnormalities. It is to be noted that the apparent uniformity of meiosis in Auchenorrhyncha could be due to the small number of species which have been studied in any detail. The incidence of meiotic abnormalities and their relationship with different spermatogenic parameters was assessed in the leafhopper species *Alebra albostriella* and *A. wahlbergi* (Kuznetsova et al. 2013). Several isolated populations of these species in Greece sampled from different food plants, such as *Castana sativa* Mill., *Fagus sylvatica* L., *Quercus cerris* L., *Acer opalus* Mill., and *Ulmus* spp., showed a great deal of meiotic abnormalities in males, including end-to-end non-homologous chromosomal associations, heterozygous translocation chains, univalents, anaphasic laggards, and aberrant sperms. The primary causes of abnormal chromosome behavior in studied populations, whether those are male-specific meiotic mutations or some environmental mutagens, remained unknown. Also it is not known whether these meiotic abnormalities may play a role in the genome diversity and karyotype evolution of the genus *Alebra*. The resolution of the issues will have to await further studies (Kuznetsova et al. 2013).

New approaches to cytogenetic studies

White (1978) estimated that over 90% of all speciation events are accompanied by karyotypic changes. Current evidence shows that chromosome numbers in Auchenorrhyncha are quite often remarkably conservative within a group despite the theoretical capacity of holokinetic chromosomes for fusion and fragmentation. It is to be noted, however, that, in general, cytogenetic studies of Auchenorrhyncha use standard techniques, providing evidence for chromosome numbers, sex chromosome mechanisms, and, in outline, the behaviour of chromosomes during meiosis. Nevertheless, for a student of auchenorrhynchan cytogenetics (as well as for an investigator of any other holokinetic group), the main challenge is the identification of individual chromosomes and chromosomal regions. This information would result in considerable progress in the field because it will allow identification of the interchromosomal and, what is more important, intrachromosomal rearrangements involved in the evolution of holokinetic organisms.

Chromosome banding is a staining technique to reveal differentiation within chromosomes as a series of reproducible cross-bands. Besides the identification of individual chromosomes in a karyotype, the bands tell a good deal about fundamental aspects of the chromatin organization and compartmentalization of the genome. These techniques have had an invaluable impact on plant and animal cytogenetics but still are very little used in Auchenorrhyncha. In this group, a number of studies have applied some conventional techniques, such as C-banding, AgNOR-banding, and DNA base specific fluorochrome-banding. C-banding characteristically reveals the extent and location of heterochromatic segments (C-bands), which contain highly

condensed, repetitive and largely transcriptionally silent DNA. Fluorochrome-banding mainly involves GC-specific antibiotic chromomycin A_3 (CMA_3) and AT- specific 4-6-diamidino-2-phenylindole (DAPI) to detect variation in base composition along the chromosomes. AgNOR-banding reveals the nucleolus organizer regions (NORs), containing the genes that code for ribosomal RNA. These techniques have proved their utility for comparative purposes at the generic level. For example, the C-banding technique showed that taxonomically related species sharing the same chromosome number differ often in chromosome constitution due basically to the accumulation of many rearrangements since divergence from the common ancestor. For instance, differences in C-banding pattern were described between the delphacids *Nilaparvata lugens* and *Calligypona pellucida* Horváth (Noda and Tatewaki 1990), between the cicadas *Tibicen bihamatus* Motschulski and *Platypleura kuroiwae* Matsumura (Perepelov et al. 2002), between the species of the spittlebug genus *Philaenus* (Kuznetsova et al. 2003, Maryańska-Nadachowska et al. 2008, 2012), and between the species of the family Issidae (Kuznetsova et al. 2009b, 2010). The issid species *Hysteropterum albaceticum* Dlabola and *Agalmatium bilobum* Fieber, both with 2n = 26 + X(0) in males, were shown to differ considerably in the amount of C-heterochromatin, which appeared clearly more abundant in the first of these species. The species differed also in C-heterochromatin distribution along the karyotypes and its ability to stain with DAPI and CMA_3 (Kuznetsova et al. 2009b). On the other hand, the application of AgNOR-banding showed that *H. albaceticum* and *A. bilobum* were similar in having NORs located sub-terminally in the largest pair of autosomes. It should be noted, that such a location of NORs seems to represent the most common pattern in Auchenorrhyncha as a whole (Kuznetsova et al. 2003, 2009b, 2010, Maryańska-Nadachowska et al. 2006, 2012).

