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Karyotype differentiation and male meiosis in European clades of the spider genus *Pholcus* (Araneae, Pholcidae)

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Abstract

Haplogyne araneomorphs are a diverse spider clade. Their karyotypes are usually predominated by biarmed (i.e., metacentric and submetacentric) chromosomes and have a specific sex chromosome system, X_1X_2Y. These features are probably ancestral for haplogynes. Nucleolus organizer regions (NORs) spread frequently from autosomes to sex chromosomes in these spiders. This study focuses on pholcids (Pholcidae), a highly diverse haplogyne family. Despite considerable recent progress in pholcid cytogenetics, knowledge on many clades remains insufficient including the most species-rich pholcid genus, *Pholcus* Walckenaer, 1805. To characterize the karyotype differentiation of *Pholcus* in Europe, we compared karyotypes, sex chromosomes, NORs, and male meiosis of seven species [*P. alticeps* Spassky, 1932; *P. creticus* Senglet, 1971; *P. dentatus* Wunderlich, 1995; *P. fuerteventurensis* Wunderlich, 1992; *P. phalangioides* (Fuesslin, 1775); *P. opilionoides* (Schrank, 1781); *P. silvai* Wunderlich, 1995] representing the dominant species groups in this region. The species studied show several features ancestral for *Pholcus*, namely the $2n♂ = 25$, the X_1X_2Y system, and a karyotype predominated by biarmed chromosomes. Most taxa have a large acrocentric NOR-bearing pair, which evolved from a biarmed pair by a pericentric inversion. In some lineages,

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the acrocentric pair reverted to biarmed. Closely related species often differ in the morphology of some chromosome pairs, probably resulting from pericentric inversions and/or translocations. Such rearrangements have been implicated in the formation of reproductive barriers. While the X_1 and Y chromosomes retain their ancestral metacentric morphology, the X_2 chromosome shows a derived (acrocentric or subtelocentric) morphology. Pairing of this element is usually modified during male meiosis. NOR patterns are very diverse. The ancestral karyotype of *Pholcus* contained five or six terminal NORs including three X chromosome-linked loci. The number of NORs has been frequently reduced during evolution. In the Macaronesian clade, there is only a single NOR-bearing pair. Sex chromosome-linked NORs are lost in Madeiran species and in *P. creticus*. Our study revealed two cytotypes in the synanthropic species *P. phalangioides* (Madeiran and Czech), which differ by their NOR pattern and chromosome morphology. In the Czech cytotype, the large acrocentric pair was transformed into a biarmed pair by pericentric inversion.

Keywords
haplogyne, inversion, NOR, rDNA, sex chromosome, speciation, Synspermiata

Introduction

Spiders exhibit an enormous species diversity, paralleled by high karyotype diversity. However, despite considerable recent progress (e.g., Král et al. 2006, 2013, 2019; Araujo et al. 2012; Kořínková and Král 2013; Ávila Herrera et al. 2021), our knowledge of spider cytogenetics is still fragmentary. Most data on spider chromosomes concern entelegyne araneomorphs, which include the large majority of the described spider species. The cytogenetics of the other clades (mesotheles, mygalomorphs, haplogyne araneomorphs) is much less understood (Kořínková and Král 2013; Ávila Herrera et al. 2021).

Haplogyne araneomorphs ("haplogynes") consist of the Synspermiata clade and two families, Filistatidae and Hypochilidae (Wheeler et al. 2017; Shao and Li 2018). Haplogynes currently include more than 6150 described species placed in 20 families (based on data of World Spider Catalog 2022). Haplogynes exhibit a considerable karyotype diversity. Their diploid numbers range from 2n♂ = 5 (*Afrilobus* sp., Orsolobidae) to 2n♂ = 152 (*Caponia natalensis* O. Pickard-Cambridge, 1874, Caponiidae), which are the lowest and highest diploid numbers in spiders, respectively (Král et al. 2019). Their karyotypes are composed of monocentric (i.e., standard) chromosomes except for the superfamily Dysderoidea whose chromosomes are holokinetic (holocentric) (Král et al. 2019). Holokinetic chromosomes lack a localized centromere (Mola and Papeschi 2006). Karyotypes of haplogynes with monocentric chromosomes are usually predominated by biarmed (i.e., metacentric and submetacentric) chromosomes (Král et al. 2006; Ávila Herrera et al. 2021). Furthermore, the prophase of the male first meiotic division includes the so-called diffuse stage (Kořínková and Král 2013), characterized by a considerable decondensation of autosomes and overcondensation of sex chromosomes (Benavente and Wettstein 1980; Král et al. 2006; Ávila Herrera et al. 2021). Haplogynes exhibit a variety of sex chromosome systems. Male sex chromosomes include one or several elements that do not recombine during meiosis and

are presumably nonhomologous. The peculiar X_1X_2Y system has been found in seven families (Král et al. 2006, 2019; Ávila Herrera et al. 2016, 2021; Paula-Neto et al. 2017; Araujo et al. 2020). It is probably ancestral for araneomorph spiders including haplogynes (Paula-Neto et al. 2017; Ávila Herrera et al. 2021). The ancestral structure of the X_1X_2Y system probably comprises two large metacentric X chromosomes and a metacentric Y microchromosome, which display a specific achiasmatic end-to-end pairing during male meiosis (Ávila Herrera et al. 2021). The origin of the X_1X_2Y system is unresolved. In some clades, it has converted into other sex chromosome systems (Král et al. 2006, 2019; Ávila Herrera et al. 2016, 2021). Besides non-recombining elements, spider sex chromosomes probably also contain a chromosome pair formed by the chromosomes X and Y, which recombine and show a very low level of differentiation (cryptic sex chromosome pair, CSCP) (Kořínková and Král 2013). Haplogynes also vary greatly in the number and location of nucleolus organizer regions (NORs) (Král et al. 2006; Ávila Herrera et al. 2021). These structures contain genes for 18S, 5.8S and 28S rRNA (Sumner 2003). The number of NORs ranges from one to nine; their position is usually terminal; and they spread frequently from autosomes to sex chromosomes (Král et al. 2006; Ávila Herrera et al. 2021).

The present study focuses on the cytogenetics of pholcid spiders (Pholcidae), the most diversified haplogyne family with monocentric chromosomes. This family currently comprises almost 1900 described species in 97 genera (World Spider Catalog 2022). Pholcids occur on all continents except Antarctica. Most species inhabit tropical and subtropical regions; some species are synanthropic (Huber 2011). From a cytogenetic point of view, pholcids are the best-explored group of haplogynes. A total of 64 species have been karyotyped, including 11 species determined to genus level only (based on The Spider Cytogenetic Database 2022). Despite this, our knowledge on karyotype evolution remains insufficient for many pholcid clades, including the most species-rich genus, *Pholcus* Walckenaer, 1805 (with currently more than 350 species; World Spider Catalog 2022). To reduce this gap, we studied the differentiation of karyotype, sex chromosomes, and NORs as well as the course of male meiosis in the dominant species groups of *Pholcus* present in mainland Europe, Crete, and Macaronesia. Nucleolus organizer regions have previously been studied in few spider species. More comprehensive data on the evolution of these structures are only available from pholcids (Ávila Herrera et al. 2021).

We paid specific attention to the Macaronesian clade of *Pholcus*. Macaronesia consists of five volcanic archipelagos in the Atlantic Ocean, west of the Iberian Peninsula and northwestern Africa. *Pholcus* is among the most species-rich genera of Macaronesian spiders. The Macaronesian clade currently includes more than 20 described species that are largely restricted to the Canaries and Madeira (Dimitrov and Ribera 2007; Dimitrov et al. 2008; Huber 2011). This clade exhibits an enormous diversification rate, among the highest found in spiders (Dimitrov et al. 2008).

Our aim is to determine the fundamental traits of karyotype evolution in European clades of *Pholcus*. Based on our new findings and on previously published data, we explore the congruence of individual karyotype markers with published phylogenies and discuss the possible evolutionary implications of karyotype transformations.

Material and methods

Spider specimens

Information on the studied species (number of analyzed specimens, their sex, and locality data) is given in Table 1. Voucher specimens are deposited in the Zoological Research Museum Alexander Koenig, Bonn (Germany).

Table 1. Species studied, with specimen number, sex, and geographic origin. Abbreviation: sad = subadult.

Taxon	Individuals	Locality	GPS Coordinates (Latitude, Longitude)
P. crypticolens/opilionoides species group			
P. creticus	4♂	Greece, Crete, Topolia, Topolia cave	35.4119, 23.6817
	2♂	Greece, Crete, Stavros, Lera cave	35.5908, 24.1023
P. opilionoides	4♂	Czech Republic, Veselí nad Lužnicí	49.1506, 14.6930
P. phalangioides species group			
P. alticeps	8♂	Czech Republic, Chomutov	50.4527, 13.4166
P. phalangioides	1♂	Portugal, Madeira, Santana	32.8043, -16.8855
Macaronesian species group			
P. fuerteventurensis	2♂	Spain, Canariens, Fuerteventura, Giniginamar	28.2024, -14.0734
P. dentatus	1 sad ♂, 1♂	Portugal, Madeira, Achadas da Cruz	32.8390, -17.1907
P. silvai	2♂	Portugal, Madeira, Levada das 25 fontes	32.7611, -17.1374

Preparation of chromosomes, determination of karyotype

Chromosome preparations were obtained from testes of adult males by a modification of the spreading technique described by Dolejš et al. (2011). The gonads were dissected and immersed into a hypotonic solution (0.075M KCl) for 20–25 min at room temperature (RT). Hypotonization was followed by two fixations in ethanol:acetic acid (3:1) for 10 and 20 min (RT), respectively. Subsequently, tissue was placed in a drop of 60% acetic acid on a clean slide and quickly shredded with a pair of tungsten needles to obtain a cell suspension. Finally, the slide was placed on a warm (40 °C) histological plate. The drop of dispersed tissue evaporated while being moved constantly by a tungsten needle. Slides were stained using 5% Giemsa solution in Sörensen buffer (pH 6.8) for 28 min (RT). They were studied under an Olympus BX 50 microscope equipped with DP 71 CCD camera (Olympus, Tokyo, Japan). To construct the karyotype, the morphology of metaphase II chromosomes was analyzed. Sister metaphases II (5 plates) were evaluated using the IMAGE TOOL 3.0 software (https://imagetool.software.informer.com/3.0/). Relative chromosome length was estimated as a percentage of the total chromosome length of the haploid set (TCL). This set also included sex chromosomes X_1, X_2, and Y. Karyotypes were assembled using the COREL PHOTO PAINT X3 programme. Determination of the sex chromosome system was based on data from male meiosis (segregation of sex chromosomes and their behavior in prophase and metaphase I). The X_2 and Y chromosomes were similar in size. Therefore,

we used a paired samples Wilcoxon test to analyse their size difference. It was impossible to distinguish the CSCP from autosomes by light microscopy. Therefore, the CSCP and autosomes are referred to collectively as chromosome pairs.

Detection of nucleolus organizer regions (NORs)

The NOR pattern was determined by fluorescent in situ hybridisation (FISH) with a 18S rDNA probe from *Dysdera erythrina* (Walckenaer, 1802) (Dysderidae) (see Ávila Herrera et al. 2021 for details of probe). Whereas the previously common method of NOR-detection by silver staining only visualizes NOR sites transcribed during the preceding interphase (Miller et al. 1976), NOR detection by a rDNA probe gives more accurate results. The probe was generated following Sadílek et al. (2015). The 18S rRNA gene fragment was amplified by polymerase chain reaction (PCR) from genomic DNA using forward and reverse primers 5′-CGAGCGCTTTTATTAGACCA-3′ and 5′-GGTTCACCTACGGAAACCTT-3′, respectively. The PCR product was extracted using the Wizard SV Gel and PCR Clean-Up System (Promega), re-amplified by PCR, and labeled with biotin-14-dUTP by nick translation using a Nick Translation Kit (Abbott Molecular).

FISH was performed with the biotinylated 18S rDNA probe as described by Fuková et al. (2005). Chromosome preparations were pre-treated with 100 µg/ml RNase A in 2× saline-sodium citrate (SSC) buffer (1 h, 37 °C). Chromosomes were denatured (3 min 30 s, 68 °C) by 70% formamide in 2×SSC. The probe mixture contained 20 ng of 18S rDNA and 25 µg of salmon sperm DNA (Sigma-Aldrich, Burlington, MA, USA) in 5 µl of 50% formamide and 5 µl of 20% dextran sulphate per slide. Biotin labelled 18S rDNA was detected with Cy3-streptavidin (Jackson ImmunoRes. Labs Inc., West Grove, PA, USA), with signal amplification by biotinylated antistreptavidin and Cy3-streptavidin (Vector Labs Inc., Burlingame, CA, USA). The preparations were counterstained with Fluoroshield containing 4′,6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich, Burlington, MA, USA). Considering the sensitivity of pholcid chromosomes to denaturation, two procedures were used to reduce this process. First, the slides were placed in an incubator for 1 hour (60 °C) before the experiment. Second, denaturation time was reduced (3 min). Preparations were observed under the Olympus IX81 microscope (Olympus, Tokyo, Japan) equipped with an ORCA-AG monochromatic camera (Hamamatsu, Hamamatsu, Japan). The images were pseudocolored (red for Cy3 and light green for DAPI) with Cell^R software (Olympus Soft Imaging Solutions GmbH, Münster, Germany).

Results

Karyotype data

The male karyotype of all species studied had 25 predominantly metacentric chromosomes and the X_1X_2Y system ($2n♂ = 25, X_1X_2Y$). The X_1 was the longest element of the set. Chromosomes X_2 and Y were medium-sized elements of similar size. Chromosome pairs decreased gradually in length (Suppl. material 1).

Pholcus crypticolens/opilionoides species group

The chromosome pairs of the males of *P. creticus* comprised five metacentric (nos 1, 5–8), four submetacentric (nos 2,4,9,10), one subtelocentric (no. 11), and one acrocentric pair (no. 3). Sex chromosomes were metacentric except for the acrocentric X_2 (Fig. 1A). Lenghts of the X_2 and Y chromosomes differed significantly (paired samples Wilcoxon test, W = 0, P < 0.001). The Y chromosome was longer than the X_2 (Suppl. material 1). This species had two chromosome pairs with a terminal NOR each (Fig. 2C). The morphology of these pairs is unresolved.

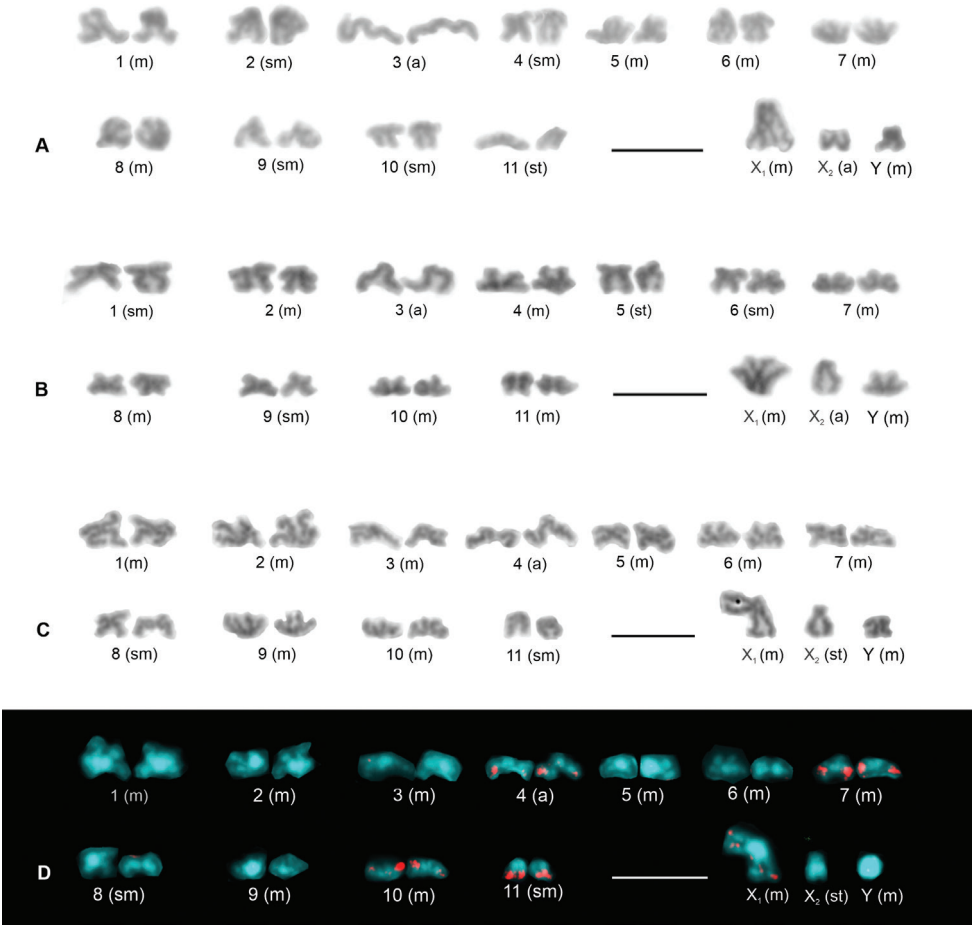


Figure 1. *Pholcus*, *crypticolens/opilionoides* and *phalangioides* groups, male karyotypes (**A–C** stained by Giemsa **D** FISH). Based on sister metaphases II **A** *P. creticus* **B** *P. alticeps* **C, D** *P. phalangioides* (Madeira) **C** standard karyotype **D** karyotype, detection of NORs. Prepared from the same plate as the standard karyotype. Note four chromosome pairs with terminal NOR (nos 4,7,10,11) and the X_1 chromosome with NOR at both ends. Pairs nos. 7, 10, and 11 are biarmed, pair no. 4 is acrocentric. NORs are localized at the long arm of these pairs. Scale bars: 10 μm.

The chromosomes of the males of *P. opilionoides* exhibited the same morphology as in populations studied previously (Ávila Herrera et al. 2021). They were metacentric except for five submetacentric chromosome pairs (nos 2–6) and an acrocentric X_2 chromosome. The lengths of the X_2 and Y chromosomes differed significantly (paired samples Wilcoxon test, W = 0, P < 0.001). The Y was shorter than the X_2. We succeeded in determining the NOR pattern in one specimen. The karyotype contained three biarmed chromosome pairs bearing a terminal NOR each. One pair was heterozygous for a NOR cluster. Furthermore, a small NOR was also detected at each end of the X_1 chromosome (Fig. 2A, B).

Pholcus phalangioides species group

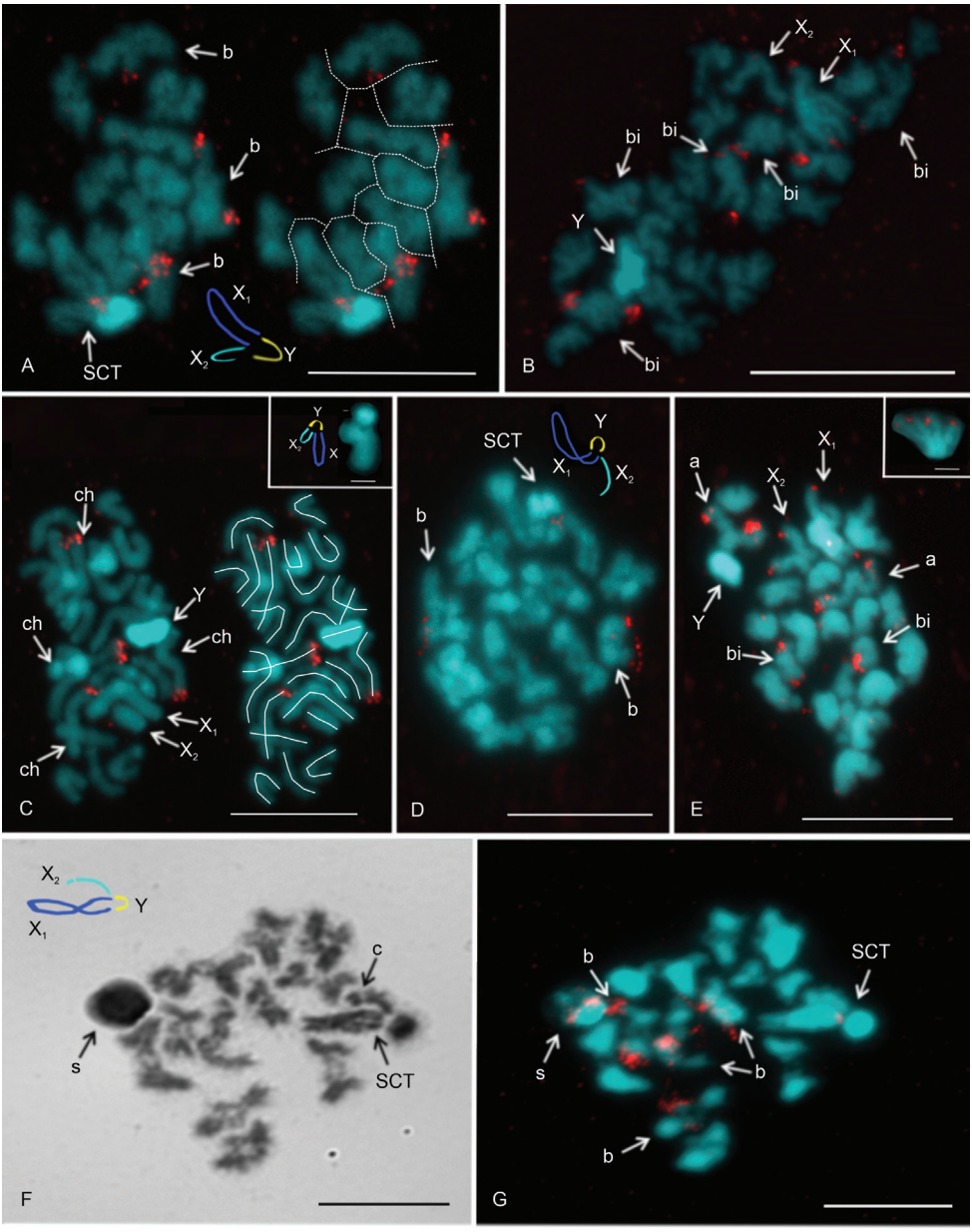
The male karyotype of *P. alticeps* consisted of metacentric chromosomes except for three submetacentric (nos 1,6,9), one subtelocentric (no. 5), and one acrocentric (no. 3) chromosome pairs as well as the acrocentric X_2 chromosome (Fig. 1B). The lengths of the X_2 and Y chromosomes did not differ significantly (paired samples Wilcoxon test, W = 1, 0.10 < P < 0.20). The karyotype included two chromosome pairs with a terminal NOR locus each. While one NOR-bearing pair was formed by small biarmed chromosomes, the other one consisted of large acrocentric chromosomes with a NOR at the end of the long arm. The karyotype contained three terminal sex chromosome-linked NORs (two on the X_1 chromosome and one at the end of the long arm of the X_2 chromosome) (Fig. 2D, E).

