# Partial mtCOI-sequences of Balkanic species of *Pseudochazara* (Lepidoptera: Nymphalidae, Satyrinae) reveal three well-differentiated lineages

Kornél Takáts & Morten Mølgaard

**Summary**: In this paper we present 'barcode' haplotypes of mitochondrial cytochrome c oxidase subunit I (mtCOI) of *Pseudochazara* species (Lepidoptera: Nymphalidae) occurring in Balkan Peninsula. Our phylogenetic reconstruction indicates 3 well-differentiated lineages within the 7 species: the '*mamurra*-group' (*Pseudochazara geyeri*, *P. graeca*, *P. amymone*), the '*mniszechii*-group' (*P. mniszechii*, *P. cingovskii*, *P. orestes*) and a third lineage comprising only *P. anthelea*. In lineages containing multiple members, the number of differences between pairs in nucleotide positions generally were considerably low: *amymone-graeca*: 1 (0.15%), *mniszechii-orestes*: 2(0.30%), *mniszechii-cingovskii*: 3(0.46%) and *orestes-cingovskii*: 3 (0.46%). The genital studies of male specimens largely supported the lineage relations mentioned above. The divergence of wing colouration could change much more rapidly than that of mtCOI sequences, influenced by external environmental factors such as the colour of the substrate.

**Key words**: *Pseudochazara*, barcode haplotypes, lineages, genetic differentiation, endemic species, wing colouration, substrate.

## Introduction

DNA sequence analysis is gaining an increasing importance in establishing of phylogenetic relationships between taxa at different levels (Chen and Maiden, 2010; Parr *et al*., 2012). A practical reason for this is the decreasing cost of DNA sequencing and the fact that aligned sequences are easier to compare and evaluate. Based on the identical or different homologous positions we obtain a more precisely quantifiable and thus more objective picture on the degree of relationship, that is, the genetic distance between the taxa concerned. The study of morphology of the external and genital characters of butterflies and moths should remain an essential tool of systematic analysis, however. These methods especially can rely on the examination of „similarities" in certain structures and patterns to reveal the relationship primarily between closely related taxa. At the same time, these similarities may be resulted from convergent evolution, moreover, evaluating the degree of similarity is an inherently subjective process (Windsor and Felder, 2014).

Different genes and DNA sequences change differently over time, according to the function of the given DNA segment. The genome of eukaryotes is not compact (Ridley, 2006), meaning that most sequences do not encode proteins. These parts, e.g, may contribute to the regulation of gene expression but some of them lost their function completely (called pseudogenes) (Vanin, 1985). The non-protein coding sequences tolerate mutations much better, due to the less evolutionary pressure on them. For example, the difference between the protein coding DNA of humans and chimpanzees is approximately 0.1%, while this value is 4%, 40-times larger regarding the non-coding sequences (CSAAC, 2005).

Genes that encode proteins can also have remarkable differences in their susceptibility to mutation (Patthy, 1999). This mainly depends on the size and flexibility of the functional sequence space of the protein product encoded. Some protein sequences are extremely conservative, such as the DNA-packing histone proteins in the nucleus. Their amino acid sequences have hardly changed during the evolution of different organisms (Cox *et al*., 2005). These slowly evolving genes are not suitable for identification of specimens and estimating phylogenies at species level.

The mutation rate of the mtCOI gene is rapid enough to allow the identification of specimens on the species level with high reliability (Hebert *et al*., 2003; Zahiri *et al.,* 2014); in addition, being a mitochondrial gene, this sequence is also suitably conservative, therefore low intraspecific variability can be expected. As the mitochondrial genome is transmitted only via maternal line, the comprising nucleotide sequences are not subjected to the recombinatory events occurring during sexual reproduction (Saccone *et al*., 1999). These favourable properties of the mtCOI gene was the reason why researchers started to build a database of the nucleotide sequences of a shorter, 658 bp

region (i.e. „barcode”-region) of it (Barcode of Life Data Systems = BOLD) for identification purposes. This explains why there are significantly more barcode sequences known in Lepidoptera and also in other animal taxa than any other genes. The BOLD database does not only collect barcode sequences: on 14 December 2015, exactly 976.444 sequences out of 1.031.374 butterfly and moth genes were barcode sequences. This significant dominance and easy accessibility of barcode sequences incited researchers to apply also these relatively short sequences in their phylogenetic analyses (Nazari *et al*., 2009; Yuan *et al*., 2015).

It is generally accepted that at least several, properly chosen genes have to be analysed in combination in order to establish an accurate phylogenic reconstruction, since first of all the nodes of a gene tree reflect the mutation events of the given gene, not in turn the speciation events (Brown, 2002). When investigating Lepidoptera species, the most commonly used genes except the fore-mentioned mtCOI are the nuclear genes carbamoyl phosphate synthetase domain protein (CAD) (Reiger *et al*., 2012; Kawahara *et al*., 2009), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Zahiri *et al*., 2012; Yuan *et al*., 2015), elongation factor 1-α (EF-1A) (Braby *et al*., 2006) and the wingless genes (Kawahara *et al*., 2009). In our case we wish to support our phylogenetic reconstruction based on mtCOI sequences with morphological data obtained from male genitalia studies.

*Pseudochazara* species belong to Satyrinae subfamily of Nymphalidae and are the members of a Palearctic butterfly genus. The range of this genus extends from Mongolia, Northern Tibet and Transbaikalia through Central Asia, Asia Minor, and the Balkan Peninsula to the Iberian Peninsula and the Atlas Mountains in North Africa. Most species live in Central Asia (Tien Shan, Hindukush, the Persian Plateau) and Asia Minor. Exactly a dozen species occur in Europe, but three of them restricted to the Caucasus: *P. alpina* (Staudinger, 1878), *P. daghestana* (Holik, 1955), and *P. pelopea* (Klug, 1832) (Tshikolovets, 2011). *P. euxina* (Kusnezov, 1909) is endemic to the Crimean Peninsula and *P. hippolyte* (Esper, [1784]) lives on the South of Iberian Peninsula, although the latter species has also a wide area in Asia. Here, it is important to note that some authors treat the Spanish populations of *P. hippolyte* as separate species under the name of *P. williamsi* (Romei, 1927) (Settele *et al*., 2008). The remaining 7 species occur in the Balkan Peninsula (Fig. 1.), 3 of them can be found in Asia Minor as well.

From the few publication about *Pseudochazara* species, we should emphasize the paper of Gross, where he gives the detailed description of the wing patterns, androconial scales, geographical range and habitats of most species (Gross, 1978). Based on this and the taxonomical considerations of other

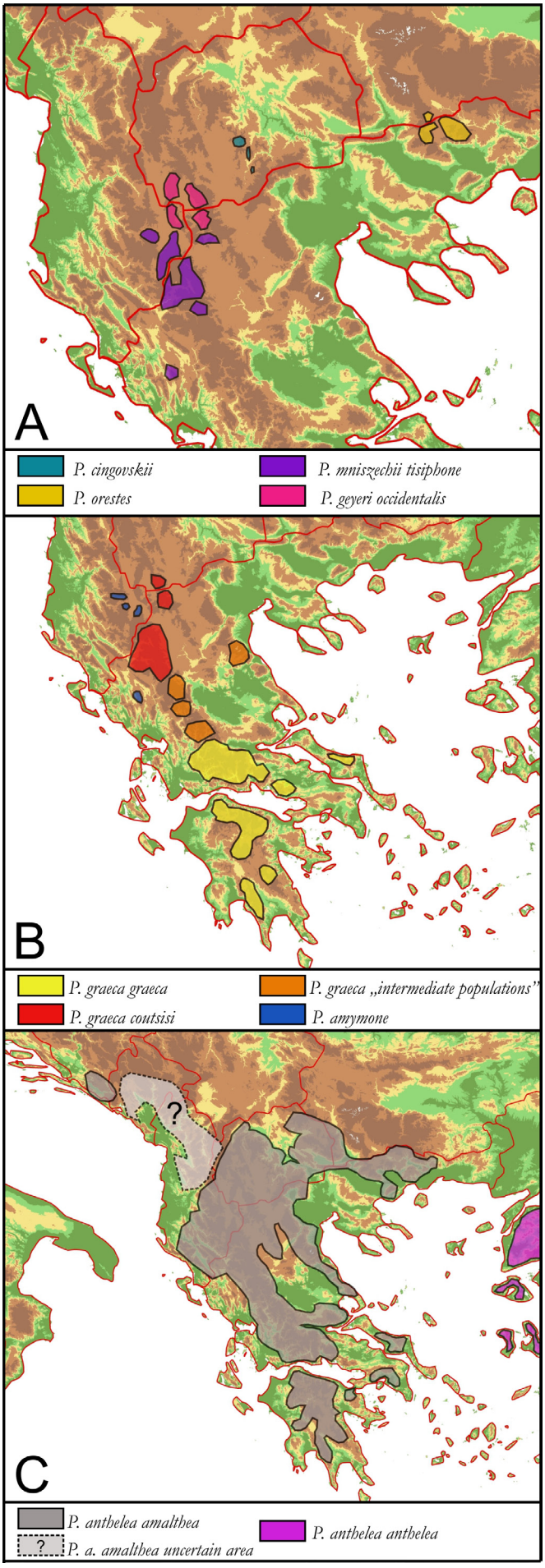


Fig. 1. Schematic distribution maps of *Pseudochazara*-spp. occurring in the Balkan Peninsula. A: *P. cingovskii*, *P. orestes*, *P. mniszechii tisiphone* and *P. geyeri occidentalis*, B: *P. graeca graeca*, *P. graeca coutsisi*, their intermediate populations and *P. amymone*, C: *P. anthelea*.