In the last few decades, the ability to identify individual chromosomes in a karyotype has been markedly improved by the development of molecular cytogenetic techniques. These include, for example, fluorescence *in situ* hybridization (FISH) to locate the positions of different genes and specific DNA sequences on chromosomes, comparative genomic hybridization (CGH) for analyses of genome homology, genomic *in situ* hybridization (GISH) to identify alien chromosomes or segments, and immunofluorescence to detect the location and relative abundance of the proteins. Some of these techniques have been applied to economically important holokinetic species (Mandrioli et al. 2003, Mandrioli and Borsatti 2007, Marec et al. 2010, Grozeva et al. 2010, 2015), but have not yet been developed specifically for Auchenorrhyncha. The only exceptions are the Southern hybridization of genomic DNA with a telomeric probe and FISH of chromosomes with telomeric and ribosomal probes, which have been applied successfully to several auchenorrhynchan species (Frydrychová et al. 2004, Maryańska-Nadachowska et al. 2013, Golub et al. 2014, Kuznetsova et al. 2015a).

Telomeres are defined as the regions of the chromosomal ends that are required for complete replication, meiotic pairing, and stability of a chromosome (Zakian 2012). The molecular structure of telomeres is characterized by a tandem repeat of a short DNA sequence that is diversely differentiated in eukaryotes. Comparative

analysis of these repeats (motifs) in various groups of organisms showed that they are evolutionarily stable, and, having once appeared during evolution, define taxa and phylogenetic branches of high rank (Traut et al. 2007). Frydrychová et al. (2004), using the Southern Hybridization technique, demonstrated the presence of telomeric TTAGG sequences in the genome of *Calligypona pellucida* (Delphacidae). However, this technique is capable to reveal a sequence but not its chromosomal location within a genome. In contrast, *in situ* hybridization (FISH) is a technique that allows precise localization of specific DNA sequences on chromosomes.

Maryańska-Nadachowska et al. (2013) and Golub et al. (2014) pioneered in applying FISH to Auchenorrhyncha. *In situ* hybridization with the telomeric (TTAGG) and 18S rRNA gene probes was used to study eight species of the genus *Philaenus*, Aphrophoridae (Maryańska-Nadachowska et al. 2013) and *Mapuchea chilensis*, Myerslopiidae (Golub et al. 2014). In most eukaryotic genomes, ribosomal DNA (rDNA) consists of tandemly repeated arrays of three genes (18S, 5.8S, and 28S) encoding nuclear rRNA and separated by internal spacers (Hillis and Dixon 1991). These arrays make up the nucleolus organizing regions (NORs) and can be found clustered in one or several regions of the genome. First, the telomeric repeat probe confirmed that the chromosome ends of *Philaneus* spp and *M. chilensis* are composed of the $(TTAGG)_n$ nucleotide sequence, a common motif of insect telomeres. This motif was reported in the vast majority of evolutionary lineages in Arthropoda and is suggested to represent an ancestral sequence of telomeres in insects (Sahara et al. 1999, Frydrychova et al. 2004, Lukhtanov and Kuznetsova 2010). Second, the 18S rRNA gene probe showed that in *M. chilensis* 18S rDNA loci are placed on a medium-sized pair autosome and that *Philaneus* species differ from one another in both number and location of major ribosomal gene loci in their karyotypes. Thus, the application of *in situ* hybridization technique to *Philaenus* species showed an extensive reorganization of their genomes: the ribosomal genes changed repeatedly their relative position along the chromosomes indicating that a large number of rearrangements probably occurred during or soon after the species formation.

Evolutionary relationships revealed from chromosome data

Given that chromosomes represent morphology at small scale, they can be used in phylogenetics in the same way as other morphological characters and can contribute to clarifying the systematics and phylogeny of a particular group.

Chromosome data have contributed to establishing the evolutionary relationships in several different ways which, except for rare occasions (e.g. Blackman 1980, Emeljanov and Kirillova 1990, 1992, Gokhman 2006, Angus and Tatton 2011; for some interesting references see also Lukhtanov and Kuznetsova 2010), are usually ignored by entomologists. As in other insect groups, cytogenetic data have been applied at both higher and lower rank levels in taxonomic and/or phylogenetic studies in Auchenorrhyncha.

Evolution at and above family level. Cytogenetic data placed in a phylogenetic context can provide insights into chromosome evolution within a higher rank taxon.