The karyotype of the single male of *P. phalangioides* from Madeira consisted of metacentric chromosomes except for two submetacentric (nos 8 and 11) and one acrocentric pair (no. 4) as well as a subtelocentric X_2 (Fig. 1C). The lengths of the X_2 and Y chromosomes did not differ significantly (paired samples Wilcoxon test, W = 2, 0.10 < P < 0.20). Three biarmed (nos 7,10,11) and one acrocentric chromosome pairs (no. 4) contained a terminal NOR each, which was placed at the end of the long arm. Beside this, a NOR was also found at each end of the X_1 chromosome (Figs 1D, 2F, G).

Macaronesian species group

The karyotype of *P. fuerteventurensis* from the Canaries was composed of metacentric chromosomes except for one submetacentric (no. 1) and one acrocentric pair (no. 5) as well as an acrocentric X_2 chromosome (Fig. 3A). The lengths of the X_2 and Y chromosomes did not differ significantly (paired samples Wilcoxon test, W = 5, P > 0.2). *P. fuerteventurensis* had a single large acrocentric NOR-bearing pair containing a NOR at the end of the long arm. A NOR was also placed at the end of the long arm of the X_2 chromosome (Fig. 4A–C).

In *P. dentatus* from Madeira, the chromosome pairs were metacentric except for two submetacentric (nos 7 and 11) and one acrocentric pair (no. 3). The sex chromosomes had a metacentric morphology except for the acrocentric X_2 (Fig. 3B). The lengths of the X_2 and Y chromosomes differed significantly (paired samples Wilcoxon test, W = 0, P < 0.001). The X_2 was longer than the Y (Suppl. material 1).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G

The chromosome complement of the second Madeiran species, *P. silvai*, had metacentric chromosomes except for one submetacentric (no. 8), one subtelocentric (no. 10), one acrocentric pair (no. 4), and an acrocentric X_2 chromosome (Fig. 3C). The lengths of the X_2 and Y chromosomes differed significantly (paired samples Wilcoxon test, W = 0, P < 0.001). The Y was larger than the X_2 chromosome (Suppl. material 1).

Both Madeiran species showed the same NOR pattern, namely a single locus at the end of the long arm of the acrocentric pair (Fig. 4D–I).

Sex chromosome behavior in male germline

In general, the behavior of the sex chromosomes was characterized by positive heteropycnosis (i.e., more intensive staining) and association (i.e. close proximity of chromosomes without pairing) which transformed into pairing in some phases. The specific behavior of sex chromosomes was initiated as early as in spermatogonial mitosis. Sex chromosomes often exhibited positive heteropycnosis and a loose association in spermatogonial prophases, metaphases, and anaphases (Fig. 5A, B). During metaphase (Fig. 5A) as well as on anaphase half-plates (Fig. 5B), they were often placed in the middle of the plates. They remained overcondensed and positively heteropycnotic during premeiotic interphase, early prophase I (leptotene-pachytene), and diffuse stage. During this period, they often formed a body on the periphery of the plate (Fig. 5C, D). Bivalents were fuzzy and spherical during the early diffuse stage (Fig. 5C). However, towards the end of the diffuse stage, they showed chiasmata and their morphology was similar to that found during late prophase I (Fig. 5D). During late prophase I (diplotene-diakinesis) and metaphase I, the condensation of

Figure 2. *Pholcus*, *crypticolens*/*opilionoides* and *phalangioides* groups, males, NOR detection **A–E, G** FISH **F** Giemsa staining **A, B** *P. opilionoides* **A** diplotene. Three bivalents contain NOR. There is also a signal on the sex chromosome trivalent. Y chromosome overcondensed. Note the scheme of sex chromosome pairing and scheme of the plate (particular elements separated by a dotted line) **B** two fused sister metaphases II. Note the terminal signal on five biarmed elements belonging to chromosome pairs. Odd number of chromosomes with signal suggests that NOR locus of one chromosome pair is heterozygous for NOR cluster. The X_1 chromosome includes NOR at both ends **C** *P. creticus*, mitotic metaphase. Two chromosome pairs contain a terminal NOR. Y chromosome overcondensed. On the right side: scheme of the plate (particular chromosomes marked by a line). Inset: metaphase I, sex chromosome trivalent (without signal). Note the scheme of sex chromosome pairing **D, E** *P. alticeps* **D** metaphase I. Two bivalents contain NOR. There is also signal on the sex chromosome trivalent. Y chromosome overcondensed. Note the scheme of sex chromosome pairing **E** two fused sister metaphases II, Y chromosome overcondensed. NOR bearing elements: one pair of biarmed chromosomes (a terminal NOR), one pair of acrocentric chromosomes (a terminal NOR at long arm), X_2 chromosome (a terminal NOR at long arm), X_1 chromosome (NOR at both ends). Inset: X_1 chromosome (from another plate), note the NOR at both ends **F, G** *P. phalangioides*, Madeira, metaphase I. Four bivalents include a NOR. There is also a signal on the sex chromosome trivalent. Note the scheme of sex chromosome trivalent. Abbreviations: a = chromosome of the acrocentric pair bearing NOR, b = bivalent containing NOR, bi = chromosome of a biarmed pair bearing NOR, c = centromere, ch = chromosome bearing NOR, s = sperm nucleus, SCT = sex chromosome trivalent, X_1 = X_1 chromosome, X_2 = X_2 chromosome, Y = Y chromosome. Scale bars: 10 μm except for insets (5 μm).

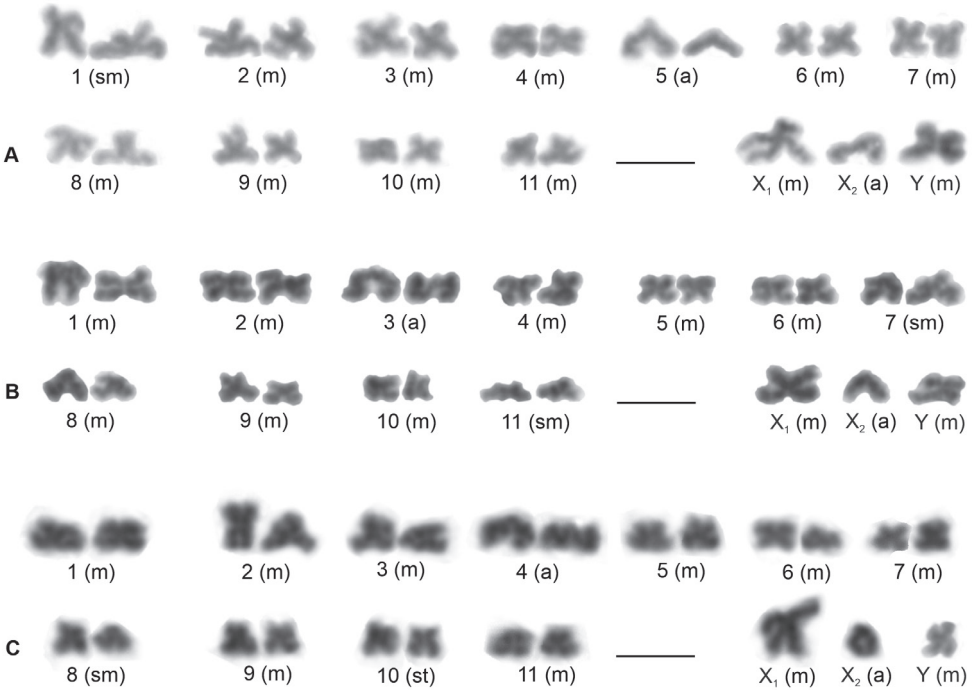

Figure 3. *Pholcus*, Macaronesian group, male karyotypes, stained by Giemsa. Based on sister metaphases II **A** *P. fuerteventurensis* **B** *P. dentatus* **C** *P. silvai*. Scale bars: 10 μm.

the sex chromosomes decreased. The Y chromosome was often more condensed than the X chromosomes and bivalents (Fig. 5E). The pattern of heteropycnosis also varied during metaphase II. While in the Madeiran species the sex chromosomes usually exhibited none or only indistinct heteropycnosis (Fig. 6A), they were often positively heteropycnotic in *P. fuerteventurensis* from the Canaries and in species from mainland Europe (Fig. 6C). The Y chromosome often showed a more intensive staining than the X chromosomes. All species were characterized by sex chromosome heteropycnosis during anaphase II whereas heteropycnosis of the X_2 chromosome was indistinct in some plates (Fig. 6B).

In the premeiotic interphase, the association of sex chromosomes transformed into sex chromosome pairing. The mode of sex chromosome pairing was most apparent during late prophase and metaphase I. Both ends of the metacentric sex chromosomes, X_1 and Y, took part in pairing (Fig. 5E, F). The pairing pattern of the monoarmed X_2 chromosome differed among species. In *P. creticus* (and in some plates of *P. alticeps* and *P. dentatus*), both ends of the X_2 chromosome were involved in pairing (Fig. 5F). The same pattern of pairing was found in *P. opilionoides* during early diplotene (Fig. 2A). After that, pairing was restricted to the long arm of the X_2 chromosome. In other species, only the long arm of the X_2 chromosome was involved in pairing, by its end (Fig. 5E); this pattern was also observed in the absence of hypotonization. The X chromosomes were usually arranged in parallel during anaphase I, metaphase II, and anaphase II (Fig. 6B). The Y chromosome was placed in the middle of the half-plates during anaphase II (Fig. 6B).

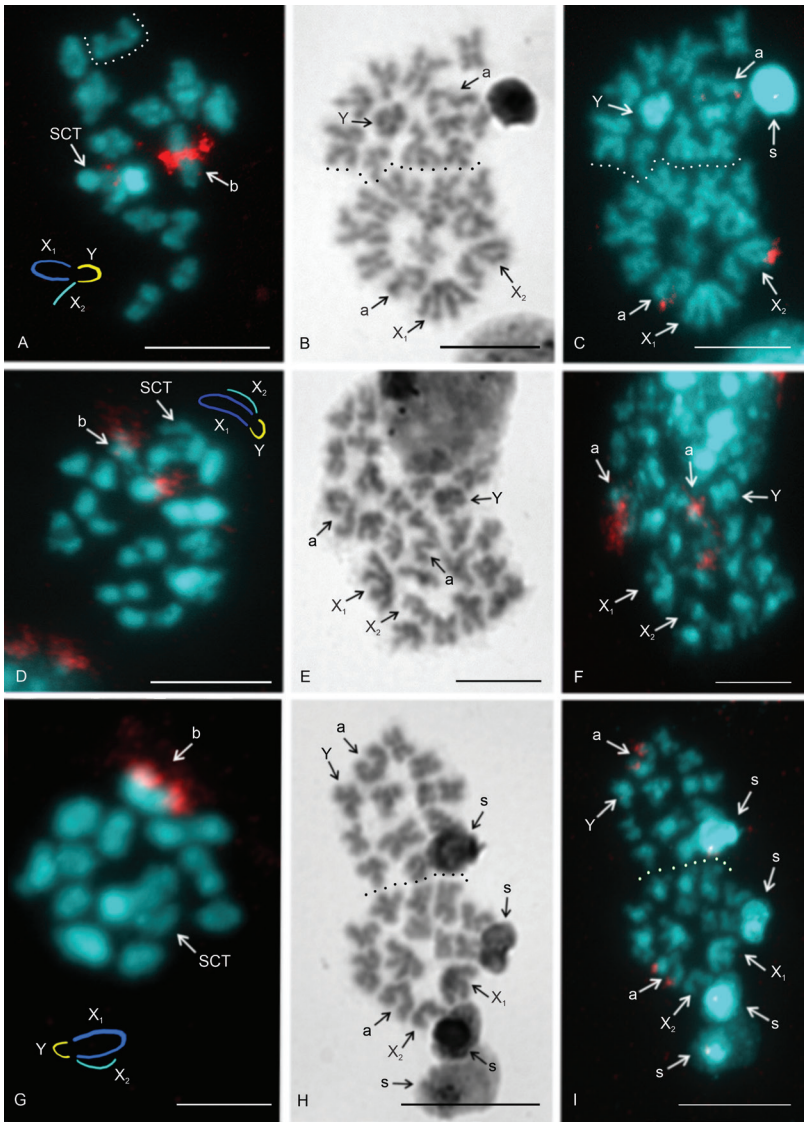

Figure 4. *Pholcus*, Macaronesian group, NOR detection **A, C, D, F, G, I** FISH **B, E, H** Giemsa staining **A–C** *P. fuerteventurensis* **A** metaphase I (a bivalent belonging to another plate is separated by a dotted line). One bivalent contains a NOR. There is also a signal on the sex chromosome trivalent. Note the scheme of sex chromosome pairing **B, C** two sister metaphases II separated by a dotted line. Note two terminal NORs, one on the long arm of the acrocentric pair and another one on the long arm of the acrocentric X_2 chromosome **D–F** *P. dentatus* **D** metaphase I, one large bivalent contains a terminal NOR. Note the scheme of sex chromosome pairing **E, F** two fused metaphases II. Long arm of the acrocentric pair contains terminal NOR. Sister chromatids of chromosomes of this pair are sometimes associated by NOR clusters (see the right chromosome of the pair) **G–I** *P. silvai* **G** metaphase I, one bivalent involves a terminal NOR. Note the scheme of sex chromosome pairing **H, I** two metaphases II separated by dotted line. Long arm of the acrocentric pair contains terminal NOR. Abbreviations: a = chromosome of the acrocentric pair bearing NOR, b = bivalent containing NOR, s = sperm nucleus, SCT = sex chromosome trivalent, X_1 = X_1 chromosome, X_2 = X_2 chromosome, Y = Y chromosome. Scale bars: 10 μm.

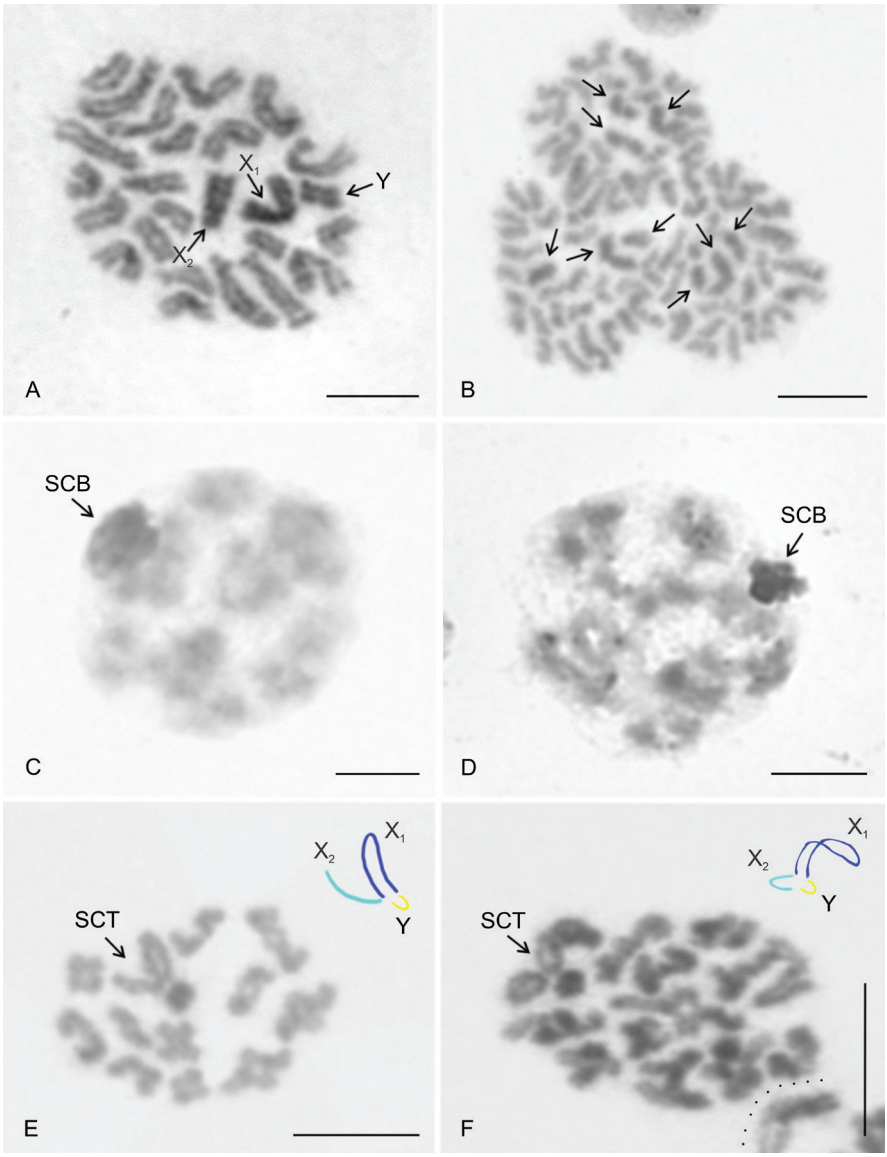

Figure 5. *Pholcus*, males, sex chromosome behavior at spermatogonial mitosis and first meiotic division, Giemsa staining **A** *P. dentatus*, spermatogonial metaphase. Note the association of positively heteropycnotic sex chromosomes in the middle of the plate **B** *P. silvai*, early spermatogonial anaphase, three half plates. Sex chromosomes exhibit a slight positive heteropycnosis and are placed in the middle of the half plates. Sex chromosomes are marked by arrows **C** *P. fuerteventurensis*, early diffuse stage. Sex chromosomes form a positively heteropycnotic body on the periphery of the nucleus **D** *P. silvai*, late diffuse stage. The sex chromosome body on the periphery of the nucleus exhibits positive heteropycnosis **E** *P. fuerteventurensis*, diakinesis (11 bivalents and a X_1X_2Y trivalent). The Y chromosome stained more intensively than the X chromosomes. Note the scheme of sex chromosome pairing **F** *P. alticeps*, diplotene (11 bivalents and a X_1X_2Y trivalent). Edge of another diplotene separated by dotted line. Note the scheme of sex chromosome pairing. Abbreviations: SCB = sex chromosome body, SCT = sex chromosome trivalent, X_1 = X_1 chromosome, X_2 = X_2 chromosome, Y = Y chromosome. Scale bars: 10 μm.

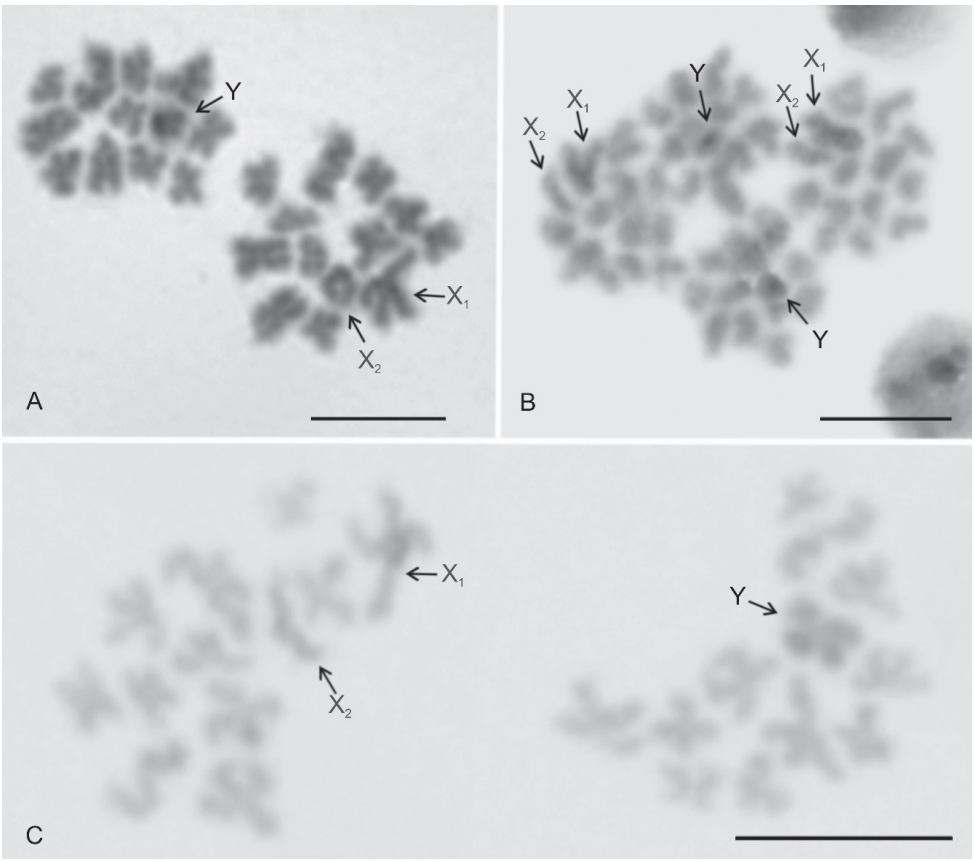

Figure 6. *Pholcus*, males, sex chromosome behavior in second meiotic division, Giemsa staining **A** *P. silvai*, two sister metaphases II. Metaphase II containing the X chromosomes is composed of 13 chromosomes. Metaphase II containing the Y chromosome comprises 12 chromosomes **B** *P. alticeps*, two sister anaphases II. Chromosomes X_1 and Y display positive heteropycnosis. The X chromosomes are associated. The Y chromosome is placed in the middle of the half plates **C** *P. fuerteventurensis*, two sister metaphases II. Plate containing the X chromosomes is incomplete (1 chromosome missing). Note the positive heteropycnosis of the sex chromosomes. Abbreviations: X_1 = X_1 chromosome, X_2 = X_2 chromosome, Y = Y chromosome. Scale bars: 10 μm.

Discussion

Pholcids are the most diversified family of haplogyne spiders with monocentric chromosomes and a suitable model group to study karyotype evolution. Their distribution is worldwide, and the available molecular phylogeny is the most comprehensive among all major spider families (Eberle et al. 2018). They are currently the best-explored family of haplogynes from a cytogenetic point of view. Closely related species often differ in their karyotypes, suggesting the involvement of chromosome rearrangements in the formation of interspecific barriers (Ávila Herrera et al. 2021).