references we can presume that the species occurring in the Balkan Peninsula belong to 3 different groups. *P. anthelea* (Hübner, [1824]) is not mentioned in details by Gross, because the valvae and androconial stripes of the males fundamentally differ from those of other species. Later Aussem (1980) reports that this sharply distinct stripe in the cell of the forewing of males of *P. anthelea* and *P. thelephassa* (Geyer, [1827]) is in fact not an androconial stripe, as the androconial scales can only be found adjacent to this. *P. amymone* Brown, 1976 have been considered alternately as a separate species (Pamperis, 2009; Gascoigne-Pees *et al*., 2014; Cuvelier and Mølgaard, 2015) and as a subspecies of *P. mamurra* (Herrich-Schäffer, [1846]) (Eckweiler, 2004; Tshikolovets, 2011). Higgins in his book of 1975 treated *P. graeca* (Staudinger, 1870) also as a subspecies of *mamurra* (Higgins, 1975). The currently accepted *P. mniszechii tisiphone* Brown, 1980 was originally described under the name of *P. cingovskii tisiphone* Brown, 1980. The remaining two Balkan species, *P. geyeri* (Herrich-Schäffer, [1846]) has *graeca*-like external characters, while *P. orestes* de Prins & van der Poorten, 1981 rather resembles *tisiphone* and *cingovskii* in its appearance.

This paper aims to clarify the evolutionary relationship between the *Pseudochazara*-species of the Balkan Peninsula based on their mtCOI barcode sequences.

## Materials and methods

**Examined specimens.** Applied abbreviations: Private persons: GS: Gábor Simonics; KT: Kornél Takáts, MGP: Martin Gascoigne-Pees, MM: Morten Mølgaard. Institutions: HNHM: Hungarian Natural History Museum, Budapest. Others: P: photographed, G: male genitalia dissection, S: sequenced.

*P. amymone*: 4♂♂ (18-VII-2015, Albania, Boboshtiçë, leg. GS) | 1♂ (18-VII-2015, Albania, Boboshtiçë, leg. KT, P (Fig. 3M), G (Fig. 7D), S) | 1♂ (18-VII-2013, Albania, Gjergjeviçë, leg. MM, P (Fig. 3D)) | 2♀♀ (18-VII-2015, Albania, Boboshtiçë, leg. GS) | 1♀ (18-VII-2013, Albania, Boboshtiçë, leg. MM, P (Fig. 3D)) | 1♀ (18-VII-2013, Albania, Gjergjeviçë, leg. MM, P (Fig. 9A)); *P. anthelea amalthea*: 1♂ (05-VII-2010, Greece, Skíti, leg. KT, G (Fig. 7G) | 1♂ (08-VII-2012, Greece, Eptachori, leg. KT, P (Fig. 3H,L), S) | 3♂♂ (29-VI-1994, Greece, Kalavrita, leg. MM) | 3♀♀ (29-VI-1994, Greece, Kalavrita, leg. MM, 1♀ P (Fig. 3H)); *P. cingovskii*: 2♂♂ (06-VII-1995, Macedonia, Pletvar, leg. MM, 1♂ P (Fig. 3E)) | 2♂♂ (13-VII-2001, Macedonia, Pletvar, leg. MM, 1♂ P (Fig. 3O)) | 1♀ (20-VII-2015, Macedonia, Pletvar, leg. KT, P (Fig. 3E,O), S) | 2♀♀ (13-VII-2001, Macedonia, Pletvar, leg. MM); *P. geyeri occidentalis*: 1♂ (02-VIII-1936, Macedonia, Ohrid, leg. Viktor Mayer, coll. HNHM, P (Fig. 3C), G (Fig. 7F)) | 1♂ (26-VII-2013, Macedonia, Mt. Galicica, leg. MGP, S) | 3♂♂ (05-VIII-1997, Greece, Mt. Malimadi, leg. MM, 1♂ P (Fig. 3N)) | 4♀♀ (05-VIII-1997, Greece, Mt. Malimadi, leg. MM, 1♀ P (Fig. 3C)); *P. graeca coutsisi*: 1♂ (08-VII-2012, Greece, Katara-pass, leg. KT, P (Fig. 3B,J), G (Fig. 7E)) | 3♂♂ (25-VII-1993, Greece, Katara-pass, leg. MM) | 1♂ (24-VII-2013, Greece, Katara-pass, leg. MM) | 1♀ (08-VII-2012, Greece, Katara-pass, leg. KT, P (Fig. 3B,J), S) | 1♀ (25-VII-1993, Greece, Katara-pass, leg. MM, P (Fig. 9B)) | 3♀♀ (24-VII-2013, Greece, Katara-pass, leg. MM); *P. graeca graeca*: 2♂♂ (05-VII-1994, Greece, Mt. Chelmos, leg. MM) | 1♂ (08-VII-1994, Greece, Mt. Chelmos, leg. MM, P (Fig. 3I)) | 1♂ (15-VII-1994, Greece, Mt. Taygetos, leg. MM, P (Fig. 3A)); 1♀ (29-VII-1995, Greece, Kaliakouda, leg. MM) | 2♀♀ (19-VII-1997, Greece, Mt. Chelmos, leg. MM) | 2♀♀ (22-VII-1997, Greece, Mt. Parnassos, leg. MM, 2♀♀ P (Fig. 3A,I)); *P. mniszechii tisiphone*: 3♂♂ (18-VII-2015, Albania, Boboshtiçë, leg. KT, 1♂ P (Fig. 3F,P), G (Fig. 7B)) | 3♀♀ (18-VII-2015, Albania, Boboshtiçë, leg. KT, 1♀ P (Fig. 3F,P), S); *P. orestes*: 3♂♂ (06-VII-2009, Greece, Mt. Falakro, leg. KT, 1♂ P (Fig. 3G), G (Fig. 7C), S) | 2♂♂ (20-VII-1993, Greece, Mt. Falakro, leg. MM) | 2♀♀ (20-VII-1993, Greece, Mt. Falakro, leg. MM, 1♀ P (Fig. 3K) | 1♀ (29-VI-1998, Greece, Ohiro, leg. MM, P (Fig. 3G)).

**DNA-extraction, barcode amplification by PCR**. Two legs from each specimen were homogenized with pestle for DNA-extraction, to which we used DNEasy Blood and Tissue kit (Qiagen). At first, a proteinase K treatment was applied on 56°C for overnight, then we followed the protocol of the manufacturer of the kit. As last step the DNA bound to the column was eluated in 2x100 µl Elution Buffer. The first fractions with higher DNA-concentrations were used for the PCR-reactions. The standard LepF1 (5'-ATTCAACCAATCATAAAGATATTGG-3') and LepR1 (5'-TAAACTTCTGGATGTCCAAAAAAT CA-3') primers (Hebert *et al*., 2004; Ivanova and Grainger, 2006) were applied for amplification of the 658 bp barcode region of mtCOI by PCR. The reactions were carried out in 50 µl final reaction volumes containing 2-8 µl template DNA from the first elution fractions, 5 µl 10x Pfu buffer + MgSO4, 1 µl 10 mM dNTPs, 1 µl Pfu DNA polymerase (2.5 U/µl, Thermo Scientific) and 1-1 µl 10 pmol/µl of each primer. In certain cases, addition of 1 µl 50 mg/ml BSA-solution was also necessary for the appropriate yields. At each amplification the following program was used: 3 min at 94°C for initial denaturation; 35 cycles of 30 sec at 94°C, 30 sec at 49°C, 1 min at 72°C; final extension step: 72°C, 5 min. The successfulness of reactions was checked by running 4-6 µl of products on 1,5% agarose gels stained with SYBR Safe (Invitrogen). PCR products were purified and in certain cases concentrated with GenElute PCR Clean-Up Kit (Sigma-Aldrich) and sequenced by BIOMI Kft. (Hungary).