A number of successful examples of this approach have been made in Auchenorrhyncha (e.g., Emeljanov and Kirillova 1990, 1992, Kuznetsova et al. 2009a, Maryańska-Nadachowska et al. 2012). One of those deals with the planthopper subfamily Orgeriinae (Dictyopharidae). This group comprises 192 species in 37 genera of four tribes: the Palaearctic ones Ranissini (7 genera, 43 species), Colobocini (1 genus, 1 species), and Almanini (20 genera, 104 species), and the Nearctic tribe Orgeriini, with 37 species in 10 genera (Emeljanov 1980, Emeljanov et al. 2005). For construction and substantiation of the phylogeny of Orgeriinae, three types of characters were used: morphological (Emeljanov 1980), incomplete cytogenetic (Kuznetsova 1986, Kuznetsova et al. 2009a), and preliminary molecular (Emeljanov et al. 2005). As a whole, chromosome complements of 30 species (more than 15% of the total species recognized in Orgeriinae), belonging to 17 genera (almost one-half of the total) and all tribes, except for the African monotypic tribe Colobocini, are currently known. Chromosome numbers being combined with some anatomical data (testis structure in terms of the number of testicular follicles) provided a strong support for the monophyly of Orgeriinae and the recognition of two tribes, Ranissini and Almanini. All Ranissini were shown to have 26 autosomes, a simple sex chromosome mechanism of an X(0) type (2n = 26 + X), and male testes each composed of 6 follicles. On the other hand, species belonging to Almanini were described as having a pair of autosomes less, a secondary neo-XY sex determining system (2n = 24 + neo-XY), and 4 follicles per male testis. The karyotype of Ranissini was suggested to have evolved by the fusion of two autosome pairs in an ancestral karyotype of 2n = 28 + X(0) (inherent to the second subfamily Dictyopharinae). The karyotype of Almanini, in its turn, had originated from that of Ranissini by an X-autosome fusion. Besides the gradual reduction of the total number of autosome pairs, there is apparently a trend towards the reduction of the number of testicular follicles in the evolution of Dictyopharidae. Thus, karyotype and testis structure both suggest a "basal" position of Ranissini within Orgeriinae. The states of these characters encountered in Almanini (2n = 24 + neo-XY and 4 follicles) are treated as being derived from those of Ranissini (2n = 26 + X and 6 follicles), which is in good agreement with the morphological data (Emeljanov 1980). The point of interest is the tribe Orgeriini. The latter shows a number of morphological apomorphies and, therefore, is considered to be one of the most advanced tribes within Orgeriinae (Emeljanov 1980, Emeljanov et al. 2005). Despite of this, three recently studied species of Orgeriini (*Orgerius rhyparus* Stål, *O. ventosus* Ball & Hartzell, and *Deserta bipunctata* Ball) were found to share the same karyotype and testis structure as the basal tribe Ranissini (Kuznetsova et al. 2009a).

Additional examples showing the significance of chromosome data for the systematics and phylogenetics of Auchenorrhyncha are given below. As noted above, Fulgoromorpha differ distinctly from Cicadomorpha in chromosome numbers (Fig. 8). The planthopper families Fulgoridae and Dictyopharidae are also a good case in point. Emeljanov (1979) identified a number of important morphological differences that support the discreteness of these families. Fulgoridae and Dictyopharidae are also distinguished by the morphology and the assumed origin of their Y chromosomes

(Kuznetsova et al. 2009a; see also sections **"Sex determining systems"** and "**Polymorphism for B-chromosomes"**). Opinions vary broadly on the phylogenetic position of the planthopper family Tettigometridae. Although this family is mainly accepted as the most basal one within Fulgoroidea, some morphological and molecular evidence suggest that it is a relatively derived lineage among fulgoromorphans (see for references Yeh et al. 2005 and Urban and Cryan 2007). The currently available chromosome data seem to be consistent with this opinion: all hitherto studied species of Tettigometridae display the evolutionarily derived neo-XY sex chromosome system (Kuznetsova and Kirillova 1990, Kirillova 1993).

Evolution below family level. Cytogenetic data also provide useful information about lower rank taxonomic relationships. For example, in the leafhopper *Rhopalopyx preyssleri* Herrich-Schäffer, Halkka (1959) discovered two types of populations in Finland, i.e. with 2n = 14 + X and with 12 + XY in males respectively. He treated these differences in terms of a common polymorphism for an X-autosome translocation resulting in the formation of a neo-XY system. However, shortly afterwards, Vilbaste (1962) showed that the putative "12 + XY race" of *R. preyssleri* was in fact *R. adumbrata* Sahlberg.

Whitten (1965) described variation in chromosome number in different populations of a leafhopper species in Australia. An examination of male genitalia by J.W. Evans revealed that two morphologically distinct species were present. The 2n=11 group belonged to a species which, subsequent to Whitten's (1965) paper, was separated from the genus *Deltocephalus* as *Alodeltocephalus longuinquus* Kirkaldy, while the remaining chromosomal groups were morphologically uniform and all included in the new species, *A. draba* (Evans 1966).