Here we focus on karyotype differentiation of the genus *Pholcus*. Previously published cytogenetic data concern seven species determined to species level and

two species determined to genus level only (The Spider Cytogenetic Database 2022). With five newly studied species, our study increases the number of cytogenetically analyzed *Pholcus* species to 14. However, karyotype data of three species are in all probability incorrect (Table 2). These data are analysed in detail by Ávila Herrera et al. (2021). The karyotyped representatives determined to species

Table 2. Summary of *Pholcus* cytogenetic data. Doubtful data in bold. In most of these cases, it is possible to deduce probable correct information (in parentheses). †see Ávila Herrera et al. (2021: 22) for discussion of sex chromosome system. ‡See Ávila Herrera et al. (2021) for discussion of sex chromosome system (p. 23) and morphology of chromosome pairs (p. 21). §See Ávila Herrera et al. (2021) for discussion of number of chromosome pairs (p. 18) and sex chromosome system (p. 22). Abbreviations: a = acrocentric, bi = biarmed, CP = chromosome pair, m = metacentric, p = short chromosome arm, q = long chromosome arm, SC = sex chromosome, SCS = sex chromosome system, sm = submetacentric, st = subtelocentric, t = terminal, ? = unknown.

Taxon	2n	SCS	Chromosome pairs: number, morphology	Sex chromosome morphology	NOR number (CP/SC)	NOR-bearing CPs: number, morphology (NOR location)	NOR-bearing sex chromosomes: morphology (NOR location)	References
bicornutus species group								
P. pagbilao	23	X_1X_2Y	7m+3sm	X_1m+X_2a+Ysm	5/0	3 bi (t);1 bi (1 NOR p, t + 1 NOR q, t)		Ávila Herrera et al. 2021
crypticolens/*opilionoides* species group								
P. creticus	25	X_1X_2Y	5m+4sm+1st+1a	X_1m+X_2a+Ym	2/0	2 (t)		this study
P. crypticolens†	**24** (25)	**X_1X_20** (X_1X_2Y)	most or all m	$X_1?+X_2?$				Suzuki 1954
P. manueli‡	25	**X0** (X_1X_2Y)	**11a**	**Xsm**				Wang et al. 1997
P. opilionoides	25	X_1X_2Y	6m+5sm	X_1m+X_2a+Ym	3/2	3 bi (t)	X_1 m (1 NOR p, t + 1 NOR q, t)	Ávila Herrera et al. 2021, this study
guineensis species group (+ *P. bamboutos*)								
P. bamboutos	23	X_1X_2Y	most bi	X_1m+X_2m+Ym				Ávila Herrera et al. 2021
P. kindia	23	X_1X_2Y	8m+1sm+1st	X_1m+X_2m+Ym				Ávila Herrera et al. 2021
Macaronesian species group								
P. dentatus	25	X_1X_2Y	8m+2sm+1a	X_1m+X_2a+Ym	1/0	1a (q, t)		this study
P. fuerteventurensis	25	X_1X_2Y	9m+1sm+1a	X_1m+X_2a+Ym	1/1	1a (q, t)	X_2 a (1 NOR q, t)	this study
P. silvai	25	X_1X_2Y	8m+1sm+1st+1a	X_1m+X_2a+Ym	1/0	1a (q, t)		this study
phalangioides species group								
P. alticeps	25	X_1X_2Y	6m+3sm+1st+1a	X_1m+X_2a+Ym	2/3	1 bi (t); 1a (q, t)	X_1 m (1 NOR p, t + 1 NOR q, t); X_2 a (1 NOR q, t)	this study
P. phalangioides (Czech cytotype)	25	X_1X_2Y	9m+2sm	X_1m+X_2sm+Ym	3/3	3 bi (t)	X_1 m (1 NOR p, t + 1 NOR q, t); X_2 sm (q, t)	Král et al. 2006, Ávila Herrera et al. 2021
P. phalangioides (Madeiran cytotype)	25	X_1X_2Y	8m+2sm+1a	X_1m+X_2st+Ym	4/2	3 bi (q, t); 1 a (q, t)	X_1 m (1 NOR p, t + 1 NOR q, t)	this study
species determined to the genus level only								
Pholcus sp. (India)§	**26**(?)	**X_1X_20** (X_1X_2Y)						Sharma and Parida 1987
Pholcus sp. (Kazakhstan)	25	X_1X_2Y	7m+3sm+1a	X_1m+X_2st+Ym				Ávila Herrera et al. 2021

level represent five of the clades proposed for the genus (Huber et al. 2018), namely the *P. bicornutus*, *P. crypticolens/P. opilionoides*, *P. guineensis*, *P. phalangioides*, and Macaronesian groups.

Diploid numbers and morphology of chromosome pairs

The ancestral pholcid karyotype probably consisted of 15 chromosome pairs and the sex chromosomes X_1, X_2, and Y (Ávila Herrera et al. 2021). Like many other spider groups (Suzuki 1954; Král et al. 2006, 2013), some pholcid clades show a trend towards a decrease in chromosome number (Ávila Herrera et al. 2021). This is also probably how the ancestral karyotype of the subfamily Pholcinae has evolved with its 11 chromosome pairs and sex chromosomes X_1, X_2, and Y. This karyotype is also ancestral for *Pholcus* (Ávila Herrera et al. 2021). It was found in all karyotyped clades of the genus except for the *P. bicornutus* and *P. guineensis* groups (Ávila Herrera et al. 2021; this study). In the latter two species groups, the number of chromosome pairs decreased further to ten. This feature could be a synapomorphy of a large group within *Pholcus* comprising the Subsaharan African, Southeast Asian, and Australasian groups of this genus (Ávila Herrera et al. 2021).

The chromosome pairs of ancestral pholcids probably had a biarmed morphology (Ávila Herrera et al. 2021). Most pairs were probably metacentric. Chromosome pairs of *Pholcus* species are predominated by biarmed chromosomes except for *P. manueli* Gertsch, 1937 (Wang et al. 1997). However, the information on this species is based only on the pattern of constitutive heterochromatin. Therefore, it should be reanalyzed by determination of chromosome morphology at the mitotic metaphase or metaphase II (Ávila Herrera et al. 2021).

The karyotype of the unidentified *Pholcus* sp. from Kazakhstan reported in Ávila Herrera et al. (2021) contains a large acrocentric pair, which was supposed to be an apomorphy of this species. Kazakhstan is inhabited by representatives of the *P. crypticolens/opilionoides* and *P. ponticus* groups (Huber 2011). Our study revealed that the acrocentric pair is in fact more common in Eurasian *Pholcus* groups with the karyotype formula 25, X_1X_2Y. The pair is the third, fourth or fifth by size and its relative length ranges from 7.20 to 8.22% of TCL (Ávila Herrera et al. 2021; this study). The end of the long arm of this pair contains a NOR (see discussion on NOR evolution below). The large acrocentric pair has most probably originated by a pericentric inversion from a biarmed one. In the present study, it was found in representatives of all analyzed groups. This pattern suggests that the large acrocentric pair could be a synapomorphy of several species groups within the genus with the karyotype formula 25, X_1X_2Y. A further interesting pattern was found in *P. phalangioides*. While the cytotype from Madeira retained the large acrocentric pair, in the Czech cytotype this pair had reverted to biarmed, thus the karyotype was again composed exclusively of biarmed chromosomes. Since the chromosome pairs of the above mentioned cytotypes differed only by this reversion, it most probably resulted from a pericentric inversion. Furthermore, the reversion of an acrocentric pair to biarmed had also occurred in *P. opilionoides* whose karyotype is also formed exclusively by biarmed chromosomes. The acrocentric pair is not present in

karyotypes of the *Pholcus* lineages with the formula 23, X_1X_2Y. However, a reversion of an acrocentric pair to non-acrocentric cannot be ruled out in ancestors of these lineages. If such a scenario is correct, the large acrocentric pair would be a synapomorphy of the entire genus *Pholcus.* This marker has not been found in the sister clade of *Pholcus*, i.e. the *Micropholcus/Leptopholcus* clade (Ávila Herrera et al. 2021). However, the large acrocentric pair could even have been present in the ancestral karyotype of the *Micropholcus/Leptopholcus* clade. The karyotypes of this clade have been derived from the supposed ancestral karyotype of pholcines (25, X_1X_2Y) by multiple fusions of chromosome pairs. The large acrocentric pair could have been involved into these fusions.

Closely related species of *Pholcus* often differ by the morphology of one or several chromosome pairs. For example, *P. fuerteventurensis* from the Canaries (belonging to the Macaronesian clade) differs from species of the same clade from Madeira by the morphology of three pairs. A possible apomorphy of *P. fuerteventurensis* is the transformation of the largest chromosome pair from metacentric to submetacentric. The Madeiran species show two possible synapomorphies, namely transformations of two metacentric pairs into submetacentric or subtelocentric. The first transformation concerned the 7th pair of *P. dentatus* and the 8th pair of *P. silvai*, respectively. The second transformation concerned the 11th pair of *P. dentatus* and the 10th pair of *P. silvai*, respectively (Suppl. material 1). Even greater are the differences found between *P. opilionoides* and *P. creticus* from the *P. crypticolens/opilionoides* clade. A possible synapomorphy of these species is the change of two metacentric pairs to submetacentric (2nd and 4th pairs). While the large acrocentric pair has been retained in *P. creticus*, it has converted to biarmed in *P. opilionoides*. Moreover, both species differ by the morphology of five other chromosome pairs (Suppl. material 1). Potential synapomorphies of *P. alticeps*, *P. phalangioides* (*P. phalangioides* group) and *Pholcus* sp. from Kazakhstan include changes of two metacentric pairs into submetacentric. The first change concerned probably the 6th pair of *P. alticeps*, the 8th pair of *P. phalangioides* (the 7th pair in Ávila Herrera et al. 2021), and the 7th pair of *Pholcus* sp. The second change concerned probably the 9th pair of *P. alticeps*, the 11th pair of *P. phalangioides* (the 10th pair in Ávila Herrera et al. 2021), and the 9th pair of *Pholcus* sp.

A similar karyotype differentiation, where the morphology of one or more chromosome pairs changed while the number of chromosome pairs remained the same, has also been found in other pholcid genera (Ávila Herrera et al. 2021). These changes in morphology occurred most probably by pericentric inversions or translocations. These rearrangements leave the chromosome number unchanged and they can often result in reproductive isolation (Rieseberg 2001; Ayala et al. 2005).

Sex chromosomes

All *Pholcus* species studied so far exhibit the X_1X_2Y system (Král et al. 2006; Ávila Herrera et al. 2021, this study). Many haplogynes with the X_1X_2Y system have retained its ancestral type with two large metacentric X chromosomes and a metacentric microchromosome Y (Ávila Herrera et al. 2021).

The genus *Pholcus*, like most other pholcids with the X_1X_2Y system (Ávila Herrera et al. 2021), is conservative in having a metacentric X_1 chromosome, which is the largest chromosome of the set. In *Pholcus* species with the karyotype 25, X_1X_2Y, the size of the X_1 ranges from 9.87 to 14.37% of TCL (Ávila Herrera et al. 2021; this study). The size of the Y chromosome has increased considerably in a clade of the subfamily Pholcinae including *Quamtana* Huber, 2003, *Muruta* Huber, 2018, *Leptopholcus* Simon, 1893, and *Pholcus*. In general, the Y chromosome can increase in size by accumulation of constitutive heterochromatin, rearrangements between autosomes and sex chromosomes, or by a combination of these events (e.g., Kejnovský et al. 2009; Schartl et al. 2016). Available data suggest a major role of heterochromatin accumulation in the expansion of the pholcine Y chromosome. The Y chromosome of *P. phalangioides* is composed exclusively of constitutive heterochromatin (Král et al. 2006). A reinterpretation of karyotype data obtained by Wang et al. (1997) suggests the same composition of the Y chromosome in *P. manueli* (Ávila Herrera et al. 2021). Constitutive heterochromatin is a very dynamic part of the genome. The size of heterochromatic blocks could change even within populations (Sumner 1990). Although the Y chromosome of *Pholcus* is formed exclusively by heterochromatin, its size is relatively stable in this genus ranging from 4.77 to 7.10% of TCL except for *P. kindia* Huber, 2011 (11.72% of TCL) (Ávila Herrera et al. 2021; this study). Particular *Pholcus* species might differ by the extent of condensation in the Y chromosome, which contributes to its diversity in size. The enormous increase in size of the Y chromosome in *P. kindia* was probably caused by insertions of autosomal material (Ávila Herrera et al. 2021). Among other spiders with the X_1X_2Y system, a considerable increase of the Y chromosome size has only been found in one representative of pacullid spiders (Král et al. 2019).

The increase of Y chromosome size in pholcines has been accompanied by a reduction of the X_2 chromosome. The X_2 and Y chromosomes exhibit a similar size in the *Pholcus* clades analyzed in this study. The X_2 chromosome is the most dynamic chromosome of the X_1X_2Y system in pholcids. It exhibits a considerable diversity in size and morphology (Ávila Herrera et al. 2021). The ancestral metacentric morphology of the X_2 chromosome has changed frequently to submetacentric or even monoarmed, probably by pericentric inversions or translocations (Ávila Herrera et al. 2021). As already mentioned, these rearrangements can play a role in the formation of reproductive barriers. This effect is even stronger if the rearrangement concerns sex chromosomes (Presgraves 2008; Kitano et al. 2009; Hooper et al. 2019). The ancestral X_2 chromosome of *Pholcus* was probably metacentric as found in *P. guineensis* and *P. bamboutos* Huber, 2011 (23, X_1X_2Y). This hypothesis is supported by the biarmed morphology of the X_2 chromosome in the closest relatives of *Pholcus* (Ávila Herrera et al. 2021). During following evolution, the morphology of the X_2 chromosome gradually changed to acrocentric. This scenario is supported by the non-acrocentric morphology of this element in two species with the formula 25, X_1X_2Y, *P. phalangioides* (submetacentric or subtelocentric X_2) and *Pholcus* sp. (subtelocentric X_2). The size of the X_2 chromosome ranges from 5.53 to 6.56% of TCL in species with this formula (Ávila Herrera et al. 2021; this study).

Interestingly, Madeiran and central European specimens of *P. phalangioides* differed slightly in the morphology of the X_2 chromosome. While the X_2 chromosome of the Czech *P. phalangioides* was submetacentric (centromeric index 2.85), the X_2 of the Madeiran specimen was subtelocentric (centromeric index 3.96) (Ávila Herrera et al. 2021; this study). This change in morphology might result from chromosome rearrangement or addition of heterochromatin. The acrocentric morphology of the X_2 chromosome observed in some metaphases II of *P. phalangioides* is an artifact resulting from precocious separation of chromatids of this chromosome.

The sex chromosomes in *Pholcus* show a specific behavior in the male germline, which, like in other pholcids, includes positive heteropycnosis (more intensive staining), preferential location, and association or pairing. The association and heteropycnosis of sex chromosomes occur as early as during spermatogonial mitosis. Moreover, the sex chromosomes are usually located in the middle of spermatogonial plates, specifically on the metaphase plates (Král et al. 2006; Ávila Herrera et al. 2021; this study) and anaphase half plates (this study). Such behavior in spermatogonial anaphase has not been reported so far and it might occur in other spider species as well, not only in the taxa with the X_1X_2Y system. Due to its short duration, the spermatogonial anaphase is only rarely observed, which precludes analysis of sex chromosome behavior during this period. During the premiotic interphase in pholcids, the sex chromosome association evolves into pairing that continues up to metaphase I (Král et al. 2006; Ávila Herrera et al. 2021; this study). Chromosomes of the X_1X_2Y system are usually located at the periphery of the plate during early prophase I and diffuse stage. In contrast to that, during late prophase I and metaphase I, they tend to be in the middle of the plate. After segregation of the X and Y chromosomes, the X chromosomes are associated till the end of meiosis. The Y chromosome is usually located in the middle of half plates during anaphase II. Sex chromosomes are positively heteropycnotic only in some phases of meiosis (Ávila Herrera et al. 2021; this study).

Metacentric chromosomes of the X_1X_2Y system pair without chiasmata in male meiosis, namely by the ends of both arms (Silva et al. 2002; Král et al. 2006; Ávila Herrera et al. 2021). In some species with a non-metacentric X_2 chromosome, both chromosome ends remain involved in chromosome pairing. In other species, however, the non-metacentric X_2 chromosome only pairs by the end of its long arm (Král et al. 2006; Ávila Herrera et al. 2021; this study). In *P. creticus*, both ends of the acrocentric X_2 chromosome take part in pairing. In *P. alticeps* and *P. dentatus*, which share the morphology of the X_2 chromosome with *P. creticus*, pairing by both ends was only observed in a small proportion of the cells probably because the pairing of the shorter arm is less stable and loosens during the hypotonization and fixation step of chromosome preparation. In *P. opilionoides*, pairing of the X_2 chromosome by both ends was only observed in the early diplotene; afterwards, the chromosome paired only by its long arm. In other *Pholcus* species with a monoarmed X_2 chromosome, only the long arm of X_2 was involved in pairing (Ávila Herrera et al. 2021; this study). This pattern was observed even in the absence of hypotonization (this study), which indicates that it is not an artifact.

NORs

So far, NORs have only been detected in a low number of spider species (see Forman et al. 2013; Král et al. 2013 for references), especially by the means of FISH (see Šťáhlavský et al. 2020; Reyes Lerma et al. 2021 for references). In pholcids, however, NOR patterns have been determined recently in many species by FISH (Ávila Herrera et al. 2021), which makes it possible to contextualize our data with previous knowledge on the NOR evolution in this family. Pholcid spiders show a highly variable numbers of NORs (one to nine), which in the majority of pholcids occur on chromosome ends (Ávila Herrera et al. 2021). Their terminal position suggests that the NORs spread within the karyotype mostly by ectopic recombination, which is most effective in telomeric areas (Goldman and Lichten 1996). NOR bearing pairs in pholcids have a biarmed morphology except for the acrocentric pair found in the present study in most *Pholcus* species with the karyotype formula 25, X_1X_2Y. Unlike in other spiders, the spreading of NORs to sex chromosomes is quite common in haplogynes (including pholcids, where it has occurred at least five times) (Král et al. 2006; Ávila Herrera et al. 2021).

The ancestral pattern of the subfamily Pholcinae probably involves three chromosome pairs with a terminal NOR each. Prior to the separation of *Aetana* Huber, 2005, a NOR locus appeared on one end of the X_1. Thereafter, the NORs gradually spread to the other end of the X_1 chromosome and to the end of the long arm of the X_2, i.e., to the regions that ensure the achiasmatic pairing of the sex chromosomes. We assume that the sex chromosome-linked NORs (SCL-NORs) take part in this pairing (Ávila Herrera et al. 2021), probably together with the sequences of the Y chromosome invading the end of the X_2 (Sember et al. 2020).

Our study reveals a considerable diversity of NOR patterns in *Pholcus*. Based on data from *Pholcus* and the closely related genera, we suppose that the ancestral NOR pattern of *Pholcus* probably comprised two or three chromosome pairs with a terminal NOR locus each and three terminal X chromosome-linked loci (two on the X_1 chromosome and one on the X_2). The number of loci has then increased in some species and decreased in others (Ávila Herrera et al. 2021; this study). In *P. pagbilao* Huber, 2011, four NOR bearing pairs have been found, one of them with two terminal NORs (Ávila Herrera et al. 2021). Four NOR-bearing pairs were also found in the Madeiran cytotype of *P. phalangioides* (this study).

A reduction in the number of NORs has occurred repeatedly in *Pholcus*, both on chromosome pairs and on chromosomes of the X_1X_2Y system. Thus, the Macaronesian clade exhibits a single acrocentric NOR-bearing pair. *P. fuerteventurensis* from the Canaries retained a single SCL-NOR located at the end of the X_2 chromosome. The two Madeiran species share a degeneration/loss of SCL-NORs. In the *P. crypticolens/opilionoides* group, the reduction was more extensive in SCL-NORs than in NORs located on chromosome pairs. The pattern of *P. opilionoides* differs from the supposed ancestral pattern only by the absence of the X_2-linked NOR, while the pattern of *P. creticus* is more derived, the SCL-NORs are degenerated/lost (this study). In *P. pagbilao* (*P. bicornutus* group), the number of NOR-bearing chromosome pairs has increased to four whereas

SCL-NORs were degenerated/lost (Ávila Herrera et al. 2021). Remarkably, particular clades differ in their pattern of reduction of SCL-NORs. In the *P. phalangioides* and *P. crypticolens/opilionoides* groups, the X_2-linked NOR has been degenerated/lost first. In the Macaronesian clade, however the degeneration/loss has first affected the X_1-linked NORs (this study). The rDNA sequences responsible for achiasmatic pairing of sex chromosomes could be retained even after degeneration of SCL-NORs, as already reported from the males of *Drosophila* Fallén, 1823 (Roy et al. 2005). The reasons for the repeated degeneration of SCL-NORs in *Pholcus* are unclear. All species without SCL-NORs are island species. Island populations are frequently reduced and thus experience genetic drift, which could lead to random fixation of sex chromosomes without NORs. Moreover, genetic drift is more effective in case of sex chromosomes whose number in the population is reduced in comparison with autosomes (Johnson and Lachance 2012). Within the subfamily Pholcinae, the loss of the SCL-NORs had also occurred in a clade including *Canticus* and *Micropholcus*. In this case, the loss of these NORs has been accompanied by a conversion of the X_1X_2Y system to X0 (Ávila Herrera et al. 2021).

Karyotype diversity in *P. phalangioides*

P. phalangioides showed intraspecific diversity of the NOR pattern and chromosome morphology. Considering NORs, the Czech cytotype exhibited the supposedly ancestral pattern of *Pholcus* (Ávila Herrera et al. 2021). In the Madeiran cytotype, the number of the NOR-bearing pairs has increased to four, each pair containing one NOR locus. The NOR on the X_2 chromosome has been lost. Intraspecific variability in the NOR number has not previously been reported from pholcids, but it could be expected based on the occurrence of heterozygotes for number of NORs in some species (Ávila Herrera et al. 2021).