Table 1. The list of mtCOI sequences applied during our phylogenetic reconstruction with their sources and GenBank accession numbers.

| Taxon | Country | Locality | GenBank acc. number (other ID) | Collection date | Collector |
|---|---|---|---|---|---|
| *P. amymone* | Albania | Boboshtiçë | KU360262 | 18-Jul-2015 | K. Takáts |
| *P. mniszechii tisiphone* | Albania | Boboshtiçë | KU360263 | 18-Jul-2015 | K. Takáts |
| *P. orestes* | Greece | Mt. Falakro | KU360264 | 06-Jul-2009 | K. Takáts |
| *P. graeca coutsisi* | Greece | Katara-pass | KU360265 | 08-Jul-2012 | K. Takáts |
| *P. anthelea amalthea* | Greece | Eptachori | KU360266 | 08-Jul-2012 | K. Takáts |
| *P. cingovskii* | Macedonia | Pletvar | KU360267 | 20-Jul-2015 | K. Takáts |
| *P. geyeri occidentalis* | Macedonia | Mt. Galicica | (14-O240) | 26-Jul-2013 | M. Gasc.-Pees |
| *P. hippolyte pallida* | Russia | Altai | FJ664021 | 07-Jul-1999 | V. Lukhtanov |
| *P. hippolyte hippolyte* | Kazakhstan | Tarbagatai Mts. | FJ664018 | 22-Jun-1997 | V. Lukhtanov |
| *P. hippolyte mercurius* | Kazakhstan | Kurdai-pass | FJ664020 | 28-Jun-1993 | V. Lukhtanov |
| *P. hippolyte augustini* | Spain | Sierra de Gador | KP871026 | 24-Jun-1999 | F. Gilt |
| *P. turkestana tarbagata* | Kazakhstan | Tarbagatai Mts. | FJ664022 | 22-Jun-1997 | V. Lukhtanov |
| *P. turkestana turkestana* | Kazakhstan | Kurdai-pass | FJ664025 | 11-Jun-2000 | V. Lukhtanov |
| *P. mamurra schahrudensis* | Iran | Isfahan | DQ338598 | Unknown | Unknown |
| *Chazara briseis major* | Romania | Transsylvania | KP871075 | 30-Aug-2011 | V. Dincă |
| *Satyrus ferula ferula* | Spain | Lleida | GU676975 | 01-Jul-2007 | J. Hern.-Roldan |

**Sequences, pairwise genetic distance matrix, phylogenetic reconstruction**. Table 1 presents the list of partial cytochrome c oxidase I (COI) gene sequences used to calculation of genetic distances and phylogenetic reconstruction with GenBank accession numbers. The COI-barcode-sequence of *P. geyeri occidentalis* was still not public. Its owners, Martin Gascoigne-Pees and Vlad Dincă kindly provided this sequence for us. This sequence was formerly determined at the laboratory of Biodiversity Institute of Ontario, University of Guelph, Canada. New sequences determined for this study are deposited under KU360262-67 accession numbers.

Pairwise genetic distance values shown in Table 2 were calculated according to the Kimura 2-parameter substitution model (Kimura, 1980) using MEGA6 software (Tamura *et al*., 2013).

Phylogeny was estimated with Bayesian inference method using MrBayes v3.2.4 x64 mpi version (Ronquist and Huelsenbeck, 2003). Separate partitions were defined for three codon positions. For all partitions, four-by-four nucleotide models were selected and GTR substitution model space were sampled during the MCMC analyses (nst=mixed option) with gamma distributed rate variation across sites. The base rate and all substitution model parameters were uncoupled across the four data partitions. Default priors were used for all parameters. The analysis involved two independent runs under the default settings except the following: MCMC runs comprised 12 million generations sampled every 1000 generations with 30% burn-in. Sufficient convergence was achieved diagnosed by the average standard deviation of split frequencies between the two independent runs (<0.01) and potential scale reduction factors of the parameters (1 with <1% deviation). The support for individual clades is specified as the mean of the estimated posteriori probability values across the two independent runs. The public sequences of *Chazara briseis major* and *Satyrus ferula ferula* (GenBank accession numbers KP871075 and GU676975, respectively) were defined as outgroups. A consensus tree with 50% majority rule is presented with posteriori possibilities (Fig. 6).

Table 2. The matrix shows the pairwise number of nucleotide substitutions (below diagonal) and genetic distances according to the Kimura two-parameter model (above diagonal) between the partial mtCOI sequences of *Pseudochazara*-spp. in the Balkan Peninsula.

| | 1. | 2. | 3. | 4. | 5. | 6. | 7. |
|---|---|---|---|---|---|---|---|
| 1. *geyeri occ*. | | 0.0123 | 0.0139 | 0.0575 | 0.0557 | 0.0557 | 0.0523 |
| 2. *amymone* | 8 | | 0.0015 | 0.0541 | 0.0557 | 0.0557 | 0.0523 |
| 3. *graeca cou*. | 9 | 1 | | 0.0541 | 0.0557 | 0.0557 | 0.0523 |
| 4. *anthelea ama*. | 36 | 34 | 34 | | 0.0509 | 0.0509 | 0.0476 |
| 5. *mniszechii tis*. | 35 | 35 | 35 | 32 | | 0.0030 | 0.0046 |
| 6. *orestes* | 35 | 35 | 35 | 32 | 2 | | 0.0046 |
| 7. *cingovskii* | 35 | 33 | 33 | 30 | 3 | 3 | |

**Male genitalia studies, terminology, morphometrics, UPGMA phenogram**. The presented male genitalia slide of *P. cingovskii* is deposited in NHM, London (slide No. 23630). The other male genitalia dissections (n=1 from each taxon) were made by standard method, prepared permanent microscopic slides except *P. geyeri*, the dissected genitalia of which were preserved in glycerol microvial attached to the specimen. Genital structures were macerated in KOH, cleared in lactic acid, stained with alcoholic solution of eosin and mounted in euparal. The applied terminology follows Warren *et al*. (2008). At the morphometric analysis 6 genital characters were measured, these are the followings: 1. the angle formed by tegumen and uncus / 180°, 2. the ratio of lengths of tegumen and uncus (lengths measured on the dorsal margins of these structures), 3. the ratio of lengths of subuncus and uncus (the distances measured between the base and apex of these structures), 4. the ratio of widths of valva, the widths measured along perpendiculars to 9/10 and 4/10 valva-length (hereafter „the ratio of widths of valva"), where the latter defined as the distance between the proximo-ventral endpoint and the distal end of valva, 5. the ratio of width and length of tegumen (width was measured between the base of uncus and the ventral endpoint of the base of subuncus) 6. the ratio of lengths of tegumen and valva (Fig. 2). The distances and angles were measured by MB-Ruler v5.0 software (Bader, 2012). During the analyis of the genital similarities, Euclidean distances were calculated from the fore-mentioned 6 variables for each taxa. The UPGMA phenogram was generated based on this distance matrix by PAST v3.10 software (Hammer *et al*., 2001). Bootstrap values were calculated from 2000 replicates.

**Rearing of *P. amymone***. A female of *P. amymone* was collected on 18-VII-2015 by Gábor Simonics to obtain eggs. This female laid 49 eggs until 01-VIII-2015. The rearing of hatching larvae happened according to Gascoigne-Pees *et al*. (2014), with some exceptions: 1. every developmental stage were kept in room temperature, 2. the larvae were fed with *Festuca rubra*, 3. The tube or pot containing *Festuca* stems with radix was placed in a bigger box, whose wall was perfused with water two times every day to ensure the appropriate moisture-level. After the end of feeding larvae were moved to a box containing fine soil, because the larvae pupate in the ground. After 15 days the larvae had buried themselves, they were dug out and been put between moss layers. The first imago has hatched on 10-I-2016. At the time of delivering this manuscript, the remaining one individual was in pupa stadium.

## Results and discussion

### 1. Systematic review of taxa

The list of taxa reflects the phylogenetic reconstruction presented in Fig. 6. The descriptions of genital structures are based on examination of only one specimen from each taxon.

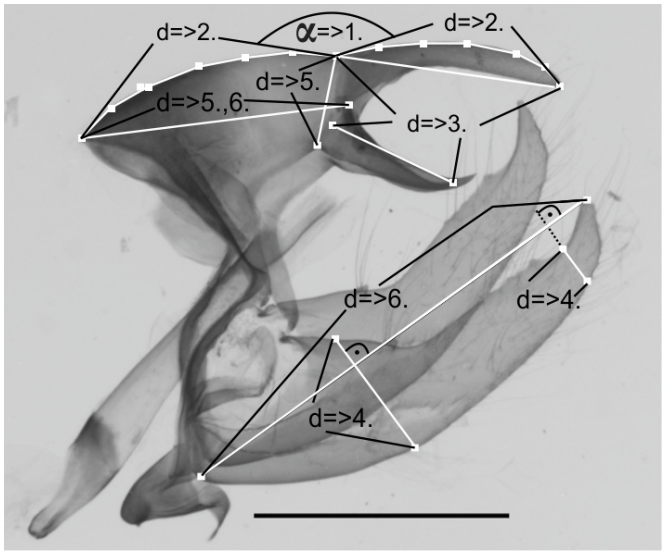


Fig. 2. Measured traits on the male genitalia. We determined ratios of distances and angles. The distances and the angle marked by numbers belong to those calculated ratios, which are described in Materials and methods under same numbers. The scale bar is 1,6 mm.