The meadow spittlebug genus *Philaenus* (Aphrophoridae) is likewise a good example. This genus has been studied using morphological (Drosopoulos and Remane 2000, Drosopoulos 2003), molecular (Maryańska-Nadachowska et al. 2010, Seabra et al. 2010) and cytogenetic (Kuznetsova et al. 2003, 2015a, Maryańska-Nadachowska et al. 2008, 2012, 2013) techniques. Numerous studies have explored the outstanding colour polymorphism and systematics of this genus (e.g. Drosopoulos and Remane 2000, Drosopoulos 2003). A total of eight *Philaenus* species are presently recognized, including the Mediterranean species *P. tesselatus* Melichar, *P. loukasi*, *P. arslani* Abdul-Nur & Lahoud, *P. signatus* Melichar, *P. maghresignus* Drosopoulos & Remane, *P. tarifa* Remane & Drosopoulos, and *P. italosignus* Drosopoulos & Remane, and the Holarctic species *P. spumarius* Linnaeus (Drosopoulos and Remane 2000). Based on morphology, the genus is currently divided into two groups: the "*spumarius*" species group and the "*signatus*" species group (Drosopoulos and Remane 2000), whereas based on larval food plant preferences, the genus is divided into three ecological groups: developing on the lily *Asphodelus aestivus* Brot., on xerophilic plants, and on various dicotyledonous and monocotyledonous plants (Drosopoulos 2003).

The results of a recent phylogenetic study of *Philaenus* using nucleotide sequences from two mitochondrial (*COI* and *CytB*) genes and one nuclear (*ITS2*) region are in general agreement both with the morphological and the food plant preferences

groupings (Maryańska-Nadachowska et al. 2010). Likewise, differences in karyotype were found to be largely in agreement with the recognized groupings proposed on the basis of morphology and on the basis of larval food plant relationships. Cytogenetic analysis has revealed that a number of *Philaenus* species share the same karyotype while some others differ in chromosome number, sex chromosome system and additional cytogenetic characters. The species feeding on *A. aestivus* were shown to have 2n = 22 + neo-XY (*P. signatus*, *P. maghresignus* and *P. tarifa*) or 2n = 20 + neo-neo-X_1X_2Y (*P. italosignus*). These species are included into the species group "*signatus*". Among the species of the "*spumarius*" group, *P. loukasi* and *P. arslani*, with larvae developing on arid plants, share 2n = 18 + neo-XY, whereas *P. tesselatus* and the polyphagous species *P. spumarius*, feeding on a wide range of dicotyledonous plants, possess 2n = 22 + X(0). It has been postulated that the ancestral karyotype of *Philaenus* is 2n = 24 + X(0) and that karyotype changes occurred several times independently in the genus (Maryańska-Nadachowska et al. 2012, 2013).

In conclusion, it may be said that one of the most important ways of increasing the taxonomic and phylogenetic inferences based on chromosome data is to enlarge sampling of taxa. Considerable progress in our understanding of the cytogenetics of Auchenorrhyncha will come from the development and application of new molecular cytogenetic techniques, which appear clearly advantageous for revealing important markers in holokinetic chromosomes. These techniques are expected to provide useful insights into the genome constitution and mechanisms of karyotype evolution in this large group of Hemiptera.

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Achiasmate male meiosis in two *Cymatia* species (Hemiptera, Heteroptera, Corixidae)

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Abstract

The karyotype and male meiosis, with a particular focus on the presence or absence of chiasmata between the homologs, were studied in the water boatman species *Cymatia rogenhoferi* (Fieber) and *C. coleoptrata* (Fabricius) (Corixidae, Cymatiainae). It is shown that the species have 2n = 33 (28A+2m+X_1X_2Y) and 2n = 24 (20A+2m+XY) respectively, post-reduction of sex chromosomes, and achiasmate meiosis of an alignment type in males. Cytogenetic and some morphological diagnostic characters separating *Cymatia* Flor from the rest of Corixidae are discussed.

Keywords

karyotype, m-chromosomes, sex chromosome post-reduction, spermatocyte meiosis, morphology, Nepomorpha, Corixoidea