The karyotype differences between the Czech and Madeiran cytotype were, however, more profound. They also differed in the morphology of some chromosomes. The chromosome pairs of the Madeiran cytotype showed the original pattern; they included a large acrocentric pair, which has changed to biarmed in the Czech cytotype. Furthermore, both cytotypes differed to some extent in the morphology of the X_2 chromosome. Intraspecific differences in chromosome morphology have not been previously reported from pholcids. Whether the presence of different cytotypes is in any way related to the apparent *COI* polymorphism in this species (documented in the sequences deposited at NCBI) is unknown. The status of both cytotypes should be further analysed using larger samples and approaches of integrative taxonomy.

Conclusions

We present new data on karyotypes and meiotic division of seven species of the genus *Pholcus* (Pholcidae) from Europe. The selected species represent several different species groups within the region whose relationships among each other remain largely

unknown. The male karyotype is composed of 25 chromosomes with a X_1X_2Y sex chromosome system. The sex chromosomes pair without chiasmata during male meiosis. The karyotypes are predominated by biarmed chromosomes. The karyotypes of most species contain an acrocentric chromosome pair, which has changed to biarmed in some taxa. This marker is either a synapomorphy of the species groups included in this study or a synapomorphy of the genus *Pholcus*. Closely related species usually differ in the morphology of one or several chromosome pairs, which suggests the operation of pericentric inversions and/or translocations. Such rearrangements have been implicated in speciation. The chromosomes X_1 and Y show a metacentric morphology. By contrast, the X_2 chromosome is usually acrocentric. NOR patterns are very diversified. In the ancestor of *Pholcus*, these structures were located both on chromosome pairs and on sex chromosomes. Sex chromosome-linked NORs could be involved in the pairing of sex chromosomes. Most of the analyzed species show a specific pattern of NORs. Nucleolus organizer regions have often been degenerated/lost during evolution. Remarkably, the loss seems to preferably affect SCL-NORs. The reason for this phenomenon is unclear. The rDNA sequences crucial for sex chromosome pairing might remain unaffected by the degeneration. *P. phalangioides* yielded two cytotypes, which differ in their chromosome morphology and NOR pattern. Some of the detected chromosome changes appear phylogenetically informative. Although the Macaronesian clade shows a very high rate of speciation, species of this lineage do not differ substantially in the number of chromosome changes from other analyzed lineages of *Pholcus*. However, this conclusion needs to be corroborated by an analysis of more species and species groups.

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Supplementary material 1

Species studied, male karyotype data (including standard deviation)

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Data type: Table (MS Excel file)

Explanation note: Abbreviations: parameters = parameters used to describe chromosome morphology [CI = centromeric index, RCL = relative chromosome length (% of TCL)], specimens = number of specimens used to obtain data (*specimens from Stavros were analysed). Chromosome morphology is indicated by background colour of a box (pink: metacentric, brown: submetacentric, dark blue: subtelocentric, light blue: acrocentric).

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RESEARCH ARTICLE

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Integrative analysis reveals cryptic speciation linked to habitat differentiation within Albanian populations of the anomalous blues (Lepidoptera, Lycaenidae, *Polyommatus* Latreille, 1804)

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Abstract

The Balkan Peninsula is one of the greatest hotspots for biodiversity in Europe. While the region has been investigated thoroughly, some parts remain understudied and may still harbour undiscovered diversity, even in well-studied organisms such as Lepidoptera. Here we investigated the group of the so-called anomalous blue butterflies, also known as 'brown complex' of the subgenus *Agrodiaetus* Hübner, 1822 including the taxa of the entire *Polyommatus aroaniensis* (Brown, 1976) species complex. This species complex is distributed in the southern part of the Balkan Peninsula and known to be represented by three closely related allopatric species, differentiated by their chromosome numbers (n) and mitochondrial (mt) DNA. These are *P. aroaniensis* sensu stricto (Southern Greece, Peloponnese, n=47–48; mt haplogroup *aroa1*), *P. timfristos* Lukhtanov, Vishnevskaya et Shapoval, 2016 (Central Greece, Attika, n=38, *aroa2*) and *P. orphicus* Kolev, 2005 (North-Eastern Greece, Southern Bulgaria, n=41–42, *orph1*).

Based on an analysis of chromosomal, molecular and morphological markers, we demonstrate that a fourth taxon of this species complex exists in Albania. This taxon possesses the mt haplogroup *aroa3*, which is the most differentiated within the entire *P. aroaniensis* species complex, and the karyotype (n=42–43), which differs by one fixed chromosome fission from *P. orphicus*. The Albanian taxon seems to be ecologically specialised (habitat on dark-coloured, ophiolitic substrate soils) and differs in colouration (wing

reflectance) from the other taxa of the *P. aroaniensis* species group. Based on the evidence here presented and following the current view of the taxonomy of the group, we propose considering the Albanian taxon as a new species, here described as *Polyommatus lurae* **sp. nov.** At the contact zone between the new species and *P. orphicus*, in addition to typical ones, we detected specimens with haplogroup orph2, karyotype n=43 and intermediate morphology, which seem to represent *P. lurae* × *P. orphicus* hybrids.

Keywords
biodiversity, chromosome number, *COI*, conservation, DNA barcoding, karyotype, mitochondrial marker, protected species, wing colour morphometrics

Introduction

The so-called anomalous blues of the subgenus *Agrodiaetus* Hübner, 1822 constitute a distinct lineage within the species-rich genus *Polyommatus* Latreille, 1804 (Talavera et al. 2013a). The subgenus *Agrodiaetus* is distributed throughout the Western Palaearctic and Central Asia. With over 120 species described, this subgenus is a striking example of explosive radiation in the last three million years (Kandul et al. 2004; Kandul et al. 2007; Talavera et al. 2013b). Sexes are often dimorphic, with females usually brown in colour, and with males displaying colours such as blue, white, silver, orange, violet, or brown on the dorsal part of the wings, which is probably a signature of the reinforcement of pre-zygotic isolation (Lukhtanov et al. 2005; Dincă et al. 2017).

In Europe, most *Agrodiaetus* species are restricted to the southern warm parts of the continent, and most of them are endemic to relatively small areas. The Balkans are one of the richest European regions for subgenus *Agrodiaetus* (Kudrna et al. 2015) and, currently, the following taxa of *Agrodiaetus* with brown males are recognised as present in this region: *Polyommatus admetus* (Esper, 1783), *Polyommatus ripartii* (Freyer, 1830) and those under the so-called *Polyommatus aroaniensis* Brown, 1976 species complex. In this paper, we focus on the latter species complex, which remains still insufficiently studied, especially in underexplored regions like Albania.

The first species of "brown" *Agrodiaetus* described as endemic to the Balkans was *P. aroaniensis* (Brown, 1976). After Coutsis (1972) attracted attention to a population of "*P. ripartii"* on Mt. Chelmos in Southern Greece, noting that in "about half of the specimens the white streak on the hindwing underside, always present in *ripartii*, was completely absent". Brown (1976) wrote about this population from Chelmos as an "unrecognized *Agrodiaetus* sp. similar and often sympatric with *ripartii* [*pelopi*] in Greece" (Kolev and van der Poorten 1997). The haploid chromosome number initially identified by Brown (1976) as n=15–16 was later corrected to n=47–48 (Lukhtanov et al. 2003). Apart from the unique chromosome number, typical *P. aroaniensis* is characterized by a coffee-brown colour with a perceptible reddish hue and by the lack of darker marks along the margins. While *P. aroaniensis* was apparently first discovered based on the absent or vestigial white streaks on the hindwings, it was later discussed that this trait was only valuable for about 60% of the specimens in the population (Kolev

and van der Poorten 1997). Using the white stripe state (absent, reduced or prominent presence) as an identification trait for *P. aroaniensis* turned out to be difficult, as has been recently shown by the misidentification of *P. aroaniensis* in Croatia. Indeed, some specimens lacking this white stripe were initially determined as *P. aroaniensis* based only on this morphological trait, and were later corrected into *P. ripartii* by the use of the mitochondrial gene *COI* for identification (Lovrenčić et al. 2016). Yet, the distribution of *P. aroaniensis* seems to be much broader and at present it is still not fully understood where the limits of distribution lie, especially for the northern distribution range in Albania, North Macedonia and Bulgaria.

Then, almost three decades later, Lukhtanov and Wiemers (2003) described a new species of the brown *Agrodiaetus* from the extreme east of Turkey (Van province): *Polyommatus dantchenkoi* (Lukhtanov et Wiemers, 2003). About 2000 km to the west, another taxon with the same chromosome number (n = 41–42) was found in the Bulgarian Rhodope Mountains in the border area with Greece. This new taxon was initially considered a subspecies of the Turkish taxon: *Polyommatus dantchenkoi orphicus* Kolev, 2005 (Kolev 2005), but is now recognized as a separate species following the latest nomenclature by Wiemers et al. (2018): *Polyommatus orphicus* Kolev, 2005. The relationship to the taxon *Polyommatus eleniae* Coutsis et J. de Prins, 2005 (Coutsis and De Prins 2005) described in the same year from Northern Greece in almost the same area is still somewhat confusing in the available literature. Tshikolovets (2011), who treats *P. orphicus* as a subspecies (*Polyommatus dantchenkoi orphicus*), writes about *P. eleniae*: "With the same chromosome number as *dantchenkoi* but differing in reduced marginal spots on the underside of hindwings." In contrast, Kudrna et al. (2011) states: "*Polyommatus orphicus* is a newly discovered supposedly distinct species reported so far from Bulgaria (Sliven, Smolyan), Greece (Mt. Falakro) and Macedonia (Kozjak, Rudina, Asandjura) by Wiemers et al. (2009) and M. Wiemers (pers. comm.)". And "*P. eleniae* Coutsis et Prins, 2005, is a junior subjective synonym of *P. orphicus*; the printed dates of publication being 01.12.2005 and 07.06.2005 respectively. Both taxa appear to be identical" (Kudrna et al. 2011; Kudrna 2019). While the markings of the withe stripe on the underside of the hindwing are less pronounced for *P. eleniae*, synonymy with *P. orphicus* has been proposed by Vishnevskaya et al. (2016) based on identical results of genetical markers, mitochondrial (*COI*) and nuclear (*ITS2*), and karyology (n=41–42). This view has been accepted in the checklist by Wiemers et al. (2018), which we follow in this paper.

The above shows that the systematics of the "brown" *Agrodiaetus* is complex. Indeed, more recently, Vishnevskaya et al. (2016) demonstrated that *P. aroaniensis* in Greece actually contained two cryptic species. By applying a combined analysis of mitochondrial and nuclear markers and karyotype, *P. timfristos* Lukhtanov, Vishnevskaya et Shapoval, 2016 was described from Mt. Timfristos and adjacent Parnassos mountains with a different karyotype (n=38) and mitochondrial haplogroup compared to *P. aroaniensis* s.s. In summary, the latest accepted systematics considers the presence of three species of the *Polyommatus aroaniensis* species complex in the Balkans: *P. aroaniensis* s.s., *P. orphicus* and *P. timfristos*.

Generally, identification of species in the subgenus *Agrodiaetus* remains challenging because of considerable geographic and individual variability in habitus (Dincă et al. 2013), e.g. the state of the white stripe on the underside of hindwings. However, modern identification techniques based on genetic markers in combination with karyological data, often obtain reliable differentiation between *Agrodiaetus* taxa (Lukhtanov and Dantchenko 2002; Wiemers 2003; Kandul et al. 2004; Lukhtanov et al. 2006; Vila et al. 2010). Besides, at species level, subtle fixed variations in traits such as wing colour are still useful for identification in the *Agrodiaetus* subgenus and other *Polyommatus* sp(p)., especially male dorsal wing colour (Lafranchis 2004). Indeed, new morphometric techniques based on measurements of wing colour and its reflectance pattern have been tested for species identification and delimitation. For example, remarkably good correlations with genetic markers have been found for *Lysandra bellargus* and *Polyommatus icarus* (Kertész et al. 2021; Piszter et al. 2021) and this colour morphometric technique can also be useful for discriminating between species of the *Agrodiaetus* subgenus.

Thus, an integrative analysis combining multiple markers and techniques may be the best solution to resolve complex systematics and to uncover potential cryptic diversity in the *Agrodiaetus* subgenus (Lukhtanov and Dantchenko 2017; Kertész et al. 2021). By using such an integrative approach, based on combined molecular, cytogenetic, and colour morphometric analyses, we here demonstrate that a fourth taxon within the entire *Polyommatus aroaniensis* species complex is present in Albania.

Methods

Sample collection and storage

All butterflies were collected by L. Parmentier at the different biotopes investigated in Albania, in the provinces of Korçë, Elbasan, Dibër and Kukës. Collected samples were put in glassine envelopes in the field, a unique code assigned to each and stored in cooled plastic boxes. At different sites, a selection of fresh male samples was kept alive, until the posterior part of the abdomen was removed for karyological analysis. Taking into account the possibility of multiple cryptic species within a local area even in well-studied European butterflies (Dincă et al. 2011; Hinojosa et al. 2022), multiple individuals were collected in each place, paying special attention to the specimens with unusual or intermediate morphology (Vishnevskaya et al. 2016). Unless otherwise noted, all collected samples were imagoes that were spread and dried to be used for the analysis of habitus, and all are stored in L. Parmentier's private collection (LPAcoll, Zulte, Belgium). Legs used for DNA analysis are stored in R. Vila's Collection (RVcoll, Institute of Evolutionary Biology, Barcelona, Spain) and karyotype plates in the Zoological Institute of the Russian Academy of Sciences. Pictures of biotopes were taken with an Iphone 6 and butterflies with a Canon 70D and a 100mm macro lens.

DNA extraction and sequencing

In order to elucidate the genetics of the subgenus *Agrodiaetus* in Albania, we analysed DNA of 41 Albanian specimens (males and females). To put them in context, we mined 19 additional *COI* sequences from GenBank, a subset where the most similar sequences to the Albanian ones were included and overlapped at least 650 base pairs (bp) of the cytochrome c oxidase subunit (*COI*). DNA extraction was done following the protocol described in Dincă et al. (2017). The primers LepF1 and LepR1 (ATTCAACCAATCATAAAGATATTGG and TAAACTTCTGGATGTCCAAAAAATCA respectively) were employed for the *COI* amplification, obtaining a full DNA barcode fragment of 658 base pairs (bp). Double-stranded DNA was amplified in 25 µl volume reactions: 14.4 µl ultra-pure (HPLC quality) water, 5 µl 5X buffer, 2 µl 25mM $MgCl_2$, 0.5 µl 10 mM dNTP, 0.5 µl of each primer (10 µM), 0.1 µl Taq DNA Polymerase (Promega) and 2 µl of extracted DNA. Conditions for the PCR cycles were set as follow: first denaturation step at 92 °C for 60 s, then 92 °C for 15 s, 48 °C for 45 s and 62 °C for 150 s in 5 cycles and other 30 cycles changing the annealing temperature to 52 °C with the final extension step at 62 °C for 7 min. A 411 to 440 bp fragment at the 5' end of the nuclear *ITS2* was amplified by polymerase chain reaction using the primers ITS3 (GCATCGATGAAGAACGCAGC) and ITS4 (TCCTCCGCTTATTGATATGC) (White et al. 1990). The reactions were prepared as for *COI* but, in this case, the typical thermal cycling profile was: 95 °C for 45 s, 51 °C for 60 s and 72 °C for 60 s, for 40 cycles. PCR products were purified and Sanger sequenced by Macrogen Inc. Europe (Amsterdam, the Netherlands). All new *COI* and *ITS2* sequences have been deposited in GenBank (ON715895–ON715938 for *COI* and OP537924–OP537930 for *ITS2*) (Table 1).

Phylogenetic inference

The *COI* analysis involved 60 sequences (19 GenBank sequences and 41 own material). For the *COI* phylogeny, sequences of different length (from 647 to 657 bp) were included into the final dataset alignment. We used Geneious Prime 2019.0.3 (https://www.geneious.com) software to align the sequences and then edited them manually. The final *COI* alignment included 657 sites, with 137 variable sites and 112 parsimony-informative sites. A phylogeny was reconstructed in BEAST v2.5.0 (Bouckaert et al. 2014). Parameters were estimated using two independent runs of 30 million generations each and convergence was checked with TRACER 1.7.1 (Rambaut, 2018). A burn in of 10% was applied. Samples of *P.* (*A.*) *damon* were used to root the tree. Besides, a haplotype network of the *COI* barcode region was created in POPART v1.7 (Leigh & Bryant, 2015) using the TCS method.

Based on Wiemers et al. (2009), *ITS2* secondary structure improves phylogeny estimation of the subgenus *Agrodiaetus* and thus we combined mitochondrial and nuclear sequences to improve phylogenetic signal, in agreement with Vishnevskaya

et al. (2016). This resulted in a concatenated *COI* + *ITS2* alignment with a total of 1039 bp. Phylogeny on concatenated sequences was reconstructed in BEAST v2.5.0 (Bouckaert et al. 2014) on a subset of Albanian specimen, covering all study sites, and extra sequences mined from GenBank (9 own material and 45 extra sequences). Phylogenetic relationships were inferred using Bayesian Inference (BI), maximum likelihood (ML) and maximum parsimony (MP) analyses. The Bayesian analysis of the concatenated matrix *COI*+*ITS2* was performed using the program MrBayes 3.2 (Ronquist et al. 2012) with default settings as suggested by Mesquite (Version 3.04. http://mesquiteproject.org): burn-in = 0.25, nst = 6 (GTR + I + G). Two runs of 10 million generations with four chains (one cold and three heated) were performed. The first 25% of each run was discarded as burn-in. The consensus of the obtained trees was visualized using FigTree 1.3.1. (http://tree.bio.ed.ac.uk/software/figtree/). The samples of *P. damon* were used to root the tree. The NJ analysis of the concatenated matrix *COI*+*ITS2* was performed using the program Mega X (Kumar et al. 2018) and Tamura3-parameter+G as the optimal model (also estimated by Mega X). The samples of *P. damon* were used to root the tree. The standard nonparametric bootstrap (Felsenstein, 1985) (100 replicates) was used to evaluate statistical nodal support of the tree.

Analysis of karyotype

Testes were removed within 1 hour after collection and were stored in the 3:1 fixative for several months at +4 °C and then stained with 2% acetic orcein for 30 days at 20 °C. In total we karyotyped a selection of 15 samples representative for the different species and biotopes handled in this paper. We used a two-phase method of chromosome analysis as described in Lukhtanov and Dantchenko (2002). In the first phase, the stained testes were placed into a drop of 40% lactic acid on a slide, the gonad membranes were torn apart using fine needles and intact spermatocysts were removed and transferred into another drop of 40% lactic acid. Intact spermatocysts were studied and photographed. The first phase was most useful for counting the number of chromosome bivalents and multivalents. In the second phase, different stages of chromosome spreading were studied using a slight, gradually growing pressure on the coverslip. The second phase was most useful for studying the chromosome structure and distinguishing between bi- and multivalents. By scaling up the pressure on the coverslip, we were able to manipulate chromosomes, e.g. change their position and orientation on the slide, and consequently to resolve controversial cases of contacting or overlapping bivalents. Haploid chromosome numbers were counted at metaphase I (MI) and/or metaphase II (MII) of meiosis.

Leica DM2500 light microscope equipped with HC PL APO 100×/1,44 Oil CORR CS lens and S1/1.4 oil condenser head was used for bright-field microscopy analysis. Leica lens HC PL APO 100×/1,40 OIL PH3 was used for phase-contrast microscopy analysis.

Table 1. List of the studied samples of brown *Polyommatus* (*Agrodiaetus*) from Albania.