1. 1. *Pseudochazara cingovskii* (Gross, 1973)
Taxonomic status:
*Satyrus sintenisi cingovskii* Gross, 1973; TL: Prilep, Macedonia
*Pseudochazara cingovskii* – Gross, 1978; Tshikolovets, 2011

Diagnosis: Wingspan: 43-51 mm (males), 53-56 mm (females). Forewing of males pointed, in females more rounded, its outer margin perpendicular to the inner margin. Upperside of wings greyish brown. Submarginal bands generally pale, sandy yellow, darker and more vivid reddish yellow distally, only rarely with an altogether reddish yellow appearance. Forewing submarginal band with two white-centered black ocelli and two tiny white dots between ocelli in both sexes. Two smaller sized white dots also placed in the hindwing band, one with inner position generally with a black frame, although this might be absent, especially in females. Underside ground colour greyish, postdiscal region with more or less yellowish-brownish hue. Fringes whitish, with a greyish hue at vein endings (Fig. 3E,O and 11A).

Male genitalia: Valvae wide, their width is halved after the arched recess found at half of the length of valva. Their distal processes resemble those in *orestes* regarding their tapered shape. Tegumen well developed, elongate, although, compared to *orestes*, less straight and proximally rounded. Uncus connected to the tegumen in 149° angle at its base, mostly similar to *graeca*. Subunci wider and their bases also relatively well developed (Fig. 7A).

Distribution: This species occurs only in the Pletvar Massif and in some mountain area close to Vitolište in Macedonia (Fig. 1A).


Fig. 3. Upperside and undersides views of *Pseudochazara*-spp. occurring in the Balkan Peninsula. The conjugated pictures show males always on left side and females on right side. A–H: upperside views of *P. graeca graeca* (A), *P. graeca coutsisi* (B), *P. geyeri occidentalis* (C), *P. amymone* (D), *P. cingovskii* (E), *P. mniszechii tisiphone* (F), *P. orestes* (G) and *P. anthelea amalthea* (H). I-P: underside view of *P. graeca graeca* (I), *P. graeca coutsisi* (J), *P. orestes* ♀ (K), *P. anthelea amalthea* ♂ (L), *P. amymone* ♂ (M), *P. geyeri occidentalis* ♂ (N), *P. cingovskii* (O) and *P. mniszechii tisiphone* (P). The scale bars are 1 cm.

Habitat: Arid, rocky slopes on very light-coloured metamorphic rocks (mostly marble and gneiss) with sparse vegetation, between 700-1,700 m altitude (Fig. 11B).

Biology: Univoltine, flying period: July-August. Feeds on *Poaceae*.

Geographical variability: As the currently known distribution area of the species fails to reach 10 km$^2$, there are no geographical forms.

Notes: Its habitat is remarkably endangered by mining activities. These butterflies seem to live on a quite specific habitat type, moreover, their tendency to migrate is low and they do not reach far from their original habitats. Thus, the species is unable to populate distant areas, even if they are otherwise suitable (Verovnik *et al*., 2013).

1. 2. *Pseudochazara mniszechii tisiphone* Brown, 1980

Taxonomic status:

*Pseudochazara cingovskii tisiphone* Brown, 1980; TL: Mt. Smolikas, NW Greece

*Pseudochazara tisiphone* – Pamperis, 2009

*Pseudochazara mniszechii tisiphone* – Tolman and Lewington, 2004; Tshikolovets 2011; Cuvelier and Mølgaard, 2015

Diagnosis: Wingspan: 45-50 mm (males), 52-58 mm (females). Forewing of males pointed, in females rounded, like at *cingovskii*. Ground colour of wings brown, without any greyish hue. Submarginal band dark orange, in males generally paler and interrupted between the two white dots, wider and rather continuous in females. In females the two white-centered black ocelli of forewings more pronounced. Males usually with two submarginal spots on hind wings, in females the inner positioned rarely absent. This latter character is very useful in distinguishing from its sympatric pair, *P. amymone*. Forewing underside of males yellowish grey in basal region, in females more brownish, postdiscal region bordered with a diffuse submarginal line (that is sharp in *amymone*), being vivid yellow in females and pale in males. For further differences between these two species, see Cuvelier and Mølgaard, 2015. Hindwing underside brownish grey, with more or less yellowish or sometimes reddish hue. Fringes greyish-greyish brown, darker at the vein endings (Fig. 3F,P and 11C).

Male genitalia: Valvae wide, distal processes similar to those of *orestes*: they gradually tapered and not elongated as in *anthelea*. The dorsal margins of valvae bear only 1 arch at the distal process, reaching towards the ventral margin. The tegumen is dorsally more rounded than in *orestes*, but straighter than in *geyeri*. The uncus is connected to the tegumen in an almost straight angle (=161°) at its basis. Subunci narrower than in *orestes*, with a relatively wide base (Fig. 7B).

Distribution: NW Greece: Vernon Mts., Grammos Mts., Smolikas Massif; SE Albania: surrounding area of Boboshtiçë and Gjergjeviçë; W Turkey: Uludağ (Fig. 1A).

Habitat: Arid, rocky, treeless mountain slopes with sparse grassy vegetation, between 600-1,900 m altitude (Fig. 11D).

Biology: Univoltine, flying period: end of June-September. Feeds on Poaceae. In captivity, caterpillars can be reared on *Festuca ovina*, and they pupate 10-20 mm underground (Tolman and Lewington, 2004).

Geographical variability: The nominotypical subspecies is distributed in most regions of Asia Minor, except from the coastal areas. Compared to the *tisiphone,* the specimens have a lighter brown ground colour, and their submarginal bands are also more remarkable, not interrupted between the ocelli of the forewings of males, and it is considerably wider in females. More to the East, in NE Turkey and in the Lesser Caucasus, the specimens (ssp. *caucasica* (Lederer, 1864)) become darker again, sometimes the submarginal band of the males is very faint, while that of females is less wide and often breaks on the forewing.

Note: Local, but abundant subspecies in suitable habitats of its area. It shares its habitat with *amymone* in SE Albania, but it is significantly more common.

1. 3. *Pseudochazara orestes* de Prins & van der Poorten, 1981

Taxonomic status:

*Pseudochazara orestes* de Prins & van der Poorten, 1981; TL: Drama, Greece

*Pseudochazara orestes* – Tolman and Lewington, 2004; Pamperis, 2009; Tshikolovets, 2011

Diagnosis: Wingspan: 49-54 mm (males), 52-62 mm (females). Wingshape similar to the previous two species. Ground colour of wings light brown. Submarginal band of both sexes bright orange yellow, not interrupted between the ocelli on the forewings (only the crossing veins are brown, opposed to *tisiphone* males) and turns to a darker, more reddish hue distally on hind wings. This dichroism is not or less remarkable at *tisiphone*. Forewing ocelli more remarkable in females. Two white spots located between the ocelli. The inner one of the two hindwing submarginal spots often lacks its black frame. Male hindwing can possess a third, submarginal scale bundle. Forewing underside yellowish brown in basal and discal region, increasingly sprinkled with greyish scales towards the margins and the base; postdiscal area sandy yellow (forewing underside of *cingovskii* dominated rather by greyish hues). Hindwing underside light ashen grey, with a whitish tranversal band limited by a dark brownish grey, diffuse band. Fringes whitish and darker at the vein endings (Fig. 3G,K and 11E).

Male genitalia: Valvae wide, distal processes gradually tapered, not elongated at all. Dorsal margins

of valvae have sharp angles, curves arching towards the ventral side can only be observed proximally from the distal process. The dorsal part of the tegumen not rounded, rather elongated. Uncus connected to the tegumen in an almost straight angle (=163°) at its base. Subunci thicker compared to the previously described species and they have a wide base (Fig. 7C).

Distribution: N Greece: Falakro Massif, Mt. Orvilos; SW Bulgaria: Pirin (Fig. 1A).

Habitat: Arid, sunny, rocky limestone slopes with sparse vegetation, between 300-1,800 m altitude (Fig. 11F).

Biology: Univoltine, flying period: End of June-September. Feeds on *Poaceae*. In captivity, caterpillars can be reared with several different Poaceae plants, including *Agrostis stolonifera* (Tolman and Lewington, 2004).

Geographical variability: As the distribution of this species is relatively narrow, no subspecies are described.

Note: Its habitat over Pirgoi (Greece) at Mt. Falakro lays on limestone. A marble mine is located close to this habitat. It seems like that species adapted to calcareous substrate with light greyish stones, such as *cingovskii*, have greyish wing undersides. This greyish colour is also present in *orestes*, but mainly on the underside of the hindwing, forewing underside is rather yellowish.