Introduction

The Corixoidea, known as water boatmen, are moderately large to small aquatic insects, belonging to the true bug infraorder Nepomorpha. According to Schuh and Slater (1995), Corixoidea include the only family Corixidae, with six subfamilies: Corixinae, Cymatiainae, Diaprepocorinae, Heterocorixinae, Stenocorixinae, and Micronectinae. Nieser (2002a, b) raised Diaprepocorinae and Micronectinae to a family rank, meaning that Corixoidea is comprised of three families only: Corixidae, Micronectidae, and Diaprepocoridae. The validity of Nieser's (2002b) system was criticised by Andersen and Weir (2004), but accepted by the majority of other authors (Tinerella 2008, Grozeva et al. 2008, Konopko et al. 2010, Weirauch and Schuh 2011, Fent et al. 2011). Chromosome data is currently available for Micronectidae as well as for the corixid subfamilies Corixinae and Cymatiainae. In Micronectidae all four hitherto studied species were reported to have achiasmate male meiosis and no m-chromosomes: three species of *Micronecta* Kirkaldy, 1897 display 2n = 24 (22A+XY), while one species of *Tenagobia* Bergroth 1899 has 2n = 30 (28+XY) (Ituarte and Papeschi 2004, Grozeva et al. 2008). In Corixinae, all 30 studied has species from eight genera were shown to share common characteristics in males: chiasmate meiosis, sex chromosome post-reduction, presence of a pair of m-chromosomes, and karyotype with 2n = 24 (20+2m+XY) (Ueshima 1979, Waller and Angus 2005, Bressa and Papeschi 2007). Cymatiainae consist of two genera, *Cymatia* Flor, 1860 with dispersed Holarctic and Oriental distributions and the monotypic *Cnethocymatia* Jansson, 1982 from northern Australia and New Guinea (Štys and Jansson 1988). For the only studied species of Cymatiainae, *Cymatia bonsdorffi* (Sahlberg, 1819), the karyotype with 2n = 26 (24 + XY) was reported with no information on m-chromosomes and presence/absence of chiasmata in male meiosis (Slack 1938, Southwood and Leston 1959).

In meiosis, the chiasmata are known to tie homologous chromosomes together until their separation in the reductional division. However, in some animal groups, instead of chiasma formation, an achiasmate type of meiosis is observed, being, as a rule, restricted to the heterogametic sex (White 1973). In true bugs, when achiasmate meiosis presents, it seems to be stable and marks taxa at the rank of family (Grozeva et al. 2008a). Until the present time, this meiotic pattern has been found in seven families of Heteroptera, belonging to the infraorders Nepomorpha, Leptopodomorpha and Cimicomorpha (see Kuznetsova et al. 2011 for references).

In the present paper, the karyotype and male meiosis of other two *Cymatia* species, *C. rogenhoferi* (Fieber, 1864) and *C. coleoptrata* (Fabricius, 1777), were studied. The focal point of this work was to clarify the presence or absence of chiasmata in spermatocyte meiosis of these species.

Material and methods

Five males of *Cymatia rogenhoferi* and two males of *C. coleoptrata* were collected by light trap and hydrobiological net in different localities (Table 1). Males of *C. coleoptrata* were fixed in 3:1 fixative (96% ethanol:glacial acetic acid mixture) in the field

Table 1. Material used for chromosome analysis

Species	Number of analysed males	Locality and date of collection
Cymatia rogenhoferi	5	Kazakhstan, Taukum Sands, near Topar River, eastern from Topar Village, 363m a. s. l., 45°02'12"N, 074°58'33"E, light trap, 31.05.2015, leg. N. Simov and F. Konstantinov
C. coleoptrata	1	Bulgaria, Danube River, marsh Malak Preslavets, 20m a. s. l., 44°05'43"N, 026°50'23"E, 13.07.2014, leg. D. Stoianova
C. coleoptrata	1	Bulgaria, Danube River, Srebarna lake, 13m a. s. l., 44°06'47"N, 027°03'34"E, 12.07.2014, leg. D. Stoianova