GenBank nr *COI* barcode	GenBank nr ITS2	LPA coll code	RV coll code	Karyo–type ID	Species	Sex	Chromo–some number	Mt haplo-group (lineage)	Locality	Remark biotope: soil type
ON715909	–	17-70-01	17E536	–	*P. orphicus*	F	–	*orph2*	Valikardhë region, Zerqan	Pure karst soil
ON715910	–	17-70-02	17F269	–	*P. orphicus*	M	–	*orph2*	Valikardhë region, Zerqan	Pure karst soil
ON715911	OP537928	18-70-K75	18D275	K75	*P. orphicus*	M	n=42	*orph2*	Valikardhë region, Zerqan	Pure karst soil
ON715912	–	18-111-K11	18D211	–	*P. orphicus*	M	–	*orph2*	Valikardhë	Pure karst soil
ON715913	–	18-111-K48	18D248	–	*P. orphicus*	M	–	*orph2*	Valikardhë	Pure karst soil
ON715914	–	18-111-K18	18D218	–	*P. orphicus*	M	–	*orph2*	Valikardhë	Pure karst soil
ON715915	–	18-111-K93	18D293	–	*P. orphicus*	M	–	*orph2*	Valikardhë	Pure karst soil
ON715916	–	18-111-K94	18D294	–	*P. orphicus*	F	–	*orph2*	Valikardhë	Pure karst soil
ON715917	–	18-111-K95	18D295	–	*P. orphicus*	F	–	*orph2*	Valikardhë	Pure karst soil
ON715918	–	18-111-K25	18D225	–	*P. orphicus*	M	–	*orph2*	Valikardhë	Pure karst soil
ON715919	OP537929	18-111-K78	18D278	–	*P. orphicus*	F	–	*orph2*	Valikardhë	Pure karst soil
ON715923	–	18-124-X103	22A030	–	*P. orphicus*	M	–	*orph2*	Lurë region, NW of Cidhën	Pure karst soil
ON715924	–	18-124-X104	22A031	–	*P. orphicus*	M	–	*orph2*	Lurë region, NW of Cidhën	Pure karst soil
ON715925	OP537930	18-115-K76	18D276	K76	*P. orphicus*	M	n=42	*orph2*	Lurë region, Fushë Lurë	Valley near 2nd mountain, karst soil
ON715926	–	18-115-K66	18D266	–	*P. orphicus*	M	–	*orph2*	Lurë region, Fushë Lurë	Pure karst soil
ON715927	–	18-115-K67	18D266	–	*P. orphicus*	M	–	*orph2*	Lurë region, Fushë Lurë	Pure karst soil
ON715920	–	18-115-X97	22A024	–	*P. orphicus*	M	–	*orph2*	Lurë region, Fushë Lurë	Mixed ophiolite/ karst soil
ON715921	–	18-115-X101	22A026	–	*P. orphicus*	M	–	*orph2*	Lurë region, Fushë Lurë	Mixed ophiolite/ karst soil
ON715922	–	18-115-X102	22A029	–	*P. orphicus*	F	–	*orph2*	Lurë region, NW of Arras	Mixed ophiolite/ karst soil
ON715928	–	18-115-K80	18D280	K80	*P. orphicus*	M	n=42	*orph2*	Lurë region, Fushë Lurë	Mixed ophiolite/ karst soil
ON715929	–	18-115-K84	18D284	K84	*P. orphicus*	M	n=42	*orph2*	Lurë region, Fushë Lurë	Mixed ophiolite/ karst soil
ON715930	OP537931	18-115-K85	18D285	K85	*P. orphicus*	M	n=42	*orph2*	Lurë region, Fushë Lurë	Mixed ophiolite/ karst soil
ON715905	–	17-94-3	17E542	2017–03	*P. lurae* × *orphicus* putative **hybrid**	M	n=43, 44	*orph2*	Lurë region, Gurë-Lurë	Nectaring on flowers along road
ON715910	–	18-115-K69	18D269	K69	*P. lurae* × *orphicus* putative **hybrid**	M	n=43	*orph2*	Lurë region, Fushë Lurë	Mixed ophiolite/ karst soil
ON715921	–	18-115-K83	18D283	K83	*P. lurae* × *orphicus* putative **hybrid**	M	n=43	*orph2*	Lurë region, Fushë Lurë	Mixed ophiolite/ karst soil
ON715908	–	18-115-K90	18D290	K90	*P. lurae* × *orphicus* putative **hybrid**	M	n=43	*orph2*	Lurë region, Fushë Lurë	Mixed ophiolite/ karst soil
ON715895	–	18-115-X98	22A025	–	*P. lurae* **sp. nova**	M	–	*orph2*	Lurë region, Fushë Lurë	Mixed ophiolite/ karst soil
ON715896	OP537924	18-115-K71	18D271	K71	*P. lurae* **sp. nova**	M	n=41+ trivalent	*aroa3*	Lurë region, Fushë Lurë to Qafa e Lura	Ophiolite soil+mixed, **paratype male**
ON715897	–	18-115-K73	18D273	K73	*P. lurae* **sp. nova**	M	n=42	*aroa3*	Lurë region, Fushë Lurë	Mixed ophiolite/ karst soil, **paratype male**
ON715898	OP537925	18-116-K68	18D268	K68	*P. lurae* **sp. nova**	M	n=ca.42	*aroa3*	Lurë region, Qafa e Lura	Ophiolite soil, **paratype male**
ON715899	–	18-116-K70	18D270	–	*P. lurae* **sp. nova**	M	–	*aroa3*	Lurë region, Qafa e Lura	Ophiolite soil, **paratype male**

GenBank nr *COI* barcode	GenBank nr ITS2	LPA coll code	RV coll code	Karyo–type ID	Species	Sex	Chromo–some number	Mt haplo-group (lineage)	Locality	Remark biotope: soil type
ON715900	OP537926	18-116-K77	18D277	–	*P. lurae* **sp. nova**	F	–	*aroa3*	Lurë region, Qafa e Lura	Ophiolite soil, **paratype female**
ON715903	OP537927	18-116-K81	18D281	–	*P. lurae* **sp. nova**	M	–	*aroa3*	Lurë region, Qafa e Lura	Ophiolite soil, **paratype male**
ON715901	–	18-116-X100	22A028	K82	*P. lurae* **sp. nova**	M	n=43	*aroa3*	Lurë region, Qafa e Lura	Ophiolite soil, **HOLOTYPE male**
ON715904	–	18-118-K88	18D288	K88	*P. lurae* **sp. nova**	M	n=42	*aroa3*	Lurë region, Pregj Lurë	Ophiolites, **paratype male**
ON715902	–	18-119-K79	18D279	–	*P. lurae* **sp. nova**	M	–	*aroa3*	Lurë region, Pregj Lurë	Ophiolites, **paratype male**

Morphometric measurements of habitus (dorsal wing reflectance)

Wing colour is an important trait for identification of butterflies and a species-specific characteristic (Bálint et al. 2012), an indicator of genetic variation (Wasik et al. 2014), and evidence of a changing population (Hiyama et al. 2012; Kertész et al. 2021). Observing fixed differences in wing colour of butterflies of different population can serve as a reliable tool for species identification (Bálint et al. 2010). Here we used colour measurements of dorsal wings of male *Agrodiaetus* to generate standardized RGB measurements of set specimens. In our set-up a constant light source in a darkened room was used (3 Marbul® suspension light sources of 12 W, 955 lm, 3500 K) in a triangle position at 1 m above the specimen to obtain a reproducible and uniform light source and minimize shades. RGB pictures were made on specimens positioned at an angle of 20° to the equatorial to measure maximum light reflectance generated by wing scale structures. The spectral position of the reflectance maximum of such photonic nanoarchitectures depends on the nanoscale geometric dimensions of the elements building up the nanostructure and was based on earlier experience and method described by Kertész et al. (2021). To obtain a uniform colour zone, the intervein space – which showed most reflectance variability- of the inner postdiscal zone (only between M1 and CU2 cells) was used. Per measurement a circular area of the wing was blurred in Lightroom and the average colour obtained was mapped on a disc. On this uniform colour discs (3 samples per wing), colour measurements were done generating exact RGB and HUE values using the colour picker tool. Averaged values per specimen were used for statistics. Wing colour measurements were taken only from fresh samples (worn specimens and those with minimal damage on fringe were discarded) belonging to the *Polyommatus aroaniensis* species complex collected from different Albanian localities, with habitats harbouring ophiolitic, karst and mixed substrates, as detailed in Suppl. material 1.

Statistical analysis of wing morphometrics

NMDS plots based on 27 specimens were obtained using the Adonis script (Vegan package) in R (Oksanen et al. 2016). Ellipses indicating 95% confidence intervals representing species identifications based on *COI* results (*orph2* and *aroa3*) and

karyotype (*orphicus*, *lurae*), obtaining the categories 'orphicus', 'lurae', and 'hybrid' (as a few specimens showed mitochondrial-karyotype discordance). In the analysis, substrate type was also integrated as a factor, with three categories 'ophiolite', 'karst', and 'mixed'.

Posterior statistics was done running a permutational multivariate analysis of variance, using distance matrices with the Adonis call (Vegan package) in R (Oksanen et al. 2016).

Results

Biotopes and Albanian samples

In total, 251 *Agrodiaetus* samples belonging to the *P. aroaniensis* and *P. ripartii* species complexes were analysed. Specimens were collected from 94 Albanian different sites visited and distributed from Southern up to North-eastern Albania. Only in a minor part of the sites (7/94 sites), specimens were identified as belonging to the *P. aroaniensis* species complex, mostly in provinces Dibër and Korçë. Specimens with clear *P. ripartii* traits – based on habitus descriptions given by Tshikolovets (2011) and Vishnevskaya et al. (2016) – were excluded from the analyses, after some voucher samples were barcoded to confirm our identifications (data not shown). Especially the study sites in the Dibër province, Lurë region, attracted our attention because many individuals displayed a remarkably dark habitus and mostly were completely lacking the white stripe on the hindwing underside (Fig. 1a, b). Their biotope was atypical, with a dark ophiolitic substrate (Fig. 1d). Here, in the vicinity, *Onobrychis alba*, a known foodplant of different *Agrodiaetus* species, was found growing (Fig. 1e, f). Besides, in another region, near Valikardhë village, a second cluster of study sites potentially harbouring *orphicus/aroaniensis* populations (Fig. 1c) was identified, although the habitat soil type was visibly paler and consisted of typical karst (chalk) substrate.

Phylogenetic reconstruction

The phylogeny obtained by Bayesian inference based on 658-bp of the gene *COI* (Fig. 2) recovered the *P. orphicus* and *P. aroaniensis* species groups as distinct lineages (although with moderate support), in agreement with previous studies (Wiemers 2003; Lukhtanov et al. 2005; Vila et al. 2010; Lukhtanov et al. 2015; Vishnevskaya et al. 2016). *Polyommatus orphicus orphicus* and *P. orphicus eleniae* formed together a paraphyletic cluster (orph 1), while most of the Albanian *P. orphicus* specimens formed a sister group (orph 2), albeit node supports were low. The *P. aroaniensis s.l.* clade showed three highly-supported clades (posterior probability = 1) corresponding to *P. aroaniensis* (aroa 1), *P. timfristos* (aroa 2), and another one (aroa 3) exclusively composed by Albanian specimens, hereunder described as *P. lurae* sp. nov.

Figure 1. Biotope and specimens in their natural biotopes at the Lurë region **a** *P. lurae* sp. nov uns. (sample code 18-115-K71), Albania, Dibër prov., Lurë region, 24.VII.2018 in its biotope **b** *P. lurae* sp. nov ups., same specimen **c** Albanian *P. orphicus* (sample code 18-124-X103) in its biotope on Karst substrate, Dibër prov., Lurë region, 25.VII.2018, NW of Cidhën village **d** typical biotope of *P. lurae* with dark ophiolitic soil substrate **e, f** *Onobrychis alba*, host plant for *Polyommatus* (*Agrodiaetus*) sp., growing in ophiolitic soil substrate.

To construct the haplotype network, we used 54 specimens that were collapsed in 28 haplotypes representing 7 haplogroups (Fig. 3) with *P. damon* as outgroup: 3 haplogroups for the *P. ripartii species* complex (including *P. ripartii ripartii* and *P. ripartii pelopi*), 2 for *P. orphicus* (orph 1, and orph 2 representing the Albanian lineage) and 3 for the *P. aroaniensis s. l.* clade including *P. timfristos* (aroa 2), *P. aroaniensis* (aroa 1) and *P. lurae* sp. nov (aroa 3). The latter haplogroup is clearly distinct from related taxa as can be seen in the number of nucleotide substitutions between them.

The phylogenies based on the concatenated COI+ITS2 sequences (54 specimens, same as the haplotype network) and using the BI and NJ methods are given in Suppl. material 2. The NJ analysis of the concatenated matrix (using Tamura3+G as the best model) (Suppl. material 2, Fig. 1) revealed *lurae* as a highly supported, differentiated lineage with the most basal position within the *entire aroaniensis* species complex. However, this basal position had a low support. The BI analysis of the concatenated matrix (using default settings) (Suppl. material 2, Fig. 2) revealed *lurae* as a highly supported lineage, sister to *aroaniensis* (but the support for this sister relationship was

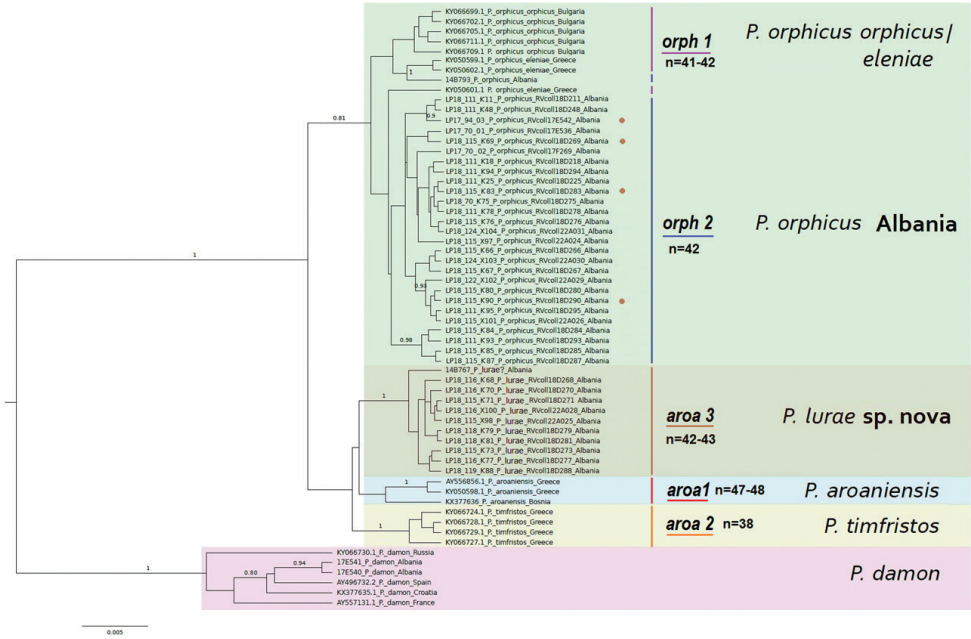

Figure 2. Bayesian inference tree based on 658-bp of the gene *COI* for the entire *Polyommatus aroaniensis* species complex. Node posterior probabilities (0.80 and higher) are written on the branches. The scale bar indicates the length of 0.005 substitutions/site. Main clades are highlighted and *COI* haplogroups and karyotype numbers indicated: *P. orphicus orphicus/eleniae* (*orph1*, n=41–42), *P. orphicus* Albania (*orph2*, n =42), *P. aroaniensis* (*aroa1*, n=47–48), *P. timfristos* (*aroa2*, n=38) and *P. lurae* sp. nov (*aroa3*, n=42–43). Brown dots next to specimens within the *orph2* clade indicate *P. lurae* × *P. orphicus* potential hybrids (n=43–44) found in the contact zone or dispersing.

very low). Generally, the concatenated alignments revealed the same topology as in the case of the COI tree, with very good support of P. *lurae* sp. nov as a well differentiated lineage, and putative sister to *P. aroaniensis*, although with lower support.

In all cases using different phylogenetic reconstruction models, and based on COI or concatenated *COI+ITS2* sequences, *P. lurae* sp. nov formed a monophyletic, well differentiated clade with very good support.

Karyotyping

Polyommatus orphicus from Albania (populations in which the only o*rph2* haplogroup is present)

In five studied samples (K75, K76, K80, K84, K85) the number of countable elements was found to be n=42 at MI and MII cells. Bivalents at MI and univalents at MII were fairly well differentiated with respect to their size; however, it was difficult to subdivide them objectively into size groups because the sizes of the elements decrease more or less linearly (Fig. 4).

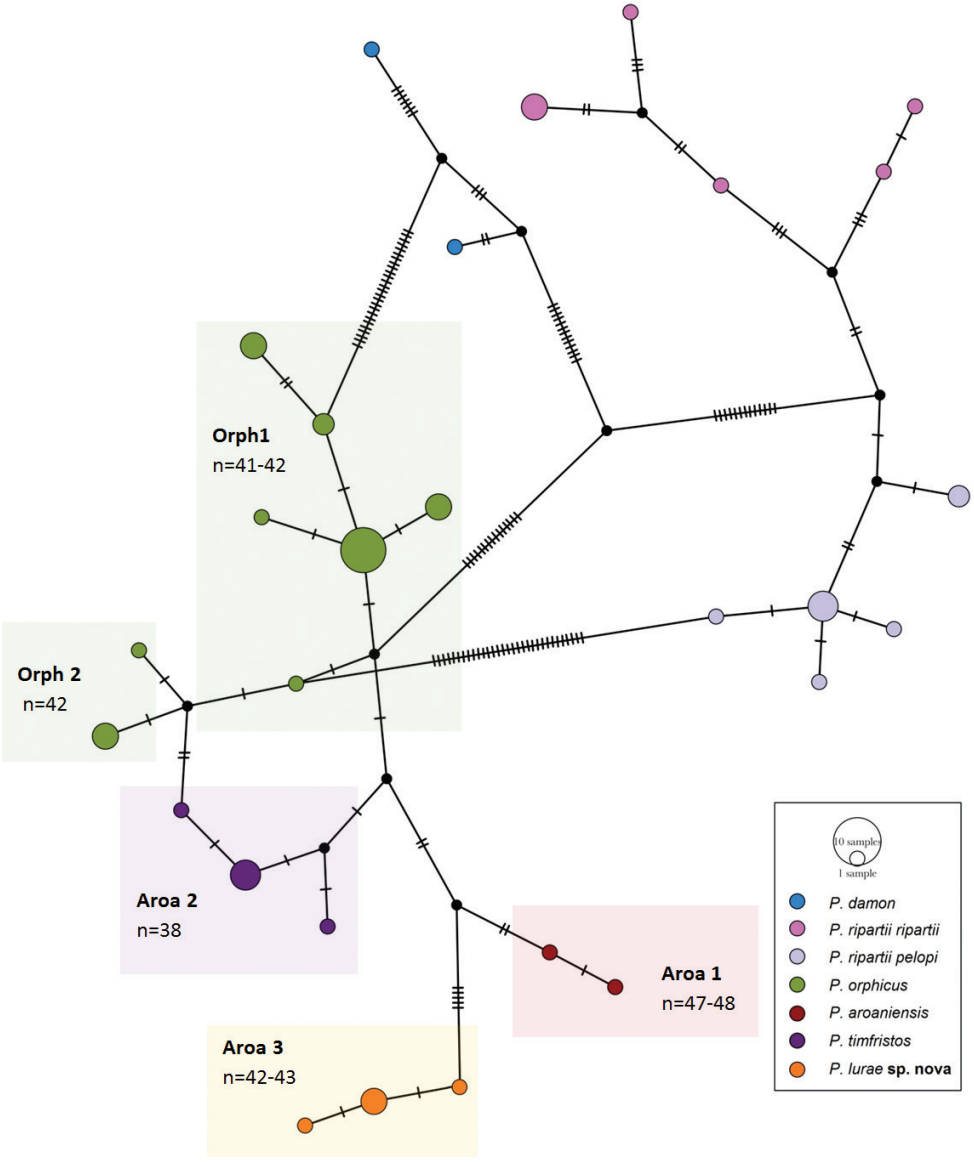

Figure 3. Haplotype network of the *P. aroaniensis* species group in relation to *P. damon* and *P. ripartii*. Coloured circles represent different taxa, as indicated in the legend; coloured boxes delimitate the haplogroups discussed in the text. Each line segment represents a mutation step, and black small circles represent "missing" haplotypes.

Polyommatus lurae sp. nov from Albania (populations in which the only *aroa3* haplogroup is present)

In five studied samples (K68, K71, K73, K81, K88), two different haploid chromosome numbers (n=42 and n=43) were observed at MI and MII cells of the 14 specimens studied. This variation was most likely caused by polymorphism for one chromosome

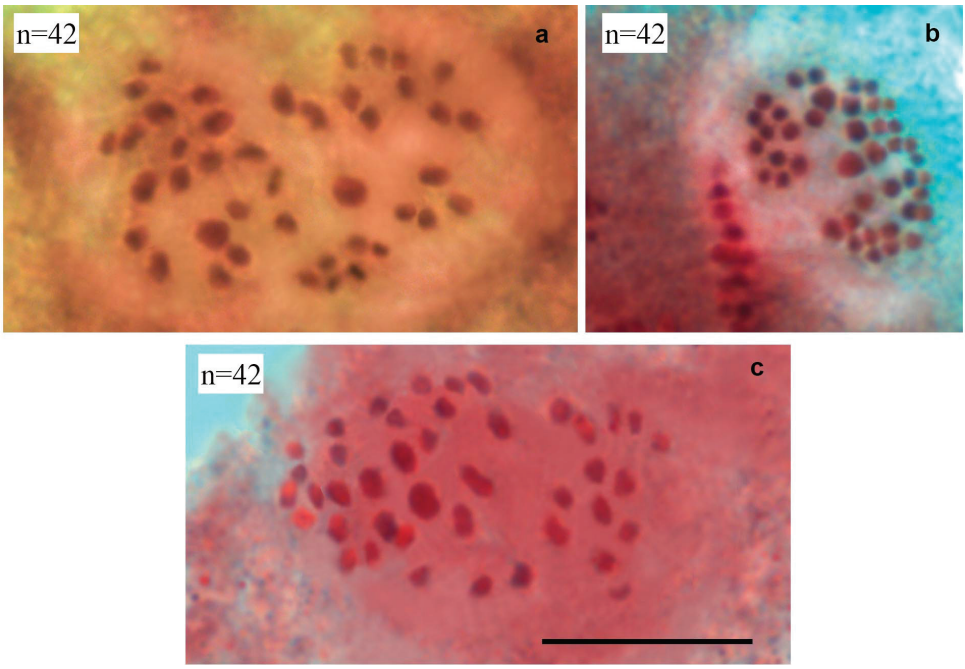

Figure 4. Karyotypes of *Polyommatus orphicus* from Albania **a** sample K75, MI, n=42 **b** sample K76, MII, n=42 **c** sample K80, MI, n=42. Scale bar: 10 μm.

fusion/fission. This polymorphism resulted in three types of MI karyotype: n=42 (homozygous for chromosomal fusion/fission, one pair of fused chromosomes; 2n=84), n=43 (homozygous for chromosomal fusion/fission, two pairs of unfused chromosomes; 2n=86) and n=42 (heterozygous for chromosomal fusion/fission, resulting in 41 bivalents and one trivalent; 2n=85). Bivalents at MI and univalents at MII were fairly well differentiated with respect to their size; however, it was difficult to subdivide them objectively into size groups because the sizes of the elements decrease more or less linearly (Fig. 5).

Contact zone with *Polyommatus lurae* × *P. orphicus* potential hybrids (area where both o*rph2* and a*roa3* haplogroups are present)

The contact zone between the two species was defined as the area where both *orph2* and *aroa3* haplogroups were found to coexist, and coincided with a mixed ophiolite/karst substrate. In this area, in addition to specimens typical to either species, a number of countable elements of n=43 at MI and MII cells was found for three samples with a mitochondrial haplogroup *orph2* (samples K69, K83, and K90). Bivalents at MI and univalents at MII were fairly well differentiated with respect to their size; however, it was difficult to subdivide them objectively into size groups because the sizes of the elements decrease more or less linearly (Fig. 6). In the sample 17E542 (also haplogroup *orph2*) a karyotype n=44 was also observed at the MI stage, along with n=43,

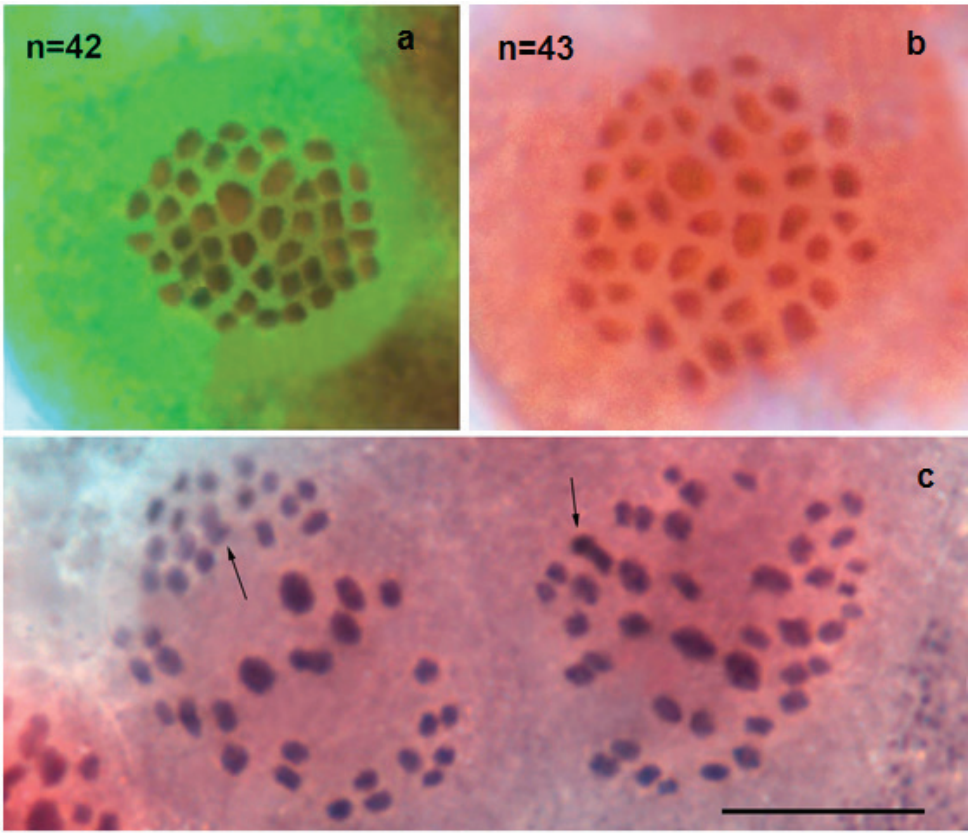

Figure 5. Karyotypes of *Polyommatus lurae* sp. nov from Albania **a** sample K73, MII, n=42 **b** sample K82, MII, n=43 **c** sample K71, two MI cells, each cell displays 41 bivalents and one possible trivalent (arrow). Scale bar: 10 μm.

most likely due to intraindividual chromosome fragmentation or a single-chromosome disjunction. This sample was found outside the contact zone nectaring on the flowers along a road and could be a dispersive specimen.