1. 4. *Pseudochazara amymone* Brown, 1976

Taxonomic status:

*Pseudochazara amymone* Brown, 1976; TL: NW Greece, Ioannina

*Pseudochaza mamurra amymone* – Gross 1978; Tolman and Lewington, 2004; Tshikolovets, 2011; Eckweiler, 2004, 2012

*Pseudochazara amymone* – Pamperis, 2009; Gascoigne-Pees *et al*., 2014; Cuvelier and Mølgaard, 2015

Diagnosis: Wingspan: 40-47 mm (males), 44-51 mm (females). Forewing of males pointed, but the outer margin rounded, even more in females. Ground colour of wings brown, usually with a lighter shade in females. Submarginal band of forewings yellowish brown, narrowly lighter than the ground colour in males and bright yellowish in females. This band remarkably wide and well-developed on hindwings of both sexes, bright orange coloured with more reddish hue on the distal areas. Submarginal band of forewings includes two white-centered ocelli with brown frame in both sexes; the white pupils often absent. Submarginal band on hindwings contains only a single ocellus in males and usually one, but rarely two ocelli in females. In males, this is a good differential character against *tisiphone* males which have two ocelli. For further differential characters between these species, see Cuvelier & Mølgaard, 2015. Forewing underside ochreous in both sexes, with more brownish and greyish sprinkling in the basal region in females. Hindwing underside mainly brown with yellowish-greyish hues. Fringes whitish, and greyish brown at the vein endings (Fig. 3D,M and 11H).

Male genitalia: Valvae relatively wide, their distal processes are shaped similarly to those of *graeca*: less narrow and tapered than in *anthelea*. Dorsal margin of the valvae bears two arches; the proximal one is deeper than that in *graeca*; this is partly because the dent-like structures proximally from the arches are much more developed than in *graeca*. Uncus connected to the tegumen in an extremely steep angle (131°) at its base. Subunci short, narrow, with a thinner base than in *graeca* (Fig. 7D).

Distribution: NW Greece: Mountains North of Ioaninna, SE Albania: Mali i Moravës Massif (Korçë) and mountains from Gjergjeviçë to Devoll Gorge (Fig. 1B).

Habitat: Arid, rocky areas with extremely sparse grassy vegetation, typically on ophiolitic substrate, between 400-1,400 m altitude (Fig. 11D).

Biology: Univoltine, flying period: end of June-August. Larva feeds on *Poaceae* (Fig. 10A), in captivity can be successfully reared on *Festuca ovina* (Gascoigne-Pees *et al*., 2014); during our rearing experiment *Festuca rubra* was used for feeding.

Geographical variability: As the species has a restricted area and its distribution is limited to a small region of the Balkan Peninsula, we cannot distinguish any subspecies.

Note: Following its description in 1976, the exact habitats of the species were kept in secret for long. This created an air of mystery around the species. Although Arnscheid and Arnscheid mention it from near Ioannina, Greece in 1981, they do not provide any details (Arnscheid and Arnscheid, 1981). Misja and Kurrizi report the species from SE Albania (Misja and Kurrizi, 1984), but these records failed to be confirmed later. Pamperis reports 4 localities in Epirus and Greek Macedonia in his work, the "Butterflies of Greece", in 1997, and this number increases to 10 in the 2009 edition of the book (Pamperis, 1997, Pamperis, 2009). However, he avoids the publication of the precise localities in order to protect the populations from collectors. In the summer of 2010, Eckweiler found a population in S Albania, but the exact location is kept in secret again (Eckweiler, 2012). Targeted searches, carried out by Verovnik *et al*. for the species during the summer of 2012 and 2013 resulted in 18 localities in Albania, and they also predicted other potential localities by using Maxent species distribution models (Verovnik *et al*., 2014).

1. 5. *Pseudochazara graeca* (Staudinger, 1870)

Taxonomic status:

*Satyrus pelopea graeca* Staudinger, 1870; TL: Mt. Parnassos and Mt. Chelmos, Greece

*Pseudochazara mamurra graeca* – Higgins, 1975

*Pseudochazara graeca* – Gross, 1978; Tolman and Lewington, 2004; Pamperis, 2009; Tshikolovets, 2011

Diagnosis: Wingspan: 41-47 mm (males), 43-53 mm (females). Forewing of males more pointed than in *amymone* males. Costal margin shows a characteristic break before the apex in males – especially in ssp. *coutsisi*. Female forewing rounder. Ground colour of wings from light to dark brown. Males usually darker. Submarginal band can be different in its appearance (see geographical variability). The two dark brown ocelli on forewing of males without white pupils or at most the costally located one can possess a weak white centre. In females either both or none of the ocelli includes white pupils. Between ocelli further spots can be observed only in exceptional cases in both sexes, but never clear white ones. Single ocellus of hindwing usually hardly developed and often absent in both sexes. Underside colour varies in a wide spectrum (see below). Fringes from light grey to whitish, unicolourous (Fig. 3A,B,I,J).

Male genitalia: Valvae wider, but their distal processes less tapered than in *anthelea* and they are not narrow. Dorsal margin of each valva have two rounded curves; the proximal one less arched than in *amymone* or *anthelea*. Uncus connected to tegumen in a steep angle (=142°) at its base. Subunci short and narrow, connecting to the tegumen with relatively wide base (Fig. 7E).

Distribution: S. Greece, Peloponnese: Mt. Chelmos, Mt. Panakhaikón, Mt. Ménalon, Mt. Taygetos; Évvia Island: Mt. Dírfis; Central- and NW Greece: Mt. Olympos, Pindos Mts. (from Smolikas Massif to Mt. Parnassos); Macedonia: Mt. Pelister (Tolman and Lewington, 2004); S Albania (?), confirmation required (Eckweiler, 2012) (Fig. 1B).

Habitat: Arid, grassy and rocky slopes on limestone or ophiolitic substrate, between 750-2,400 m altitude.

Biology: Univoltine, flying period: June-September. Feeds on *Poaceae* plants.

Geographical variability: Gross in his work of 1978 distinguishes 4 subspecies: ssp. *graeca* (Staudinger, 1870) from the Tymphristos mountain complex, ssp. *coutsisi* Brown, 1977 from the Pindos, ssp. *apollo* Gross, 1978 from Mt. Parnassos and ssp. *pelops* Gross, 1978 from Mt. Chelmos and Mt. Taygetos. The currently accepted view is that the latter two subspecies are the junior subjective synonyms of the nominotypical subspecies. Recently the validity of ssp. *coutsisi* has also been questioned by Anastassiu *et al*. (2009), as they found that the wing colouration of *P. graeca* gradually changes along a clinal gradient (see below). We can generally conclude that the specimens of ssp. *coutsisi* (Fig. 3B,J) are more brownish coloured and darker than those of ssp. *graeca* (Fig. 3A,I). Submarginal band pale yellow, whitish sandy yellow (ssp. *graeca*) or light brown, yellowish brown (ssp. *coutsisi*) with brighter, orangish or reddish colour on the distal part of hindwing band. This dichroism is less remarkable in ssp. *coutsisi*. In ssp. *graeca*, forewing underside greyish yellow in the basal region, with darker tones near the margins, while in ssp. *coutsisi* it is yellowish brown, typically darker in females. Postdiscal region of the forewing underside lighter than the basal area in both subspecies. Outer margin grey in ssp. *graeca* and brown in ssp. *coutsisi*. The predominant colour of hindwing underside is grey in ssp. *graeca*, and brown, brownish yellow in ssp. *coutsisi*.

Note: Some specimens of ssp. *coutsisi* – particularly the females – look very similar to the specimens of *P. amymone* (Fig. 9B; see below).

1. 6. *Pseudochazara geyeri occidentalis* (Rebel & Zerny, 1931)

Taxonomic status:

*Satyrus geyeri occidentalis* Rebel & Zerny, 1931; TL: Galicica Planina, Macedonia

*Pseudochazara geyeri occidentalis* – Higgins, 1975; Gross, 1978; Tolman and Lewington, 2004; Pamperis, 2009; Tshikolovets, 2011

Diagnosis: Wingspan: 35-46 mm (males), 41-47 mm (females). Forewing of males has similar shape to that of *amymone*, but more pointed and outer margin is less rounded. Female forewing wider and more rounded than that of males. Wings ground colour brown in males and light greyish brown in females. Submarginal band light brown or light brownish grey in males, while rather greyish with some yellow hue in females. Forewing ocelli have a brownish frame in males and a blackish brown in females; they have small, white pupils in both ocelli in both sexes. White spots absent in the S3 and S4 inter-venal spaces. Only one, white-centered ocellus placed on hindwing with definite contour. The sharp contrasts of wing patterns give a unique look this species among Balkanic *Pseudochazara* species: the submarginal band bordered by a thin, darker band from the basal side and with a series of darker, lunulated spots from the distal side. On the hindwing these lunulated spots definitely pointed and less arched, they delineate a zigzagged line. Besides two small dark lines placed in the forewing cell: one runs transversely at two-third of the cell and the other one closes down the cell distally. Forewing underside pale yellowish grey in both sexes with more greyish and brownish hue in the basal area and at the outer margin. The pattern-elements of the upperside appear with even higher contrasts on the underside; they are coloured black or blackish brown. Fringes checkered with sharply contrasted whitish and dark areas (Fig. 3C,N and 11G).

Male genitalia: The proximal widths of valvae are approximately the same as in *graeca*; but the distal two-third of valvae are remarkably narrow, which is unique among these species. Dorsal margin of valvae lacks the arches that are typically found in *anthelea* or *graeca*. Tegumen almost perfectly rounded dorsally. Uncus connected to tegumen in a similar angle

(=141°) as in *graeca* or in *cingovskii.* Subunci very narrow with narrow bases (Fig. 7F).