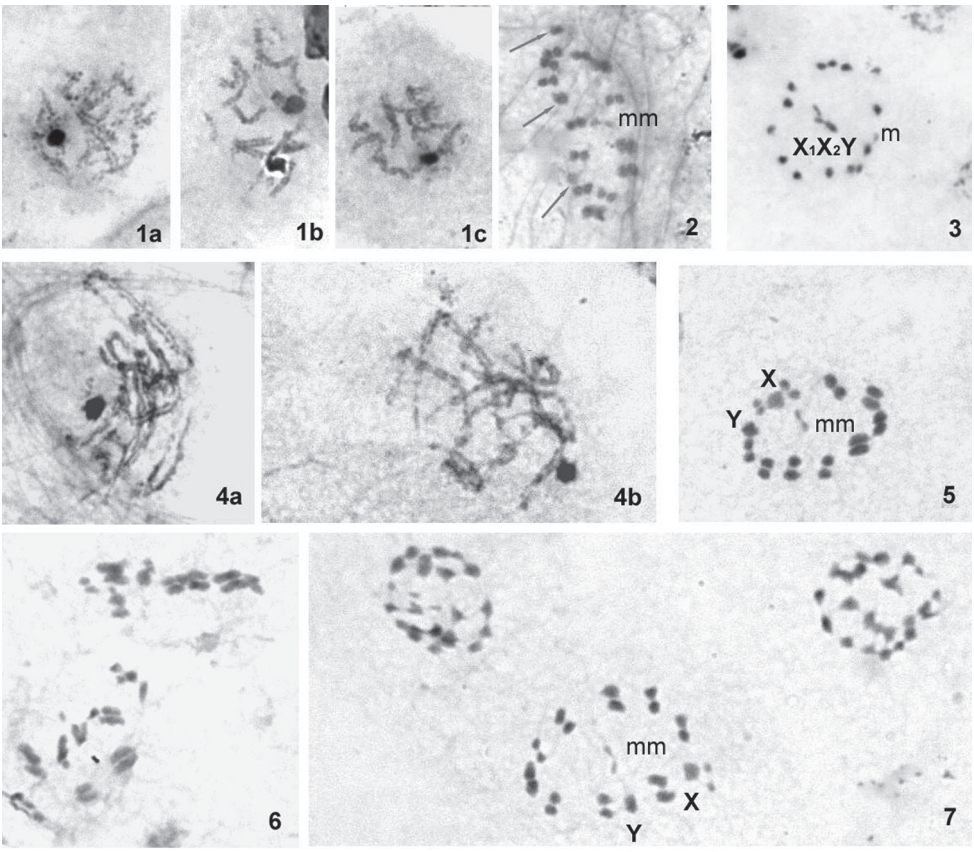
immediately after capturing. Males of *C. rogenhoferi* were fixed in the field in 95% ethanol for subsequent sequencing, and the abdomen was transferred in 3:1 fixative for chromosome analysis, as it has been done recently by Nokkala et al. (2015) for *Cacopsylla myrtilli* (W. Wagner, 1947) (Psylloidea). The gonads were dissected out and squashed in a small drop of 45% acetic acid. The cover slip was removed using dry ice. Slides were dehydrated in fresh fixative (3:1) and air dried. The preparations were stained using Schiff-Giemsa method (Grozeva and Nokkala 1996).

The chromosomes were analysed under light microscope (Axio Scope A1 – Carl Zeiss Microscope) at 100x magnification and documented with a ProgResMFcool – Jenoptik AG digital camera. All preparations and remains of the specimens are stored at the Institute of Biodiversity and Ecosystem Research (IBER), BAS in Sofia, Bulgaria.

Results

Cymatia rogenhoferi, 2n = 33 (28A+2m+X_1X_2Y)

The testes of the adult males were full of sperm, with a small number of well-synchronised dividing cells. No spermatogonial metaphases were observed. When condensing from a diffuse stage (Figs 1a, b, c), the autosomal bivalents consisted of side-by-side aligned homologous chromosomes without chiasmata, and the sex chromosomes appeared as a positively heteropycnotic body. No diplotene and diakinesis were present. At metaphase I (MI), the bivalents laid parallel to the equatorial plane, with the homologous chromosomes facing opposite poles without any sign of chiasmata. Clearly, male meiosis of this species is achiasmate. Both MI and MII were radial (Figs 2, 3). At MI, 14 autosomal bivalents and three univalent sex chromosomes (two X and one Y) formed a ring with, a pair of very small and negatively heteropycnotic m-chromosomes inside. In contrast to MI, the MII ring was formed by 14 autosomes and one m-chromosome, while the sex chromosomes formed a pseudo-trivalent placed inside the ring. The Y chromosome was clearly larger than each of the two X chromosomes (Fig. 3). The first division was thus reductional for the autosomes and m-chromosomes, but equational for the sex chromosomes (post-reduction). The chromosome formula of *C. rogenhoferi* was determined as 2n = 33 (28A+2m+X_1X_2Y).



Figures 1–6. Male meiosis in *Cymatia* species. **1–3** *C. rogenhoferi*: **a–c** early condensation stages **2** MI from the pole. The bivalents (consisting of two side-by-side aligned chromosomes facing the opposite poles) and three univalent sex chromosomes (two X and one Y) form a ring, with a pair of very small and negatively heteropycnotic m-chromosomes in its centre **3** MII. The autosomes and m-chromosome form a ring, with pseudo-trivalent of the sex chromosomes in its centre **4–7** *C. coleoptrata*: **a, b** early condensation stages **5** MI from the pole. The bivalents (consisting of two side-by-side aligned chromosomes) and two univalent sex chromosomes (X and Y) form a ring, with a pair of very small and negatively heteropycnotic m-chromosomes in its centre **6** MI from the equator.The homologous autosomes can be seen lying parallel **7** late MI and AI plates. Scale bar = 10 μm.