Distribution of species within *P. aroaniensis* complex in the Balkans

A distribution map based on current literature and predictions of the anomalous blues within the *P. aroaniensis* species complex in the Balkan peninsula is shown in Fig. 7a and a detailed map of the specimens found in Lurë region in Fig. 7b While several populations from Central and Northern Greece, as well as from other countries of the Balkan Peninsula were formerly identified as *P. aroaniensis*, based on Vishnevskaya et al. (2016) *P. aroaniensis* is only found in Southern Greece (Peloponnese). The sister taxon *P. timfristos*, of which it was then separated is currently only known from Mt. Timfristos and Mt. Parnassos in Central Greece. However, there is still a missing gap existing between *P. lurae* sp. nov and *P. timfristos* in Central to Northern Greece and it

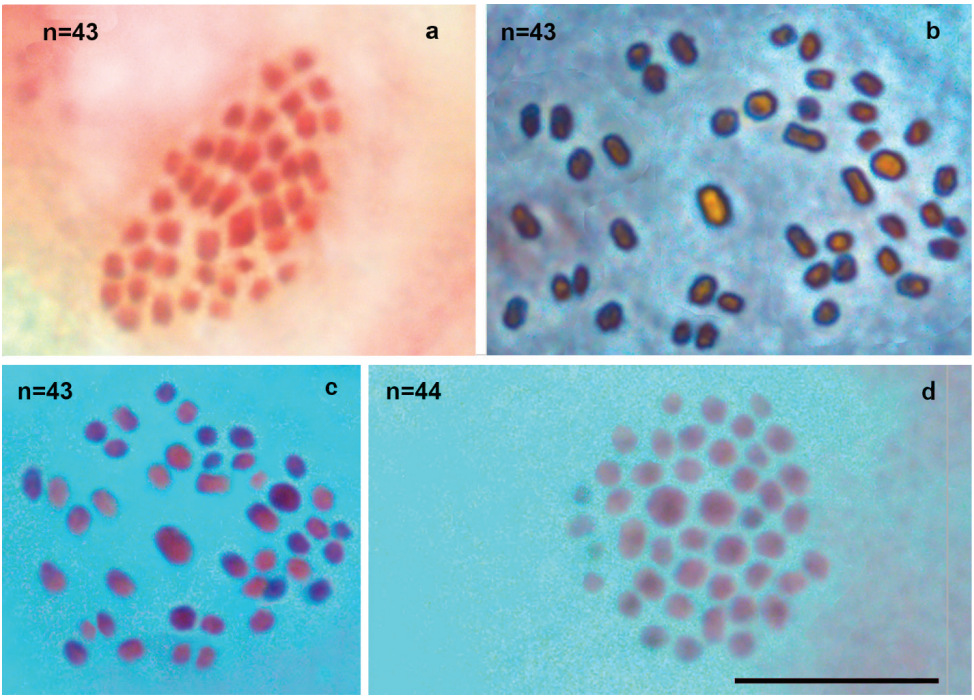

Figure 6. Karyotype of two putative hybrid specimens from the contact zone between *P. orphicus* and *P. lurae* sp. nov, where both orph2 and aroa3 haplogroups are present **a** sample K69, MII, n=43 **b–d** sample 17-094-3: **b** MI, n=43 (phase-contrast) **c** MI, n=43; **d** MI, n=44. Scale bar: 10 μm.

is not impossible that the latter can be found further northwards in suitable karst biotopes. For *P. orphicus*, the taxon is generally found in the northern parts of the Balkans (confirmed in Northern Greece and Bulgaria, and in North Macedonia). Here, we described also new populations of *P. orphicus* in Albania, and the Lurë area is the westernmost distribution of the species in the Balkans. Yet, between the Albanian populations and the former mentioned there still exist an intermediate gap of at least 500 km with insufficient distribution knowledge. Finally, in the light of the data obtained in this paper, the (possible) occurrence of *P. aroaniensis* s.l. in Kosovo, Northern Greece, North Macedonia needs to be further investigated as they could harbour unknown *P. orphicus* populations, and probably also populations of *P. lurae* sp. nov because biotopes with ophiolites exist there.

Morphometrics of male wings

The upperside wing colour is one of the main characteristic features of the anomalous blue butterflies. Vishnevskaya et al. (2016) used some external traits of the wing underside for differentiating between forms (types) of Balkan specimens under the "brown" complex, which we here reanalyse in view of Albanian taxa, here described for the first time, and identified under cryptic taxa *P. ripartii*, *P. orphicus* and *P. lurae*:

1. "*Polyommatus ripartii* type": hindwing underside with well-developed white streak, spots are small or medium-sized, marginal marking is reduced. According to Vishnevskaya et al. (2016) this type is found in different species including *P. admetus*, *P. timfristos*, *P. orphicus*, and *P. ripartii pelopi* (plates of male specimens shown in Vishnevskaya et al. (2016), which we confirm for the latter two species analysed. This type was never found in *P. lurae* specimens (data not shown).

2. "*Polyommatus aroaniensis* type": the white streak on the hindwing underside demonstrates different level of reduction. This type is found in *P. aroaniensis* s.s., *P. lurae* sp. nov (Fig. 8m). *P. timfristos*, *P. orphicus orphicus/eleniae* and Albanian *P. orphicus* (Fig. 8h). It is also found in the population of *P. ripartii* from the Crimea (Vila et al. 2010) and Croatia (Lovrenčić et al. 2016), but according Vishnevskaya et al. (2016) very rare in the Balkan peninsula while we also found some *ripartii* specimens with great reduction of the white stripe (example in Fig. 8o).

3. *Polyommatus orphicus* type: forewing underside with clear white postdiscal streak between discal spot and submarginal marking, white streak on hindwing underside is prominent, often with an additional small white streak. According to Vishnevskaya et al. (2016) this type is common in *P. orphicus orphicus* while mentioning that its most characteristic feature (the white postdiscal streak between discal spot and submarginal marking on the forewing underside) can be found in other species, e.g. *P. aroaniensis.* Only about one third of the Albanian specimens of *orphicus* showed the additional streak (data not shown), while it was never present in specimens of *P. lurae*.

4. *Polyommatus lurae* sp. nov type: forewing underside with no white postdiscal streak between discal spot and submarginal marking, white streak on hindwing underside is minimal and mostly completely lacking or invisible (Fig. 8b). This type is also found in a minor amount of Albanian *P. orphicus* (Fig. 8h). Based on pictures of the type series, also some *P. aroaniensis* specimens from Greece harbour this trait (Brown 1976).

We also analysed light reflection of male wings based on standardized colour measurements (RGB and HSV values) for 27 specimens. We focused on the species *P. orphicus* and *P. lurae*, and also included the few potential hybrid specimens (based on the atypical combination of mitochondrial haplogroup and karyotype results and always collected at the contact zone in the Lurë region. NMDS plots (Fig. 9) showed that wing reflection measurements matched significantly the phylogenetic clades *aroa3* and *orph2* (Df= 2, F=4.11, P=0.030). Specimens identified as *P. orphicus* generally showed a measurable reflectance on the postdiscal band of forewing (and hindwing) (Fig. 8h), while this trait is absent in *P. lurae* sp. nov (Fig. 8c). Interestingly, the two potential hybrids analysed showed an intermediate position in wing colour reflectance, falling in the overlapping area of the two species, as visualized by blue dots in Fig. 9. The intermediate reflectance is also noticeable on the putative hybrid specimen depicted in Fig. 8f.

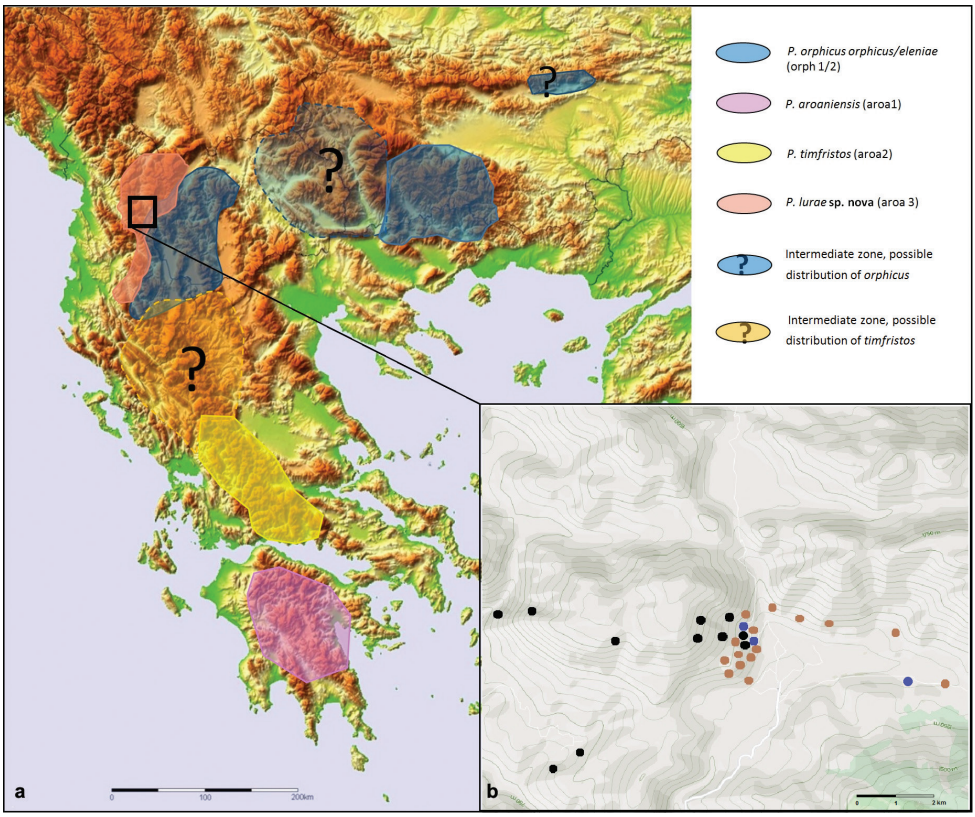

Figure 7. a distribution map based on literature and predictions of the anomalous blues within the *P. aroaniensis* species complex in the Balkan peninsula; colours correspond to the species in the legend; box is indicating the Lurë region in Albania **b** detailed map of the Lurë region with observations of *P. lurae* sp. nov (black dots) on dark ophiolites, *P. orphicus* (brown dots) and putative hybrids (blue dots) in the contact zone (blue dots) and a dispersive specimen outside suitable biotope (paler blue dot).

Next to this, a link with the soil substrate was tested and statistical analysis revealed that both species *P. lurae* and *P. orphicus* could significantly be linked with their locations harbouring typical soil substrates, i.e. dark ophiolitic versus light karts soils, respectively (Df= 3, F=4.39, P=0.014).

Taxonomy

The results showed a consensus between morphometrics, mitochondrial DNA and karyotype, in delineating three clades under the *P. aroaniensis* species complex. Two of them are generally accepted as species: *P. aroaniensis* and *P. timfristos* (Wiemers et al. 2018). Given that the genetic, morphological and karyotypic differentiation of the third clade is comparable to that between the other two species, we describe the Albanian population as a new species belonging to the *P. aroaniensis* s.l. species complex.

Figure 8. The colouration and wing pattern of *P. lurae* sp. nov, *P. orphicus* and *P. ripartii*. The letters correspond to the following species (and voucher sample codes as listed in Table 1): **a–c** *P. lurae* sp. nov **HT male** (18-115-K71) **d, f** *P. lurae* × *P. orphicus* putative hybrid (17-94-3) **g, i** *P. orphicus* (18-115-K76) **j, k PT female** (18-116-K77) **l, m** *P. lurae* **PT** (18-116-X100) **n–o** *P. ripartii* (same collecting data as 18-115-K76). Pictures of **c, f, i** were taken in natural sunlight but with same position as morphometric analysis (see in M&M section); Notice the differences in reflectance on dorsal wings in the postdiscal zone which is circled and indicated by arrow. Scale bar: 10 mm.

Polyommatus lurae **Parmentier, Vila et Lukhtanov, sp. nov**
https://zoobank.org/A561FDF8-DA47-4814-A976-C57A7F260386

Description. Typical dark ground colour of both veins and intervein space of dorsal wing sides. A character that appears useful for separation of *P. orphicus* and *P. lurae* sp. nov is the brighter yellow-greenish reflection of the former which is generally lacking in the newly described taxon. However, worn individuals of the two taxa may be indistinguishable externally and also from *P. ripartii*, which is found sympatrically in all locations studied. While Misja (2005) reports *P. admetus* from the same Lurë region, we never found *P. admetus* in sympatry with the new taxon in all Lurë locations surveyed. While the latter observation may be based on a wrong identification of the newly described taxon, also lacking white stripes on the hindwing underside, *P. admetus* is easily separated from *P. lurae*, especially because of the strongly marked underside in *P. admetus*, with a double row of small dots in the submarginal zone of underside wings, which has never been observed nor reported in literature in the taxa of the *P. aroaniensis* species complex, including *P. lurae*.

Holotype. (Fig. 8a–c) Male, field code specimen 18-116-X100, *COI* barcode number RVcoll22A028 (DNA extraction in RVcoll, Barcelona, Spain), GenBank accession number ON715901. Locus typicus: Albania, Dibër prov., Lurë region, Mountain ridge with ophiolitic soil substrate North of Cidhën near Fushë Lurë, 1250 m., 24.VII.2018, L. Parmentier leg. et coll. Holotype in LPA collection, Zulte, Belgium

Paratypes. Nine males and one female were studied in depth, with field codes of voucher specimen in LP collection (RVcoll number/ GenBank accession numbers of barcodes): LP18-115-K71 (RVcoll18D271/ ON715896), LP18-115-K73 (RVcoll18D273/ON715897), LP18-116-K68 (RVcoll18D268/ ON715898), LP18-116-K70 (RVcoll18D270/ ON715899), LP18-116-K77 (RVcoll18D277/ ON715900), LP18-116-K81 (RVcoll18D281/ ON715903), LP18-116-X100 (RVcoll22A028/ON715903), LP18-115-X98 (RVcoll22A025/ON715895), all North of Cidhën near Fushë Lurë, 1050–1600m. 23–24.VII.2018; LP18-118-K88 (RVcoll18D288/ ON715904), LP18-119-K79 (RVcoll18D279/ ON715902) Lurë region, Pregj Lurë 24.VII.2018. Additional material: 15 males, 5 females, same localities, collection dates 23- 24.VII.2018. All paratypes have red labels indicating *P. lurae* sp. nov, name of authors, signature of first author and exact localities.

Karyotype. The haploid chromosome number *P. lurae* sp. nov is determined as n=42–43 (Fig. 4).

COI **barcode sequence of the holotype.** 657 base pairs: AACATTATATTTTATTTTTTGGTATTTGAGCAGGAATAGTAGGAACATCTCTAAGAATTTTAATTCGTATGGAATTAAGAACTCCTGGATCCTTAATTGGAAATGATCAAATTTATAATACTATTGTTACAGCTCATGCATTTATTATAATTTTTTTTTATGGTTATACCTATTATAATTGGAGGATTTGGTAACTGATTAGTTCCCTTAATATTAGGAGCACCTGATATAGCCTTTCCCCGATTAAATAATATGAGATTTTGATTATTACCACCATCATTAATACTACTAATTTCTAGAAGAATTGTAGAAAATGGTGCAGGAACAGGATGAACAGTTTACCCC-

CCACTTTCATCAAATATTGCACATAGAGGATCATCTGTAGATTTAG-
CAATTTTCTCTCTTCATTTAGCAGGAATTTCTTCAATTTTAGGAGCAAT-
TAATTTTATTACAACTATCATTAATATACGAGTAAATAATTTATCTTTT-
GATCAAATATCATTATTTATTTGAGCAGTGGGAATTACAGCATTATTAT-
TACTTTTATCATTGCCTGTATTAGCTGGAGCAATTACCATATTACTAACA-
GATCGAAACCTTAATACCTCATTCTTTGACCCAGCTGGTGGAGGAGATC-
CAATTTTATATCAACATTTATTT.

Description. Males. (Fig. 8c, l, m). Forewing length 15.8–17.9 mm. ***Upperside*:** ground colour completely dark chocolate brown. Discoidal, submarginal and antemarginal markings absent on both fore- and hindwings. Veins poorly contrasting. Forewings with a developed sex brand and dark scale tuft. Fringe grayish brown. ***Underside*:** ground colour yellow-brown with ochreous to reddish coffee-milk tint. Minimal greenish blue basal suffusion. One basal black spot is present only on hindwings. Discoidal black spot is present on the forewings, but can be slightly seen on the hindwings (absent or vestigial). Postdiscal black ocelli most prominent on forewings; when present encircled by a whitish border. Postdiscal black ocelli on the hindwing small and sometimes lacking. Submarginal and antemarginal marking is absent on the forewings, and absent or vestigial on the hindwings. White streak on hindwings generally absent or very faint. Only rarely, the white streak is vestigial; no single specimen was observed with an additional short streak between postdiscal and submarginal areas of the wing, straight under the main white streak. Fringe brown, slightly darker than the underside ground colour.

***Male genitalia*.** The valva of the male genitalia of *P. lurae* sp. nov is depicted in Fig. 10. Male valves have a structure typical for other species of the subgenus *Agrodiaetus* (Coutsis (1986), Coutsis, pers. comm.). According to Kolev (2005) who studied the morphometry of the male genitalia of *P. orphicus* no overlap with *P. ripartii* was observed. As male genitalia within the *P. aroaniensis* species group do not significantly differ from each other, those from *P. lurae* may follow the same trend, but no additional analyses nor measurements have been performed.

Females. Forewing length 15.8–17.5 mm. ***Upperside*:** ground colour as in males, but lighter dark brown and without sex brand and scaletuft. Fringe greyish brown. ***Underside*:** ground colour and general design as in males but fringes lighter-coloured. Greenish blue basal suffusion almost invisible. White streak on hindwing underside mostly absent (Fig. 7j, k). If present, it demonstrates a variable level of reduction.

Life history. *Polyommatus lurae* inhabits xerothermic and xeromontane ophiolitic habitats. While in some of the localities the soil can be mixed with a minor degree of a calcareous component, *P. lurae* was never found at pure calcareous biotopes. Indeed, at such localities only *P. orphicus* was found, together with *P. ripartii*, which is in agreement with the original description of these species (Kolev, 2005). The vegetation of the type locality is sparse and dominated by low-growing grasses and flowering plants identified as *Artemisia alba* Turra and *Satureja montana* Linnaeus. Besides, other xerophilous species were observed, including scattered *Juniperus* bushes and low *Pinus nigra* trees (Fig. 1d). In all known localities *P. lurae* is syntopic with *P. ripartii*, a species widespread in the Balkans, although especially abundant in calcareous habitats (pers. obs. L. Parmentier).

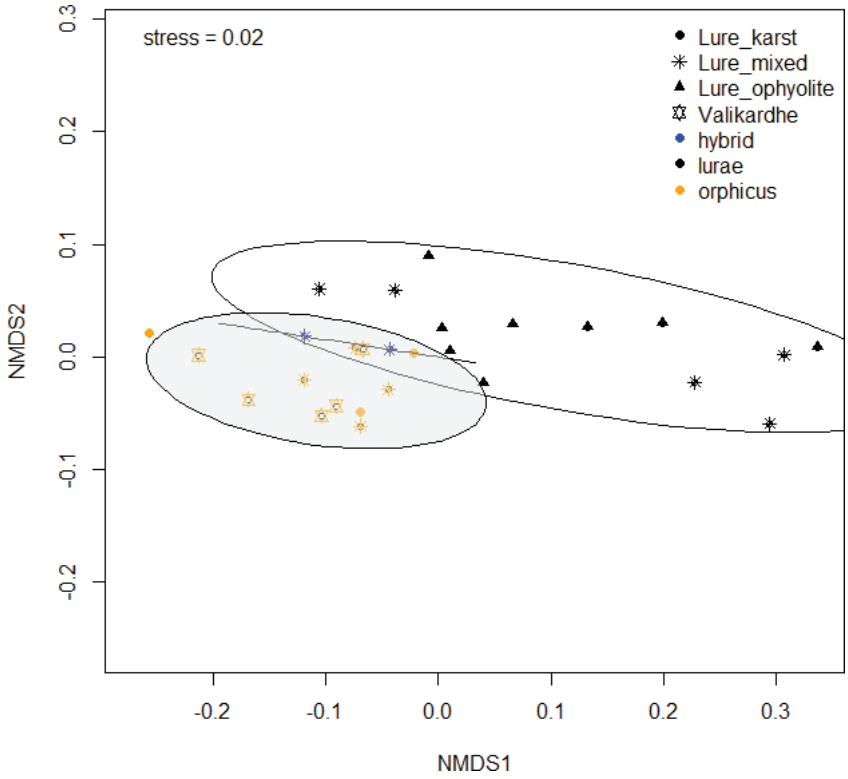

Figure 9. NMDS plot representing the morphometric analysis of dorsal male wings reflectance measurements of *P. orphicus* (orange dots) and *P. lurae* sp. nov (black dots). Stress = 0.02; specimens indicated in blue are potential *P. lurae* × *P. orphicus* hybrids collected in the contact zone and showed intermediate wing reflectance. Different symbols represent collection sites of three studied habitat with different substrate types (ophiolitic, karst, mixed). Ellipses represent 95% confidence intervals of specimen groups 'orphicus', 'lurae' and 'hybrid'.