Distribution: Similarly to several other Balkanic *Pseudochazara* species, its distribution area is remarkably small: Mt. Galicica and the Pelister Massif in SW Macedonia, the mountain range near Ohrid Lake in Albania and Mt. Malimadi and Triklarion Mts. in NW Greece (Tolman and Lewington, 2004) (Fig. 1A).

Habitat: Arid, grassy and karstic rocky slopes on limestone above the beech tree line, between 1,200-1,750 m altitude.

Biology: Univoltine, flying period: end of July-August, it starts as latest among *Pseudochazara*'s occurring in the Balkan Peninsula. Feeds on *Poaceae*. Larvae can be reared in captivity on *Festuca ovina* (Tolman and Lewington, 2004).

Geographical variability: The nominotypical subspecies (ssp. *geyeri* (Herrich-Schäffer, [1846]) can be found in Central and East Turkey and in Caucasus Minor. It has a brighter coloured wing underside, and the whitish postdiscal band on their hindwings is much narrower, compared to individuals in Balkan Peninsula (Rebel & Zerny, 1931).

Notes: The species has an extended area in Asia Minor, here it likely occupies habitats with different subtrates. The populations in the Balkan Peninsula have a quite small area, mostly on very light coloured limestone. Their wing underside is predominantly greyish, similarly to that of *cingovskii,* which also lives in habitats on whitish-light greyish substrate (although, especially on metamorfic limestone or gneiss) – this also may imply some correlation between wing colour and substrate colour. This presumed relation corresponds well with the fact that the more widespread populations in Asia Minor have a somewhat darker wing underside and their whitish postdiscal band is also narrower, which suggest that Balkan specimens are more adapted to the whitish-greyish coloured limestone substrate.

1. 7. *Pseudochazara anthelea amalthea* (Frivaldszky, 1845)

Taxonomic status:

*Hipparchia amalthea* Frivaldszky, 1845; TL: Crete

*Pseudochazara anthelea amalthea* – Higgins, 1975; Tolman and Lewington, 2004; Pamperis, 2009; Tshikolovets, 2011

Diagnosis: Wingspan: 43-48 mm (males), 49-55 mm (females). Forewing of males pointed. In females outer and inner margin perpendicular at the tornus. Wings chocolate brown. In males, a distinct stripe is present in the forewing cell, which is however not an androconial stripe, as androconial scales are found outside it (Aussem, 1980). Submarginal band runs slightly more inwards at the outer margins compared to the previously presented species; white in males and rather yellowish white in females. Hindwing submarginal band bounded by a yellowish or reddish brown area distally, mostly reduced to a postdiscal patch in females and contains a single ocellus in both sexes. Forewing with two ocelli both in males and females. Basal region of forewing underside whitish grey in males and yellowish brown in females. This is distally surrounded by white or yellowish white area of the submarginal band, then wing undersides greyish (males) or greyish brown (females) near the apex and at outer margin. Hindwing underside brownish or brownish grey. Fringes whitish on the forewing and greyish on the hindwing, but they always darker at the vein endings (Fig. 3H,L).

Male genitalia: Valvae and their distal processes are robust, well-chitinized structures, which are more intensively sclerotized than in any other species; none of the other Balkan *Pseudochazara* species have similar features on the preparations. Valvae very wide, the distal processes narrow down relatively suddenly and are long tapered. Dorsal margin of valvae bears two elongated arches. Uncus connected to tegumen in an almost straight angle (=170°) at its basis. Subunci have a rather wide basis (Fig. 7G).

Distribution: Crete, the Peloponnessos Peninsula, almost the whole continental region of Greece, excluding the lowlands of Thessaly, Greek Macedonia and Thrace, the Ionian coast and the Chalkidiki Peninsula; Albania, Macedonia, Montenegro, SW Bosnia and Herzegovina, and SW Bulgaria (Fig. 1C).

Habitat: Rocky montane areas, gorges, even woodlands if they include open areas, between 400-1,600 m altitude.

Biology: Univoltine, flying period: May-September. Feeds on *Poaceae*.

Geographical variability: The populations of *P. anthelea* in Asia Minor (=ssp. *anthelea* (Hübner, [1824]) exhibit more considerable sexual dimorphism: females have a bright orange pattern, and males have larger white areas on their wings. The females of the populations in Rhodos show transition external characters to ssp. *amalthea* (Tolman and Lewington, 2004).

Note: Based on its distribution, this species has the widest tolerance range between the Balkan *Pseudochazara*-spp.

**2. Amplicons of partial mtCOI by PCR**

Yield ratios of the ~700 bp PCR-products from specimens of the different species were rather different and slightly correlated with the age of collected specimens (Zimmermann *et al*., 2008). It has to be noted that all specimens were kept in a relaxation box until ½-2 days, and the relaxation procedure is known to make it difficult to obtain sufficient amount of intact template DNA (Knölke *et al*., 2005). In case of *P. amymone* and *P. mniszechii tisiphone* specimens collected during the summer of 2015, the reactions – performed with using 2 µl / 50 µl template DNA / PCR mix ratio – result in well detectable bands on agarose

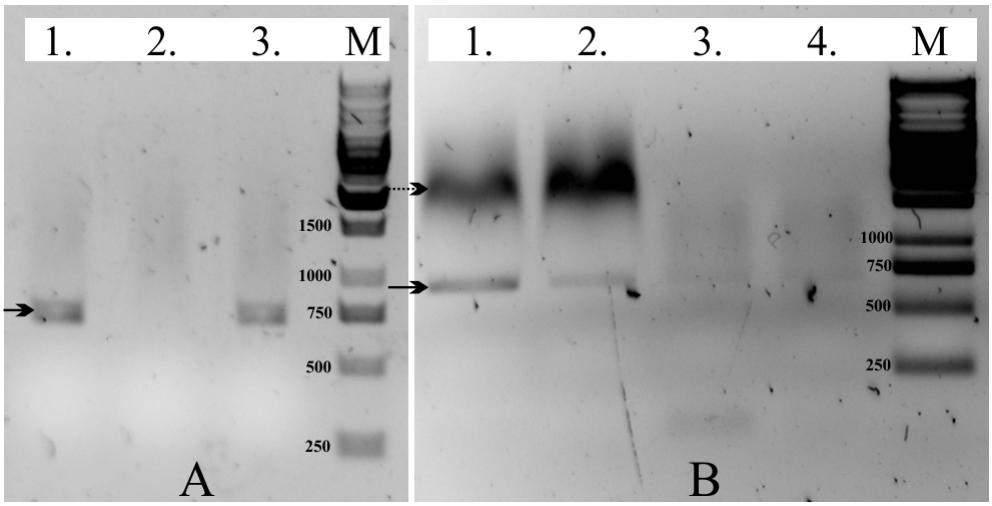


Fig. 4. A: Agarose gel image of the first PCR targeted the amplification of the mtCOI barcode region of *P. amymone* (lane1), *P. cingovskii* (lane2) and *P. mniszechii tisiphone* (lane3). 4 µl of each PCR reaction (with 2 µl template/50 µl mix) were loaded in lanes. B: Applying BSA in the amplification of barcode region of *P. anthelea* and *P. cingovskii*. Lane1: *anthelea* (+BSA), lane2: *cingovskii* (+BSA), lane3: *anthelea* (–BSA) and lane4: *cingovskii* (–BSA). Here, 6 µl of each PCR reaction (with 8 µl template/50 µl mix) were loaded in lanes. Continuous arrows indicate the mtCOI amplicons, broken arrow shows a diffuse band with higher molecular weight at the applying of BSA, lane M: DNA marker.

gel (Fig. 4A). For many species, the amount of DNA template had to be increased in order to achieve a similar template concentration (which indicated that extract eluates had different DNA-concentrations); and/or the addition of BSA was necessary. In case of *P. graeca* and *P. orestes* specimens collected in 2012 and 2009, respectively, we used 3 µl template DNA. The *P. anthelea* specimens collected in 2012 and *P. cingovskii* specimens collected in 2015 required 4x increased template amount (8 µl / 50 µl PCR) and also the addition of 1 µl 50 mg/ml BSA solution to finally result in poorly detectable bands (Fig. 4B), which were further concentrated to obtain sufficient amount for DNA sequenation. The addition of BSA resulted a diffuse band, with higher molecular weight, but this has not affected the post-PCR steps and sequencing. BSA has the affinity to bind different macromolecules on its surface, thus it can promote the separation of elongating DNA strands from the possible contamination in the sample that can inhibit the polimerase reaction (Abu Al-Soud and Radstrom, 2000). We received legs of *P. geyeri* from Jonathan Agius (leg. Isidor Saric, 01. 08. 2009., Mt. Galicica, Macedonia), but due to time limitations we could only perform a single PCR reaction with these samples that failed to result in any detectable bands. Therefore we asked for the right to use an already existing, non-public sequence from its owners. Martin Gascoigne-Pees and Vlad Dincă kindly provided this sequence for us. This barcode sequence was formerly sequenated at the laboratory of Biodiversity Institute of Ontario, University of Guelph, Canada.