C. coleoptrata, 2n = 24 (20A+2m+XY)

The behaviour of chromosomes during the first spermatocyte division was quite similar to that in *C. rogenhoferi*. Unfortunately, we found no second division stages in the two males explored here. When condensing from the diffuse stage (Figs 4a, b), the autosomal bivalents consisted of parallel aligned homologous chromosomes without traces of chiasmata, and the sex chromosomes appeared as a positively heteropycnotic body. No diplotene and diakinesis were observed. At metaphase I (MI), the bivalents were aligned parallel to the equatorial plane, with the homologous chromosomes facing op-

posite poles without any signs of chiasmata. The MI plates were radial (Fig. 5), with 10 autosomal bivalents and two univalent sex chromosomes (X and Y) forming a ring and a pair of very small and negatively heteropycnotic m-chromosomes placing inside it. The X chromosome was larger than the Y. Side by side association of homologous autosomes was still preserved at MI (Figs 5, 6) and anaphase I (AI) (Fig. 7), when the homologs moved in parallel to the opposite poles. Clearly, male meiosis of this species is achiasmate. The first division was reductional for the autosomes and m-chromosomes, but equational for the sex chromosomes (post-reduction).

The chromosome formula of *C. coleoptrata* was determined as 2n = 24 (20A+2m+XY).

Discussion

The main goal of this paper was to address the cytogenetic features of two species of *Cymatia* and compare them with those encountered within the superfamily Corixoidea. This entailed at least four related issues, namely: 1) whether the karyotypes are conservative in respect to chromosome number and sex chromosome system, 2) whether m-chromosomes are present, 3) whether the post-reduction of sex chromosomes is present, and 4) whether the chiasmata are formed in male meiosis. Both Corixidae and Micronectidae are known to be characterised by an XY sex chromosome system and an inverted sequence of X and Y chromosome divisions in spermatocyte meiosis, i.e. the sex chromosome post-reduction (for references see Ueshima 1979, Ituarte and Papeschi 2004, Waller and Angus 2005, Bressa and Papeschi 2007, Grozeva et al. 2008). Post-reduction means that the sex chromosomes behave as univalents during the first round of meiosis and undergo equational separation at AI whereas they form a pseudobivalent at MII and undergo reductional segregation at anaphase II. The species here studied, *C. rogenhoferi* and *C. coleoptrata*, were found to share the same characteristics with Corixidae and Micronectidae, i.e. an XY system and the sex chromosome post-reduction in male meiosis. Multiple X_1X_2Y mechanism found in *C. rogenhoferi* might have originated by fragmentation of the initial X chromosome. The inverted sequence of sex chromosome divisions in spermatocyte meiosis is characteristic of the Heteroptera as a whole (Ueshima 1979), with rare exceptions (e.g. Golub et al. 2015). Other cytogenetic features, including chromosome numbers and presence or absence of m-chromosomes, whose origin, nature and significance are questionable (e.g. Nokkala 1986, Kuznetsova et al. 2011), and presence or absence of chiasmata in male meiosis, are distributed variously among different taxa of Corixidae and Micronectidae.

In Corixinae, each of 30 species studied display ten pairs of autosomes, a pair of very small m-chromosomes, and X and Y chromosomes: the karyotype formula of these species can be expressed as 2n = 24 (20A+2m+X+Y). Meiosis is of a standard chiasmate type in males (Ueshima 1979, Waller and Angus 2005, Bressa and Papeschi 2007). Compared to Corixinae, the family Micronectidae is less well studied. The karyotypes are currently known in *Micronecta* (*Dichaetonecta*) *scholtzi* (Fieber, 1860), *M*. (*Micronecta*) *poweri* (Douglas et Scott, 1869), and *M*. (*Micronecta*) *griseola* Hor-

vath, 1899, each with 2n = 24 (22A+XY) (Grozeva et al. 2008), and in *Tenagobia* (*Fuscagobia*) *fuscata* (Stål, 1859), with 2n = 30 (28+XY) (Ituarte and Papeschi 2004). Based on the data available, Micronectidae differ from Corixinae in that they have alternative numbers of autosomes and no m-chromosomes. Furthermore, the species studied in Micronectidae show the achiasmate meiosis in males. *Cymatia rogenhoferi* and *C. coleoptrata* studied in this paper, were found to have 2n = 33 (28A+2m+X_1X_2Y) and 2n = 24 (20A+2m+XY) respectively and achiasmate meiosis of an alignment type in males. In another *Cymatia* species, *C. bonsdorffi* (Sahlberg, 1819), studied by Slack (1938) and later by Southwood and Leston (1959), the karyotype of 2n = 26 (24 + XY) was reported. Unfortunately, the authors provided no information on the special features of meiosis, including sex chromosomes' behaviour. Thus, on the basis of the current state of knowledge, the Cymatiainae share a presence of m-chromosomes with Corixinae, while the absence of chiasmata is shared with Micronectidae. Due to their very small size and negative heteropycnosis during meiosis, m-chromosomes are easily overlooked by researchers, and subsequently information about the distribution of these puzzling structures in different true bug taxa can hardly be used for inferences.