Distribution and biotope. The three known localities of *P. lurae* (including the type locality) are situated in the Lurë region, in the vicinity of the National Park (Parku Kombëtar Lurë-Mali i Dejës), North of the village Cidhën, along a North-Southern orientated mountain ridge and gorge at altitudes between 950 and 1.600 m (Fig. 9a). The habitats are all situated within ophiolitic soil substrates (in some localities these substrates are slightly intermixed with a minor amount of lighter karst substrate), which are not rare in some parts of Albania. In these typical ophiolitic soil substrates the presumed host plants of the genus *Onobrychis* were observed (Fig. 1e, f). However, there are as yet no observations regarding the first stages of this taxon and the larval host plant is unconfirmed.

The aforementioned ophiolitic substrates can be found in a discontinuous range from Southern Albania (Provinces Korcë, Qukës) up to the Northern part of the country (provinces Dibër, Kukës). Within Europe these rather rare substrates are present mostly

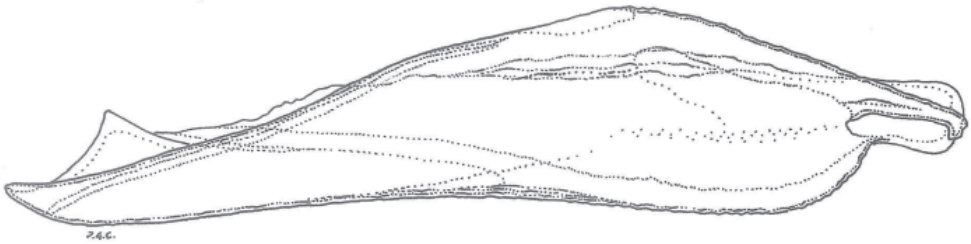

Figure 10. Valva of male genitalia of *P. lurae* sp. nov (G. Coutsis prep. 2018)

in Albania, while neighbouring countries of North Macedonia and Kosovo contain them to a minor degree. Thus, it is not impossible that the species is also present in other ophiolitic habitats where the presumed host plant is growing. Collection material from another locality in Voskopojë (Korçë prov.), situated more South, also harbouring typical dark ophiolitic soils was studied. In this locality, a single specimen (RVcoll14B767) genetically attributable to *P. lurae* was found by Sylvain Cuvelier and Morten Mølgaard, but it is not included in the type series because of the lack of karyological data and morphometrics. Additional specimens from this locality could not be found even after thorough explorations in 2018 and 2022, while only *P. ripartii* could be confirmed.

Differential diagnosis. From nominotypical *P. orphicus* the new taxon is generally distinguished by the strong reduction of a white postdiscal streak on the forewing underside, a darker colour of the upperside and underside wing, lack of wing reflectance, and less contrasting veins on the upperside. Its karyotype is different by at least one fixed chromosome fission (n=41–42) and its *COI* barcode. From *P. aroaniensis*, which is the most similar taxon externally, fresh individuals of the new taxon are distinguished by the constant presence of a typical dark ground colour of both veins and intervein space of dorsal wing sides and a generally darker colour of the upperside and underside wing (while in *aroaniensis* a warm reddish brown colour is typical). However, worn individuals may be indistinguishable externally, while they still can be identified by karyotype (n=48) and by the *COI* barcode. In the case of *P. lurae*, its dark habitus is linked to its typical environment with dark ophiolites, while the taxa *P. orphicus* and *P. aroaniensis* are generally found in biotopes with paler karst soil substrate. From the sympatric and syntopic *P. ripartii*, the new taxon is more easily distinguished by the absence of a white postdiscal streak on the forewing underside and, on average, a more reduced appearance of postdiscal spots, and on the upperside the veins are less pronounced and of a similar tone than the paler ground colour. This may be useful for discriminating even slightly worn individuals of the two taxa, while worn individuals are mostly indistinguishable externally. Yet, its karyotype (n=90) and *COI* barcode are strongly different. *P. admetus* has not been observed on the same biotopes and thus the new taxon could be separated geographically. Besides, *P. admetus* has a very distinct appearance by especially its strongly marked underside (with a double row of small dots on the marginal to submarginal zone of the underside hindwings, a trait that is lacking in the aforementioned species.

Etymology. Derivatio nominis.

The adjective *lurae* has two meanings: "ascribed to Lurë" and "surviving attacks of congeners". First, the species name is deducted from the Albanian "Lurë region, where the type locality lies, and referring to the old village Lurë e Vjetër situated in central-Eastern Albania (Dibër province). The name alludes to the fascinating history of the old Lurë village: during the Ottoman war, the village was asked 300 women by the enemies. Armed men, disguised with the *duvak*, the traditional red bridal veil, were sent instead on horseback to the Ottoman camp. As a result, the Ottomans were taken by surprise and the Lura tribe eventually won the battle. Also, this second meaning seems adequate for the taxon *lurae*: this species likely experienced periods of close contact with congener species more largely distributed in the Balkans, as is the case at present, but nevertheless has been able to avoid complete admixture and still survives in its unique ophiolitic biotope.

Discussion

Colour morphometrics, a new method for identification of cryptic *Agrodiaetus* taxa

The use of standardized light reflectance measurements to discriminate between species is a recent method used for identification (Bálint et al. 2010; Bálint et al. 2012; Wasik et al. 2014). Other morphological traits such as underside markings and prominence of the white stripe are useful, but they are not discriminative enough to unambiguously distinguish between the taxa here studied. Most of *P. lurae* specimens have a dark ground colour in the underside of the wings, and no or a very faint white stripe. However, one specimen identified as *lurae* by mitochondrial DNA, karyotype and morphometrics (colour reflectance of dorsal wing colour was typical) showed a more pronounced white stripe. Here we only used RGB an HSV values in the analysis, but more sophisticated measurements to generate full reflectance spectra and SEM graphs may be more powerful to discriminate between these taxa. Such analyses could also shed light on the physical structures that generate the typical dark colour of *P. lurae*, compared to the greenish reflectance in *P. orphicus* specimens, as has been demonstrated in other species of *Polyommatus* (Bálint et al. 2012). Besides, morphometrics on preimaginal stages could also be potentially interesting. Almost no information is available on this aspect, while recent findings showed that differences in larval morphology and in larval host plant preferences may be key in resolving the taxonomy of cryptic species (Hernández-Roldán et al. 2016; Hinojosa et al. 2022).

Karyotyping and difference with related taxa

The karyotype of *P. orphicus* was studied previously (Kolev 2005; Vishnevskaya et al. 2016) from localities in Northern Greece and Bulgaria. Two different haploid chromosome numbers (n=41 and n=42) were found to be present in these populations. The variation in chromosome numbers in these populations was explained by polymorphism

for one chromosome fusion/fission. This polymorphism resulted in three types of MI karyotype: n=41 (homozygous for chromosomal fusion/fission, one pair of fused chromosomes, 2n=82), n=42 (homozygous for chromosomal fusion/fission, two pairs of unfused chromosomes; 2n=84) and n=41 (heterozygous for chromosomal fusion/fission, 40 bivalents and one trivalent; 2n=83) (Vishnevskaya et al. 2016).

The *P. orphicus* karyotype found by us in Albania (n=42) fits into the previously described variability. At the same time, it can be assumed that in the Albanian population there is a tendency to fixation of the chromosome number n=42, although the studied data are still insufficient to consider this proved.

The taxon we describe as *P. lurae* sp. nov also exhibits intrapopulation variability in chromosome numbers (n=42, n=43; estimated diploid numbers are 2n=84, 2n=85, 2n=86) due to polymorphism for one chromosome fusion/fission, but most likely in another chromosome pair. Thus, despite chromosome polymorphism in each of the taxa *P. orphicus* and *P. lurae*, they have, most likely, a fixed difference in one chromosome pair (Fig. 11).

In the contact zone in Albania, both mitochondrial haplogroups *orph2* and *aroa3* occur together. It can be assumed that they arose as a result of hybridization, which is confirmed by the intermediate nature of the colour of the wings.

In the case of hybridization, if contacting taxa have postzygotic reproductive isolation, then hybrid individuals should represent only F1 hybrids (further hybridization is impossible due to sterility). If the hybrids are fertile, then a mixture of hybrids of different generations and the results of backcrosses should be observed.

The reconstruction of karyotypes of pure forms of *P. orphicus* and *P. lurae* sp. nov and their putative hybrids is shown in Fig. 11. As follows from this scheme, F1 hybrids should all have the same number of elements visible in the first metaphase of meiosis (n=41), despite the fact that they may include 1 to 2 complex multivalents. Such a karyotype was not observed in the putative hybrid zone. From this we conclude that, likely, there is no complete reproductive isolation between *P. orphicus* and *P. lurae*, and the observed karyotypes n=43 are the result of repeated hybridization and backcrosses. Another hypothesis to explain the pattern we observe would be that *P. orphicus* lineage orph2 in Albania displays a karyotype n=42–43 and the specimens with n=43 are not admixed, although the fact that they were only found in the contact zone and that the two specimens measured displayed intermediate morphology favour the hybridisation hypothesis.

Taxonomic position and difference from sister taxa

The data obtained demonstrate that *P. orphicus* and *P. lurae* represent two distinct phylogenetic lineages with a parapatric distribution. Indeed, both *P. orphicus* and *P. lurae* formed a highly supported monophyletic lineage based on three phylogenetic analyses (BI of *COI* barcode, ML of *COI+ITS2* and BI of *COI+ITS2*) (Fig. 2, Suppl. material 2). These two lineages are also substantially differentiated with respect to morphology (different wing reflectance), and karyotype (difference in one chromosome pair). Therefore, they can be considered species from the viewpoint of the phylogenetic species concept. These two lineages (=phylogenetic species) overlap in a small contact

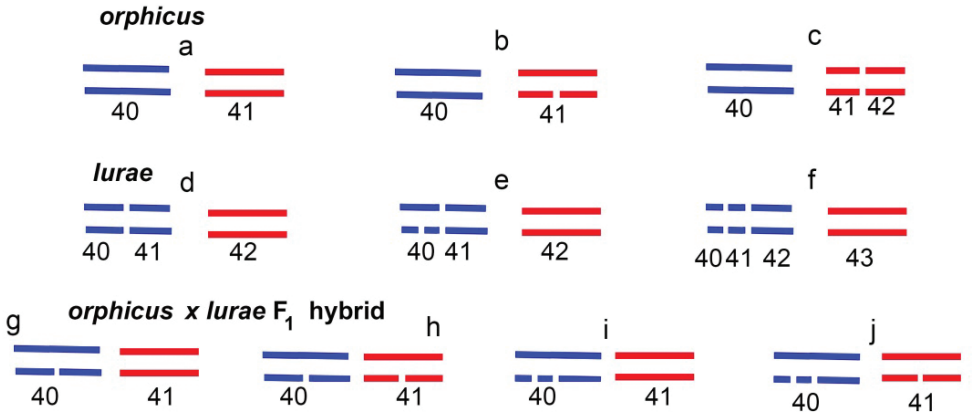

Figure 11. Scheme showing variation in number of chromosomes (lines) and visible elements (=bivalents+multivalents) in MI meiosis in *P. orphicus*, *P. lurae* and their putative F1 hybrids. **a** *P. orphicus*, homozygous for chromosomal fusion/fission, one pair of fused chromosomes, 41 visible elements **b** *P. orphicus*, heterozygous for chromosomal fusion/fission, 40 bivalents and one trivalent; 41 visible elements **c** *P. orphicus*, n=42 (homozygous for chromosomal fusion/fission, two pairs of unfused chromosomes; 42 visible elements **d** *P. lurae*, homozygous for chromosomal fusion/fission, one pair of fused chromosomes, 42 visible elements **e** *P. lurae*, heterozygous for chromosomal fusion/fission, 41 bivalents and one trivalent; 42 visible elements **f** *P. lurae*, homozygous for chromosomal fusion/fission, two pairs of unfused chromosomes; 43 visible elements **g–j** different variants of F1 hybrids. These variants include tri- and quadrivalents; however, the number of visible elements in MI remains 41.

zone in Albania, and the combination of mtDNA, karyotype and morphological data suggest that they may hybridize and no complete barrier to reproduction exist.

Theoretically, the main lineages in the *P. orphicus*, *P. timfristos*, *P. aroaniensis* and *P. lurae* subcomplex could also be interpreted as infraspecific taxa, if the polytypic species concept is applied (Vishnevskaya et al. 2016). In this case, these taxa would be subspecies under the entire *P. aroaniensis* species complex. In Albania we showed that there is a contact zone between *P. orphicus* and *P. lurae* where unusual combinations of mitochondrial and karyotype, as well as intermediate morphotypes, exist. However, none of the aforementioned taxa appear to be fully sympatric in distribution and, taken together, they form a highly supported monophyletic lineage based on analysis of *COI* sequences (Fig. 2) and the concatenated *COI+ITS2* sequences (Suppl. material 2). Under this scenario, this subspecies-complex would be considered a diverse array of allopatric populations, each of which possesses unique genetic attributes (karyotypes and molecular markers) and is distributed in a particular area within the Balkan peninsular. While one can argue that differences in chromosome numbers in the subgenus *Agrodiaetus* do not necessarily result in complete reproductive isolation and, at least in some particular cases, do not prevent interspecific hybridization and genetic introgression (Lukhtanov et al. 2015b), this does not necessarily mean that chromosomal rearrangements are irrelevant to the formation of genetic barriers between populations (Vishnevskaya et al. 2016).

Chromosome changes have been shown to be important for speciation in Polyommatina butterflies (Lukhtanov et al. 2005; Kandul et al. 2007; Talavera et al. 2013a; Vishnevskaya et al. 2016) and even a weak reduction in fertility in heterozygotes for multiple chromosomal rearrangements can result in selection against them and in the formation of a boundary between chromosomally diverged homozygous populations. More detailed studies investigating lab-controlled crosses between sister taxa and the fertility of their progeny would be interesting to shed light on this topic, as has been achieved in wood white (*Leptidea*) butterflies (Dincă et al. 2013). Recent taxonomical publications have treated *P. orphicus*, *P. aroaniensis* and *P. timfristos* as species-level taxa (Eckweiler and Bozano 2016; Wiemers et al. 2018) and our study is following this rationale also for *P. lurae* sp. nov.

Regardless of its taxonomic status as a species or subspecies, *P. lurae* represents a unique entity within the genus *Polyommatus* that deserves additional study. A better understanding of its evolutionary history and its relationship with its unique biotope and related taxa may be helpful in understanding mechanisms of chromosomal diversification within the subgenus *Agrodiaetus*, and may further elucidate the biogeography of the south Balkan and Aegean regions.

Conservation of the species and habitat

The Lurë region has become a National Park (Parku Kombëtar Lurë-Mali i Dejës) since 1966 to protect its ecosystems and biodiversity. Since 2018 by encompassing the entire section of Kunora e Lurës, its name has changed to Parku Kombëtar Lurë-Mali i Dejës, spanning an expanded area of 202.42 km^2. Despite its conservation status the area suffered massive deforestation from illegal logging and forest fires that severely affected ecosystems and it is estimated that as much as 50% of the original Lura National Park has been destroyed (Rama 2018). Moreover, it is not fully covering important biotopes such as some of the *P. lurae* biotopes.

Next to this, the first author noticed that sheep overgrazing is also affecting the ecosystems. As *Onobrychis* plants are very palatable to sheep, heavy grazing limits the growth and expansion of *Onobrychis*, sometimes leading to the extinction of the plant (Lafranchis et al. 2007). While traditional grazing by sheep is beneficial, and can help in keeping open clearings, uncontrolled and overgrazing can have a devastating impact on butterflies, and other insects such as bees, which has been increasingly reported in different parts of Europe (Kruess and Tscharntke 2002; Potts et al. 2009; Verbrugge et al. 2022) and such an evolution in Albania would be dramatic for its biodiversity, especially for ecosystems harbouring unique species diversity.

The future of various endemic species of *Polyommatus* in Europe is strongly dependent on keeping open dry clearings at montane-subalpine levels where its foodplant is growing; This is the case for *P. orphicus* and *P. aroaniensis* in Greece but even so for *P. lurae* in Albania.

As a distinct taxonomic entity occupying a very restricted area linked to a unique biotope in Albania the newly described species should be considered a candidate on the list of protected species in Albania and the whole of Europe by adding to the European red list of Butterflies (Van Swaay et al. 2010; Maes et al. 2019).

In summary, the Lurë region harbours unique endemic flora and fauna, in addition to being home for the species here described, which is currently only found very restricted and locally. Therefore, the preservation of this habitat needs being ensured. This encompasses also control of human activities as illegal logging, burning and uncontrolled grazing by livestock, all major factors that have been identified contributing to butterfly decline in Europe (van Swaay and Warren 2006). As Albania is setting up programs to be member of be the European Union, installing adequate protection legislation for its biodiversity heritage (e.g. the EU Habitats Directive 92/43/EEC) will be needed.

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Supplementary material 1

Colour measurements of wing reflectance

Authors: Laurian Parmentier, Roger Vila, Vladimir Lukhtanov

Data type: morphological

Explanation note: Deatails on colour measurements (methodology, processing) and generated data are given.

Link: https://doi.org/10.3897/compcytogen.v16.i4.90558.suppl1

Supplementary material 2

Phylogeny of concatenated *COI+ITS2* sequences based on NJ and BI reconstructions

Authors: Laurian Parmentier, Roger Vila, Vladimir Lukhtanov

Data type: docx file

Link: https://doi.org/10.3897/compcytogen.v16.i4.90558.suppl2

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RESEARCH ARTICLE

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Chromosomal polymorphism in natural populations of *Chironomus* sp. prope *agilis* Kiknadze, Siirin, Filippova et al., 1991 (Diptera, Chironomidae)

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Abstract

Species *Chironomus* sp. prope *agilis* Kiknadze, Siirin, Filippova et al., 1991 belongs to the *Ch. plumosus* group of sibling species. It was described on the basis of its karyotype and analysis of isozymes from one population in the Urals but since then no quantitative data on chromosomal polymorphism of this species have been published. The goal of this study is to broaden our knowledge of the chromosomal polymorphism and distribution of the *Chironomus* sp. prope *agilis*, which, along with the data on chromosomal polymorphism of other species from the *Ch. plumosus* group, can give us a better understanding of the connection between chromosomal polymorphism and ecological conditions of habitats. The specimens of *Chironomus* sp. prope *agilis* were found only in 8 natural populations from the Urals, Western Siberia and Kazakhstan, which allows us to conclude that the species range of *Chironomus* sp. prope *agilis* is not as wide as for most other species from *Ch. plumosus* group. An analysis of chromosomal polymorphism in these 8 natural populations of *Chironomus* sp. prope *agilis* has been performed. All of the studied populations were either monomorphic or showed very low level of chromosomal polymorphism, with 4.4–8.7% of heterozygous specimens per population and 0.04–0.08 heterozygotic inversion per larvae. The total number of banding sequences found in the banding sequence pool of *Chironomus* sp. prope *agilis* is 10. The mapping of banding sequence p'ag2B3 is presented for the first time. Besides inversions, one reciprocal translocation was found in a population from Kazakhstan, B-chromosome was found in one population from the Urals region of Russia, and heterozygosity of the level of expression of Balbiany rings in arm G was observed in several studied populations.

Keywords
banding sequence, *Ch. plumosus* group, inversion, karyological analysis, karyotype, polythene chromosome, sibling species

Introduction

The species *Chironomus* sp. prope *agilis* is one of the rarest species in the *Ch. plumosus* group of sibling species. It was first described in 1991 from the lake near Yurgamish settlement in the Urals based on its karyotype and is closest to *Ch. agilis* Shobanov et Djomin, 1988 (Kiknadze et al. 1991a). These two species differ mainly by the size of centromeric heterochromatin – medium in *Ch. agilis* but very large in *Chironomus* sp. prope *agilis* – and the dominant banding sequence in one chromosomal arm. The status of *Chironomus* sp. prope *agilis* as a separate species in the *Ch. plumosus* group of sibling species was also confirmed by isozyme analysis, which showed that genetic distances between this species and other species from the *Ch. plumosus* group correspond to values typically observed for genetic distances between sibling species in chironomids (Gunderina et al. 1988; Filippova et al. 1989, 1990).

Since its first description, no information about chromosomal polymorphism of *Chironomus* sp. prope *agilis* was published until the recent work of Kiknadze and coauthors (2016), where only information on the banding sequence pool (photos and mapping of banding sequences) of the species was presented with no quantitative data on polymorphism in studied populations. Yet the knowledge of the patterns of chromosomal polymorphism in natural populations is essential for gaining a better understanding of the connection between chromosomal polymorphism and ecological conditions of habitats, and *Ch. plumosus* group of sibling species present a great model for such studies.

Thus, the purpose of this paper is to present new data on chromosomal polymorphism in populations of *Chironomus* sp. prope *agilis* from the Russian Federation and Kazakhstan.

Material and methods

The VI instar larvae from 8 natural populations from Russia (the Urals and Siberia) and Kazakhstan were used for polytene chromosome slide preparation. Data on collection sites is presented in Table 1.

The larvae were fixed with 3:1 *v/v* of 96% ethanol and glacial acetic acid and stored at –20 °C. Polytene chromosome squashes were prepared by the routine aceto-orcein method (Keyl and Keyl 1959; Kiknadze et al. 1991b). Chromosomal mapping of arms A, C, D, E, and F was done using the mapping system created by Keyl (1962) and Devai et al. (1989), with *Ch. piger* Strenzke, 1959 as the standard karyotype. Mapping of arm B was done according to the Maximova mapping system (Maximova 1976), improved by Schobanov (1994), with *Ch. plumosus* chromosomes as the standard.

Table 1. Collection sites.