### 3. Multiple alignment, pairwise number of nucleotide substitutions, distance matrix, and phylogenetic reconstruction

Fig. 5 presents the multiple alignment of barcoding regions of mtCOI of *Pseudochazara* species in the Balkan Peninsula. From the 658 positions examined, 55 were variable and the others were conserved. Even the majority of variable positions contained nucleotide substitutions that do not change the amino acid sequence, that is, they are synonym mutations. To be specific, regarding the 7 sequences, substitution resulted in changes of the primer protein structure only in 3 cases out of the 55 positions. These positions are the following: Gly to Ser substitution at G368A in *geyeri* (in all other sequences G), Val to Ile substitution at G470A in *amymone* and *graeca* (in all other sequences G), and Ser to Asn substitution at G477A in *amymone*, *graeca* and *geyeri* (in all other sequences G). The first two of these substitutions is taking place at the first codon position, and the third substitution at the second codon position (protein sequence alignment not shown).

Table 2 presents the number of paired nucleotide substitutions of barcode sequences in *Pseudochazara* species of the Balkan Peninsula, and the genetic distance of pairs according to the Kimura-2-parameter model. By looking closely at the matrix values, we may suspect that the above mentioned species can be divided into three separate phylogenetic groups. In two groups of species (*amymone-graeca-geyeri* and *mniszechii-cingovskii-orestes*) we found rather few (1-9) difference between pairs and thus, small genetic distances. At the same time, both groups differed in approximately the same number of nucleotide substitutions (30-36) from the 7th examined species, *anthelea*, which implicated that *anthelea* represent a separate phylogenetic lineage between the previous two groups.

For the phylogenetic reconstruction with Bayesian method (Fig. 6), we also used the existing mtCOI barcode sequences, available in the GenBank, of *Pseudochazara* species occurring in distant areas. For the taxa included in the analysis and the GenBank accession numbers of their sequences, see Materials

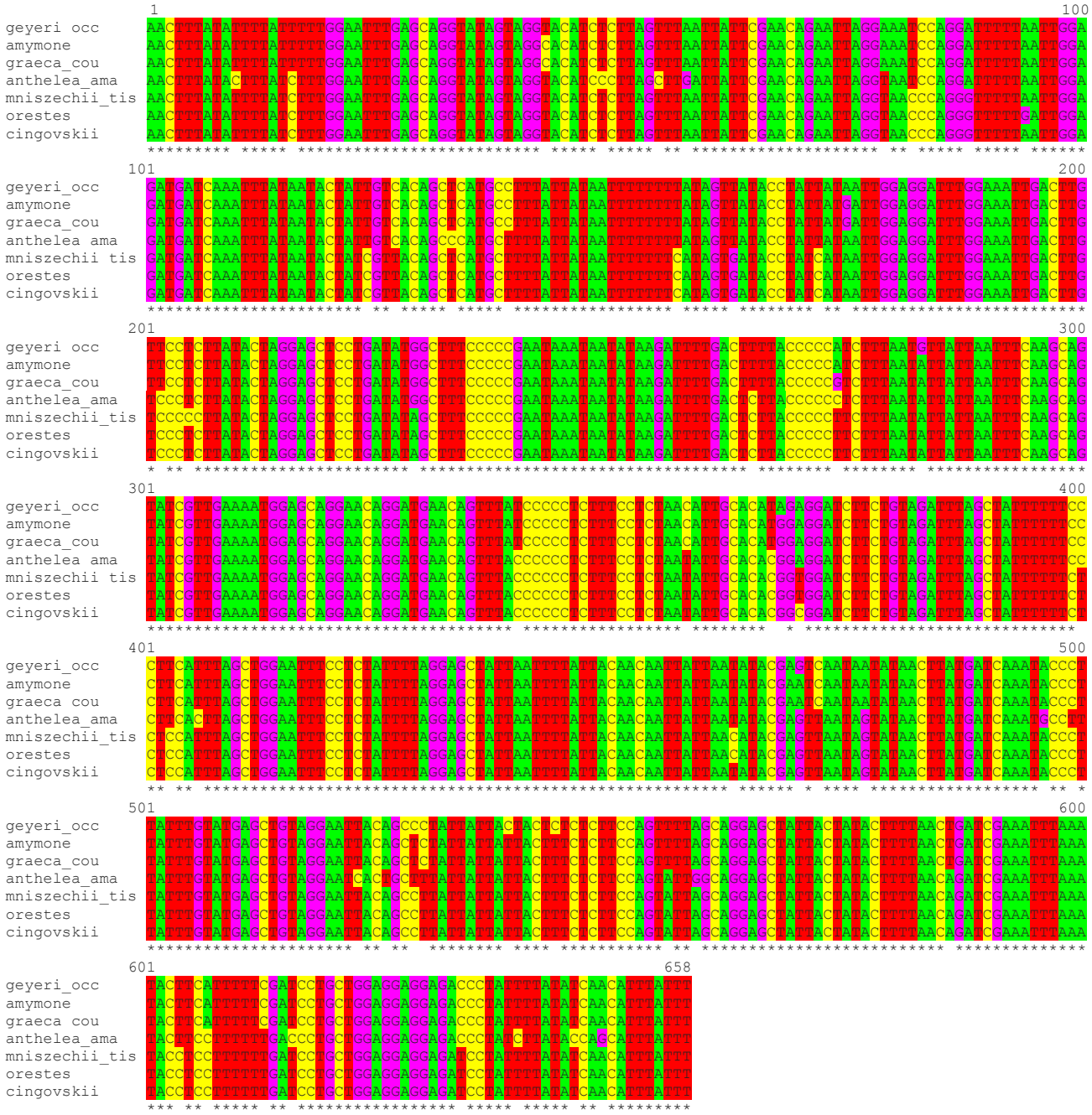


Fig. 5. Multiple alignment of mtCOI barcode regions of Balkan *Pseudochazara*-spp.

and methods, and the resulted phylogenic tree. Unfortunately, only a few taxa has publicly available barcode sequences from the *Pseudochazara* genus. We selected the public sequence of the less closely related *Chazara briseis major* and *Satyrus ferula ferula* as outgroups. We can distinguish 3 or 4 major *Pseudochazara* COI-groups on the tree that was obtained, depending on whether we treat the „B" clade separately from the „C" clade. *A posteriori* probabilities (pp) are shown at the nodes, representing the consesus values of a given clade. These have the maximal value of 1, and a clade cannot be established reliably if pp < ~0.80. Clades with the highest consensus and including *Pseudochazara* species, and *anthelea* with considerably different barcode sequence are shown with black framing on the figure. Because of its low pp value (0.58), *anthelea* cannot be reliably established, it lacks a confident sister group. According to earlier reports, its closest relative could be *P. thelephassa* (Geyer, [1827]), based on morphological characters: males have similar valva structure and a contrasted band in the cell of the forewings in both species (Gross, 1978, Aussem, 1980).

In our phylogenetic reconstruction, *P. geyeri occidentalis* is the sister taxon of *P. mamurra schahrudensis* (Staudinger, 1881), which lives mainly in Iran, with about ~1 *a posteriori* probabily value. *P. amymone* and *P. graeca coutsisi* can also be treated as sister groups, although with slight lower consensus. Finally, these 4 taxa altogether form the sister group of *P. turkestana* (Grum-Grshimailo, 1893). However, it is important to emphasize that when talking about „sister groups", „clades" and thus about monophyletic groups, we only draw conclusions based on the relationships represented on