The first (reductional in the majority of organisms) division involves several meiosis-specific events the most important being the formation of chiasmata, the points of genetic crossing-over, between homologous chromosomes. When meiosis is achiasmate and chiasmata are not formed, no diplotene or diakinesis stages can be recognised. The existence of achiasmate meiosis in phylogenetically unrelated true bug families, i.e. Micronectidae from the infraorder Nepomorpha (Ituarte and Papeschi 2004, Grozeva et al. 2008), Saldidae from the Leptopodomorpha (Nokkala and Nokkala 1983), and in several families of the Cimicomorpha (Nokkala and Nokkala 1984, Nokkala and Nokkala 1986a, b, Nokkala and Grozeva 2000, Grozeva and Nokkala 2002), argue for its repeated and independent origin in the evolution of Heteroptera. At the same time, the achiasmate meiosis in true bugs is probably of very ancient origins, since some divergence has occurred in its cytological characteristics during the evolution (Nokkala and Grozeva 2000, Grozeva et al. 2008). Consequently, true bugs evolved a diversity of achiasmate meiosis types that include a variety starting from an *alignment* type to a *colochore* type, including an intermediate type (Nokkala and Nokkala 1983, Nokkala and Nokkala 1984, Nokkala and Nokkala 1986a, b, Kuznetsova et al. 2007). Comprehensive classification of different types of achiasmate meiosis can be found in Kuznetsova et al. (2011). The most common type is achiasmate meiosis of the *alignment* type characterised by the tight side-by-side alignment of homologous chromosomes throughout prophase until MI. Meiosis of this type has been described in the Saldidae, Nabidae, Anthocoridae, Microphysidae, Corixoidea: Micronectidae (for references see Grozeva et al. 2008), and now also in another corixid group, the Cymatiainae (present study).

The Cymatiainae were erected for the first time as a separate taxon (as Cymatiini) in Corixidae on the basis of the shape and hairiness of the pala, the chitinisation of the pharynx, the length of maxillary stylets, and their position against pharynx (Walton in Hutchinson 1940). Later, the labium structure, the position of the labial sensilla, and absence of the strigil and stridulation mechanism, and the ability of sound production,

Table 2. Key diagnostic characters used to distinguish Cymatiainae from the rest of Corixidae

Cymatiainae	Corixidae
Labium without transverse sulcations	Labium with transverse sulcations
Absence of transverse pattern of distribution of the labial sensilla	Transverse pattern of distribution of the labial sensilla
Nodal furrow absent	Nodal furrow present
Pala elongate, nearly cylindrical in both sexes	Female pala spoon-shaped; male pala variable
Pala without pegs	Pala with pegs
Pala in both sexes without palm	Pala in both sexes with palm
Claw of hind leg inserted apically	Claw of hind leg inserted subapically
Absence of seta close to the claw' basis	Presence of seta close to the claw' basis
Strigil absent	Usually with strigil
Unable to stridulate	Stridulation by rubbing peg fields on the anterior femur against the side of the head, females of some species also able to stridulate
Achiasmate male meiosis	Chiasmate male meiosis

as well as some other characters of the pala and embolium (Table 2) were added to the diagnosis (Jansson 1973, 1986, Schuh and Slater 1995, Nieser 2002b, Chen et al. 2005, Hädicke 2012, Brożek 2013a, b, 2014).

In different phylogenetic studies on Corixoidea (Zimmermann 1986, Mahner 1993, Hebsgaard et al. 2004, Hädicke 2012, Brożek 2014) the position of Cymatiainae varies from being considered a sister group of Corixidae *s. str.* (Corixinae + Heterocorixinae) or a basal taxon (together with Diaprepocoridae) in the whole superfamily Corixoidea. It has repeatedly been shown that the absence of chiasmata during spermatocyte meiosis is evolutionarily stable in true bugs, and marks taxa at the rank of family (for references see Grozeva et al. 2008, Kuznetsova et al. 2011). In this context, the finding of achasmate meiosis in Micronectidae (Grozeva et al. 2008) clearly supports the familial status of this group, earlier proposed by Nieser (2002a, b). Both achiasmate meiosis and a number of morphological diagnostic characters (Table 2) distinguish Cymatiainae from the rest of Corixidae. However, more comprehensive studies on morphological and cytogenetic aspects of Corixoidea as a whole are required to decide on the rank that should be assigned to Cymatiainae. The special focus must be on the genus *Cnethocymatia* from the same subfamily, the genus *Diaprepocoris* Kirkaldy, 1897 considered the most basal taxon of Corixoidea, and the genus *Stenocorixa* Horváth, 1926 showing morphological similarities with Cymatiainae (Hebsgaard et al. 2004, Hädicke 2012, Brożek 2014).

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