Collection place	Abbreviation	Collection date	Geographic coordinates	Number of larvae
RUSSIA				
The Urals				
Kurgan region				
Lake near Yurgamish settlement	KUR-YU	27.02.1990	55°20'54.3"N, 64°28'02.9"E	80
Western Siberia				
Novosibirsk region				
Itkul Lake	NSK-IT	15.04.1993	55°04'27.3"N, 81°01'53.2"E	12
Altai territory				
Gor'koe Lake, Tumentsevo district	ALT-GT	13.05.1993	53°26'48.9"N, 81°22'47.0"E	11
Gor'koe-Peresheechnoe Lake, Egorievo district	ALT-GP	16.05.1994	51°47'04.2"N, 80°50'22.3"E	1
Gor'koe Lake, Rubtsovks district	ALT-GR	04.04.1993 17.05.1993 10.09.1993	51°37'25.3"N, 81°13'23.9"E	23
Tepliy Klyuch Lake near Yarovoe town, Slavgorod district	ALT-TK	04.07.2001	52°19'11.9"N, 83°11'24.2"E	2
Travyanoe Lake, Oskolkovo settlement	ALT-TR	08.05.1994	52°19'11.9"N, 83°11'24.2"E	1
KAZAKHSTAN				
Karasor Lake, mouth of river Tundik	KAZ-KA	23.09.1995	53°00'13.5"N, 70°50'15.7"E	64

Each banding sequence is given a short designation as follows: three-letter abbreviation of the species name (ag2 as in the first description, the species was named *Ch. agilis* 2 and the abbreviation ag2 was used in all subsequent works) followed by the name of the arm and the serial number of banding sequence in this arm (according to the order of its discovery), and prefixed by a letter indicating its geographical distribution in the genus *Chironomus* (p' for Palearctic sequences or h' for Holarctic sequences). For example, h'ag2E1 means that while *Ch.* sp. prope *agilis* itself is a Palearctic species, this banding sequence is identical to banding sequences of some other species and was found both in the Palearctic and the Nearctic and thus is a Holarctic banding sequence.

Statistical analysis was done using the program PHYLIP (https://evolution.genetics.washington.edu/phylip.html).

The following equipment of the Centre of Microscopical analysis of biological objects SB RAS in the Institute of Cytology and Genetics (Novosibirsk) was used for this work: microscope "Axioskop" 2 Plus, CCD-camera AxioCam HRc, software package AxioVision 4 (Zeiss, Germany).

Results and discussion

As all other members of the *Ch. plumosus* group of sibling species, *Chironomus* sp. prope *agilis* belongs to the "thummi" cytocomplex with a haploid number of chromosomes n = 4 and an arm combination AB CD EF G. The chromosomes I (AB) and II (CD) are metacentric, III (EF) is submetacentric, and IV (G) is telocentric (Fig. 1).

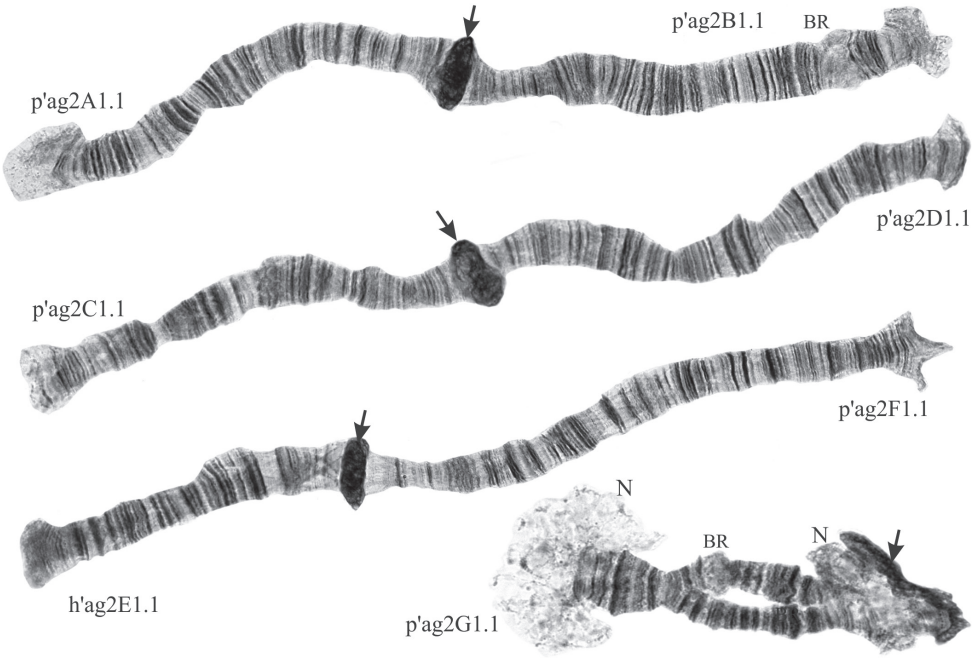

Figure 1. Karyotype of *Chironomus* sp. prope *agilis*. Centromeric regions are designated by arrows. N – nucleolus, BR – Balbiani Ring.

There are two nucleoli in *Chironomus* sp. prope *agilis* karyotype; both are situated on arm G – one on the centromeric end of the arm, the other on their opposite end near the telomere. Homologues of arm G are paired but often unconjugated at the ends in nucleolus regions. Centromeric regions are very large, which, along with two nucleoli on arm G, clearly differentiates this species from the rest of its siblings. There are three Balbiani Rings (BR) in the karyotype of *Ch. agilis*: two are situated on the arm G (usually only the one in the center of the arm is visible as the other one is often masked by the nucleolus), and the third one is on the arm B.

The revision of the mapping of main banding sequences in arms A, B, C, D, E, and F was presented by Golygina and Kiknadze previously (2008, 2012, 2018). A revised mapping of these banding sequences is shown in Figure 2. For arm E, two versions of the mapping are presented (Fig. 2e).The first one is done according to how *Chironomus* sp. prope *agilis* banding sequence should be mapped if mapping of *Ch. plumosus* (the reference species for mapping of all *Ch. plumosus* group sibling species) made by Keyl (1962) is considered to be correct (marked as KV). The second one is done according to the revised mapping of *Ch. plumosus* made by Golygina and Kiknadze (2018) (marked as GV).

As was mentioned above, *Chironomus* sp. prope *agilis* is a very rare species. Among over 200 populations of chironomids studied from Eurasia by us during the last 30 years, this species was found only in 8 (Table 1), and only in 5 of them – KUR-YU,

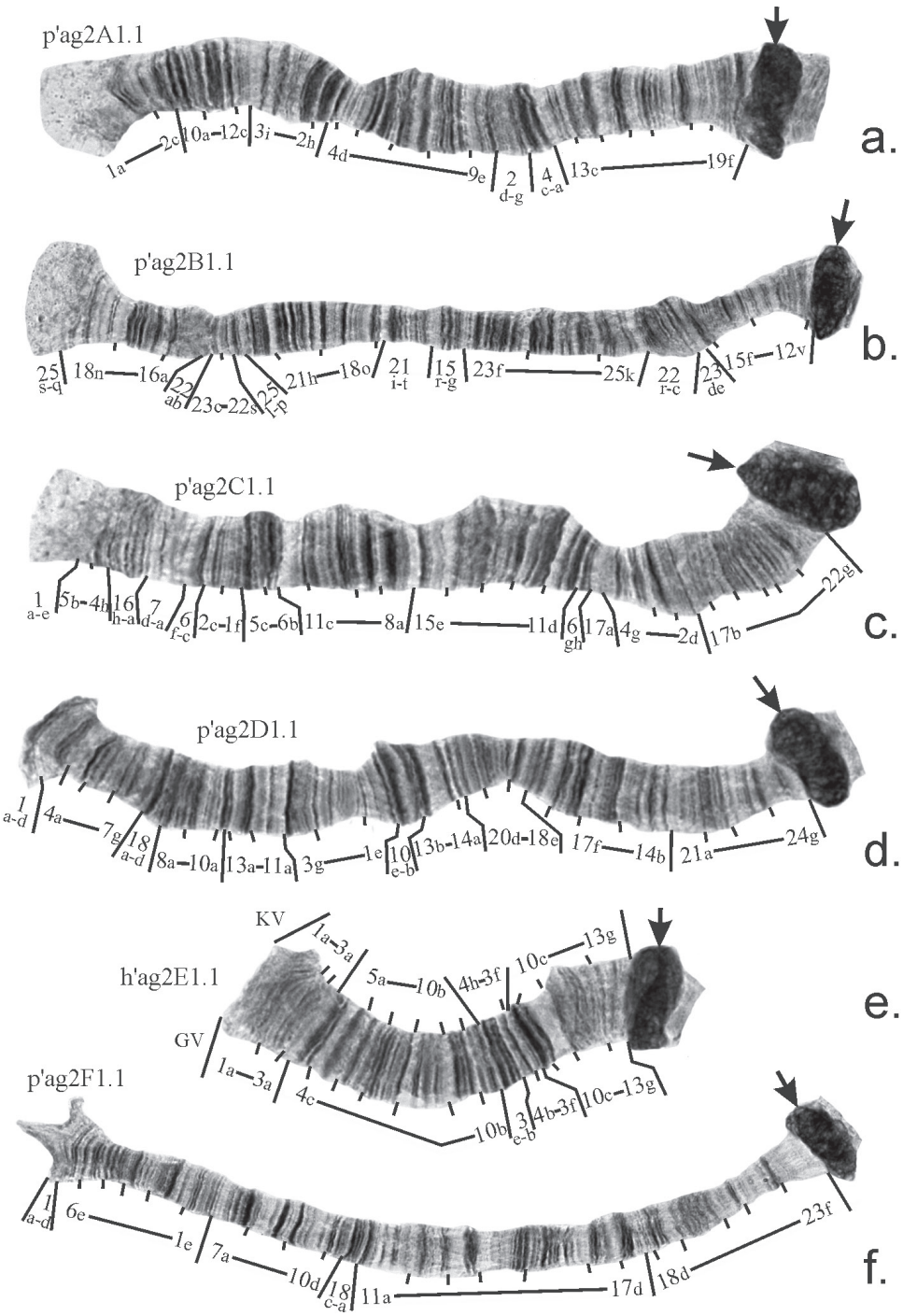

Figure 2. Mapping of main banding sequences in arms **A–F** of *Chiroomus* sp. prope *agilis*. Centromeric regions are designated by arrows. KV – version of mapping in arm E according to Keyl (1962), GV – version of mapping in arm E according to Golygina and Kiknadze (2018).

NSK-IT, ALT-GT, ALT-GR (Russia) and KAZ-KA (Kazakhstan) – we found enough larvae to perform quantitative analysis of inversion polymorphism.

The main banding sequences of *Chironomus* sp. prope *agilis* in all arms except arms B and C are identical to the main banding sequences of *Ch. agilis* (Table 2, Fig. 2). The banding sequence p'ag2B1 is identical to p'agiB2 – the alternative banding sequence of *Ch. agilis*, which is prevalent in all studied populations of this species from Siberia and the Far East. The arm C of *Chironomus* sp. prope *agilis* differs from the main banding sequence of *Ch. agilis* p'agiC1 by a large complex inversion (Table 2, Fig. 2) Previously, p'ag2C1 was considered to be unique to the species, but recent data on chromosomal polymorphism of *Ch. agilis* (Golygina and Ermolaeva 2021) have shown that a banding sequence identical to p'ag2C1 does exist in the banding sequence pool of *Ch. agilis* (p'agiC2), although up to now it has been found only once. Thus, none of the main banding sequences of *Chironomus* sp. prope *agilis* are unique to the species, so karyologically it is closer to *Ch. agilis* that was presumed previously. The main feature that differentiates the karyotypes of these two species is the size of their centromeric regions.

Inversion polymorphism was observed only in arms of chromosome I (AB), and among three inversions found, only banding sequence p'ag2A2 occurred in several populations with low frequency, the other two – p'ag2B2 and p'ag2B3 – were unique (Tables 3, 4). All three inversions were quite short, thus they could not form standard inversion loops and they are seen as unpaired regions (Fig. 3) and are easy to miss if a researcher didn't carefully inspect the entire banding pattern of an arm. Mapping of banding sequence p'ag2B3 is presented for the first time (Table 2). Thus, in total, the banding sequence pool of *Chironomus* sp. prope *agilis* at present consists of 10 banding sequences.

Besides inversions, one reciprocal translocation was found in a population from Kazakhstan (Fig. 3, d). Heterozygosity of development of BR and underdevelopment of BR on both homologs in the center of the arm G was observed in populations from

Table 2. Mapping of banding sequences of *Chiornonomus* sp. prope *agilis*.

Designation of banding sequence	Mapping of banding sequence
p'ag2A1	1a-2c 10a-12c 3i-2h 4d-9e 2d-g 4c-a 13a-19f C
p'ag2A2	1a-2c 10a-12c 3i-2h 4d-7b 4bc 2g-d 9e-7c 4a 13a-19f C
p'ag2B1	25s-q 18n-16a 22ab 23c-22s 25l-p 21h-18o 21i-t 15r-g 23f-25k 22r-c 23de 15f-12v C
p'ag2B2	25s-q 18n-16a 22ab 23c-22s 25l-p 21h-18o 21i-t 15r-o 23z-f 15g-n 24a-25k 22r-c 23de 15f-12v C
p'ag2B3	25s-q 18n-16a 22ab 23c-22s 25l-p 21h-18o 21i-t 15r-g 23f-24s 15a-f 23ed 22c-r 14r-12v C
p'ag2C1	1a-e 5b-4h 16h-a 7d-a 6f-c 2c-1f 5c-6b 11c-8a 15e-11d 6gh 17a 4g-2d 17b-22g C
p'ag2D1	11a-d 4a-7g 18a-d 8a-10a 13a-11a 3g-1e 10e-b 13b-14a 20d-18e 17f-14b 21a-24g C
h'ag2E1	1a-3e 5a-10b 4h-3f 10c-13g C † 1a-3a 4c-10b 3e-b 4b-3f 10c-13g C‡
h'ag2F1	1a-d 6e-1e 7a-10d 18c-a 11a-17d 18d-23f C

† - mapped according to Keyl (1962). ‡ - revised mapping according to Golygina and Kiknadze (2018).

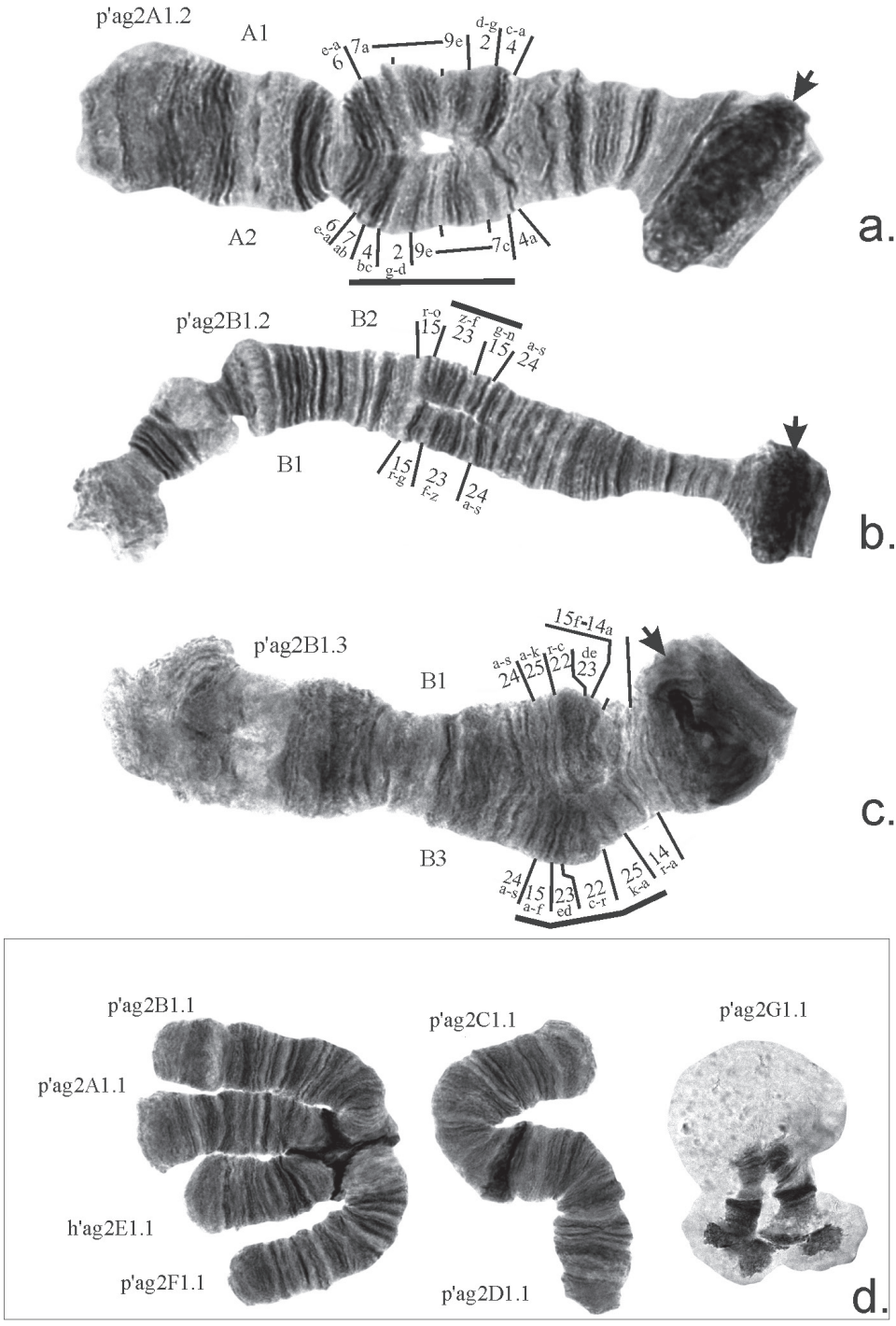

Figure 3. Chromosomal polymorphism found in populations of *Ch. agilis* **a–c** inversions in chromosome I (AB) **d** reciprocal translocation. Centromeric regions are designated by arrows. Brackets show regions of inversions.

Table 3. Frequencies of genotypic combinations of banding sequences and general characteristics of chromosomal polymorphism in populations of *Chironomus* sp. prope *agilis*.

Genotypic combination	Russia							Kazakhstan
	KUR-YU[§]	NSK-IT	ALT-GT	ALT-GP	ALT-GR	ALT-TK	ALT-TR	KAZ-KA
	80[|]	12	11	1	23	2	1	64
p'ag2A1.1	**0.962**	**0.917**	**1**	1	**0.913**	1	1	**0.984**
p'ag2A1.2	**0.038**	**0.083**	**0**	0	**0.087**	0	0	**0.016**
p'ag2B1.1	**0.987**	**1**	**0**	1	**1**	1	1	**0.984**
p'ag2B1.2	**0.013**	**0**	**1**	0	**0**	0	0	**0**
p'ag2B1.3	**0**	**0**	**0**	0	**0**	0	0	**0.016**
p'ag2C1.1	**1**	**1**	**1**	1	**1**	1	1	**1**
p'ag2D1.1	**1**	**1**	**1**	1	**1**	1	1	**1**
h'ag2E1.1	**1**	**1**	**1**	1	**1**	1	1	**1**
p'agiF1.1	**1**	**1**	**1**	1	**1**	1	1	**1**
p'agiG1.1	**1**	**1**	**1**	1	**1**	1	1	**1**
Percentage of larvae with B-chromosome	**1.3**	**0**	**0**	0	**0**	0	0	**0**
Percentage of larvae showing heterozygocity in the development of BR in arm G	**25**	**0**	**54**	0	**22**	0	0	not studied due to the bad banding structure of arm G
Percentage of larvae with undeveloped BR in arm G	**13**	**0**	**18**	0	**4**	0	0	-
Number of banding sequences	**9**	**8**	**7**	7	**8**	7	7	**9**
Number of genotypic combinations of banding sequences	**9**	**8**	**7**	7	**8**	7	7	**9**
% of heterozygous larvae	**3.75**	**8.3**	**0**	0	**8.7**	0	0	**3.1**
Number of heterozygous inversions per larvae	**0.04**	**0.08**	**0**	0	**0.09**	0	0	**0.03**

[§] - populations highlighted with bold were used for quantitative analysis of chromosomal polymorphism.
[|] - number of larvae studied.

Table 4. Frequencies of banding sequences in populations of *Chironomus* sp. prope *agilis*.[¶]

Banding sequnce	Russia				Kazakhstan
	KUR-YU	NSK-IT	ALT-GT	ALT-GR	KAZ-KA
	80[#]	12	11	23	64
p'ag2A1	0.981	0.959	1	0.957	0.992
p'ag2A2	0.019	0.041	0	0.043	0.008
p'ag2B1	0.994	1	0	1	0.992
p'ag2B2	0.006	0	1	0	0
p'ag2B3	0	0	0	0	0.008
p'ag2C1	1	1	1	1	1
p'ag2D1	1	1	1	1	1
h'ag2E1	1	1	1	1	1
p'agiF1	1	1	1	1	1
p'agiG1	1	1	1	1	1

[¶] - only populations with enough larva for quantitative analysis (more than 10 specimens) are included into this table. [#] - number of larvae studied.

the Ural and Altai regions (Fig. 1, Table 3). The genomic polymorphism in the form of an additional B-chromosome was found in one larva from the KUR-YU population from the Urals (Table 3).

Thus, *Chironomus* sp. prope *agilis* can be considered as having a very low level of polymorphism. Among all studied species from the plumosus group, with the exception of *Chironomus bonus* Shilova et Dzhvarsheishvili, 1974, which also has only a few studied populations, *Chironomus* sp. prope *agilis* is the most monomorphic. Cytogenetic distances between populations varied from 0 to 0.008.

Although there are currently no hard data on the water characteristics in the waterbodies where *Chironomus* sp. prope *agilis* was recorded (such as salinity, ion content etc.), it is possible to speculate that this species is likely adapted to life in somewhat saline waters. We suggest this conclusion as most lakes where it was found can be categorized as saline (the name 'Gor'koe' means 'bitter' and is given in the Altai region to saline lakes, and Karasor Lake in Kazakhstan is also a confirmed saline lake). It is possible that the low level of chromosomal polymorphism, as well as the rarity of these species, can also be attributed to its preference in habitats, although in order to make a firm conclusion on this matter, more studies of the species are required. At present, the species range of the *Chironomus* sp. prope *agilis* can be defined as covering the Urals, south of Western Siberia and Northern Kazakhstan.

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