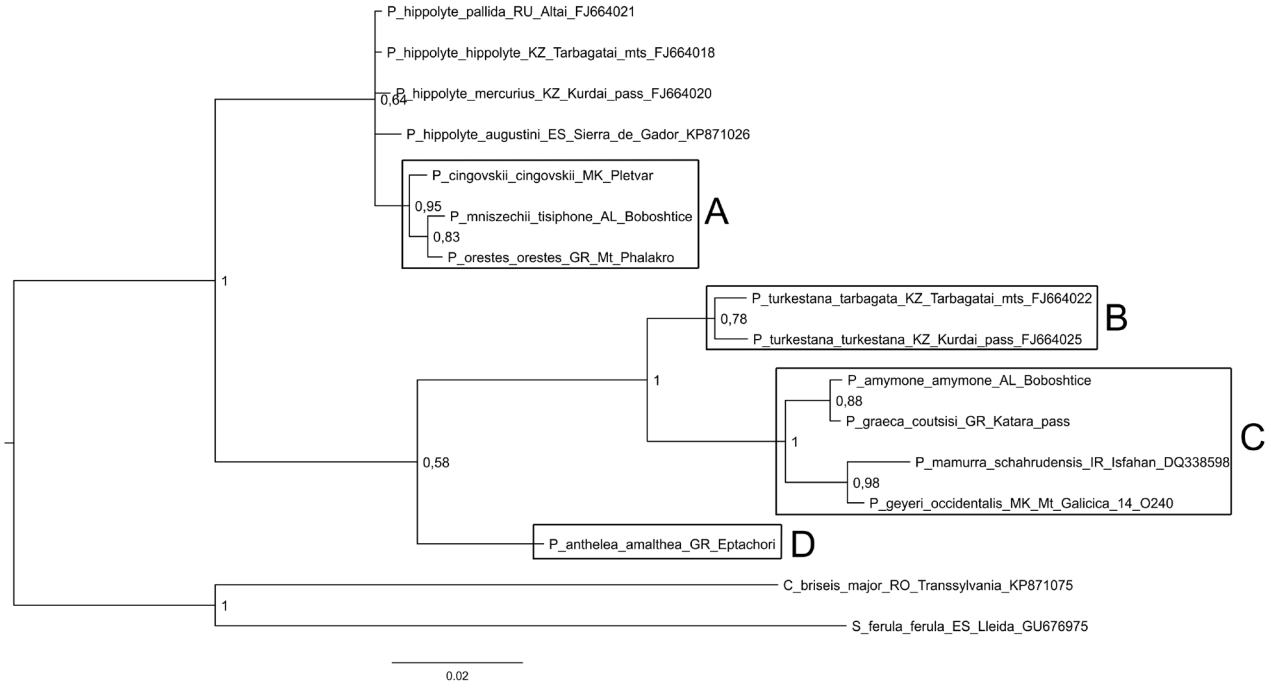


Fig. 6. Bayesian phylogenetic reconstruction based on partial mtCOI sequences. Posterior possibilities are shown at the nodes. The explanation of the framed clades and taxon see in the text.

the resulting phylogenetic tree and only concerning the taxa included in the analysis.

With regards to the „C" clade, it is worth to note that *P. amymone*, formerly treated in several reports as the subspecies of *P. mamurra* (Herrich-Schäffer, [1846]) (Gross, 1978; Tshikolovets, 2011; Eckweiler, 2012) has a closer relationship with *P. graeca coutsisi* than with *P. mamurra schahrudensis*, or at least this appears in the above presented phylogenetic reconstruction. Therefore, if we would like to keep the previous taxonomical consideration (that is, *amymone* is the subspecies of *mamurra* instead of being a separate species), then we should treat *graeca* and *geyeri* as the subspecies of *mamurra* as well. However, this is not evident, first because *schahrudensis* is treated as an individual species by several authors (Tuzov *et al*., 1997), and second, it would be favourable to know how the same sequence of the nominotypical form of *mamurra* would change the topology. Nonetheless, this „C" clade can be considered as a '*mamurra*-group' lineage.

In the „A" clade, *P. mniszechii tisiphone* became the sister taxon of *P. orestes* with an acceptable level of consensus (pp=0.83), the latter being the sister group of *P. cingovskii*. These three taxa are probably related to the widely distributed *P. hippolyte* (Esper, [1784]), but the common clade including *hippolyte*-subspecies does not have a high pp value (pp=0.64). As in the case of *mamurra* in the „C" clade, it would be valuable to know how would the sequences of the two other *mniszechii* subspecies in Asia Minor (*mniszechii mniszechii* and *mniszechii caucasica*) influence the tree topology. However, accepting the subspecies rank of *tisiphone,* we view this „A" clad as the '*mniszechii*-group' lineage.

In summary, we can conclude that our Bayesian phylogenetic reconstruction involving other *Pseudochazara* species supports our hypothesis that

Table 3. Measured values of the selected male genital variables. For the detailed description of the trait marked by asterisk, see the section 'Materials and methods'.

| | Uncus-tegumen angle / 180° | Uncus-tegumen length ratio | Subuncus-uncus length ratio | Ratio of widths of valva* | Ratio of width and length of tegumen | Tegumen-valva length ratio |
|---|---|---|---|---|---|---|
| *cingovskii* | 0.83 | 0.81 | 0.58 | 0.27 | 0.34 | 0.58 |
| *mniszechii tis.* | 0.89 | 0.91 | 0.63 | 0.29 | 0.36 | 0.52 |
| *orestes* | 0.91 | 0.86 | 0.60 | 0.33 | 0.34 | 0.56 |
| *anthelea ama.* | 0.94 | 0.94 | 0.64 | 0.21 | 0.44 | 0.45 |
| *amymone* | 0.73 | 1.02 | 0.65 | 0.18 | 0.42 | 0.48 |
| *graeca cou.* | 0.79 | 0.89 | 0.63 | 0.22 | 0.44 | 0.53 |
| *geyeri occ.* | 0.78 | 0.84 | 0.63 | 0.19 | 0.39 | 0.54 |

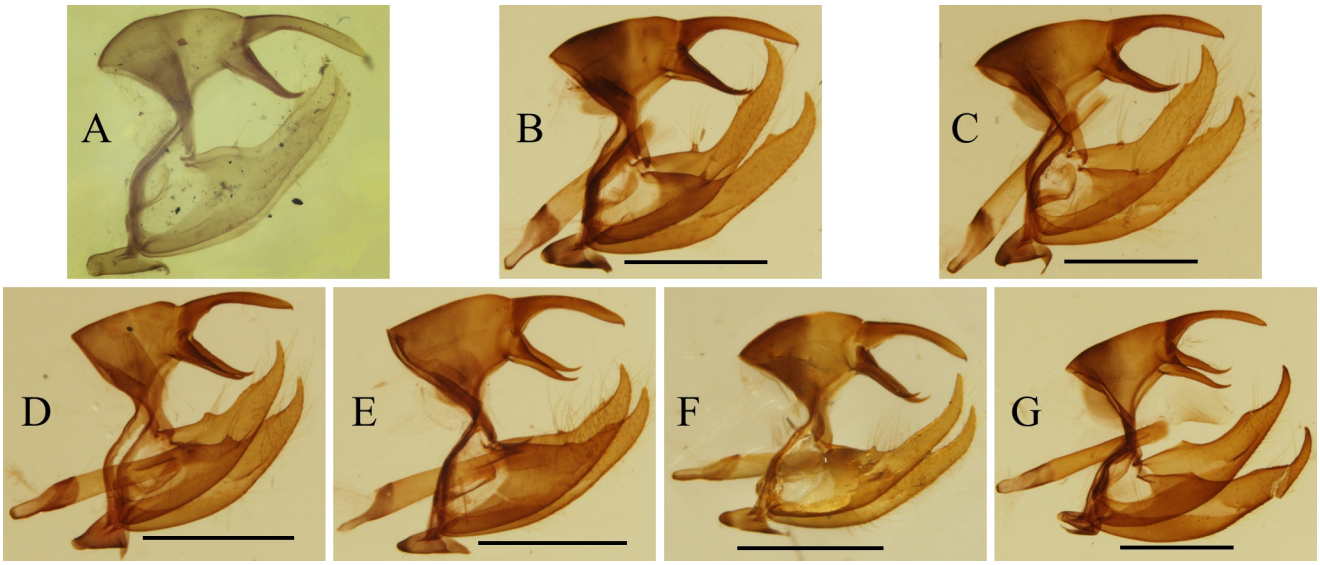


Fig. 7. Lateral views of male genital structures of the examined *Pseudochazara*-spp. A: *P. cingovskii*, B: *P. mniszechii tisiphone*, C: *P. orestes*, D: *P. amymone*, E: *P. graeca coutsisi*, F: *P. geyeri occidentalis* and G: *P. anthelea amalthea*. The scale bars are 1,6 mm.

the Balkan members of this genus can be categorized into three separate phylogenetic lineages.

**4. Male genitalia support the conclusions drawn from the phylogenetic reconstruction**

As it was mentioned in the 'Systematic review of taxa' section, it is also worth to note here that male genitalia dissections were made only from a single specimen for each taxon. Therefore, we could not clearly identify species-constant characters. When considering the general appearance of male genital structures, we can say that the members of '*mniszechii*-group' typically have a longer, tapered tegumen and wide distal halves of valvae, narrowing down only directly before their distal ending. The members of '*mamurra*-group' have relatively short tegumen, which starts with a steep angle at the base of uncus. The distal valva halves narrows gradually, dorsal margins of the valvae do not break in sharp angles. In *anthelea* especially the very robust valvae are conspicuous.

Table 3 contains the measured variables for each taxon and Fig. 8 presents the UPGMA tree based on these genital morphometric data. The obtained phenogram has a remarkably similar topology to the phylogenetic tree that based on mtCOI-sequences. The members of the „A" clade appearing in the latter (*cingovskii*, *orestes*, *mniszechii tisiphone*) form a cluster also on this phenogram with a relatively high bootstrap value, thus we can conclude that their genital structures show a high degree of similarity. Regarding the „C" clade of the mtCOI phylogenic tree, *geyeri* and *graeca coutsisi* has the shortest distance on the UPGMA phenogram, while interestingly, *amymone* fails to show close similarity to them. *Anthelea* separates definitely from the two clusters mentioned above and shows shorter distances to the members of '*mniszechii*-group'. The clear differentiation of *amymone* from every other members, supported with high bootstrap values, does not reflect the estimated evolutional relationships of the phylogenic tree obtained from mtCOI sequences and it is somewhat surprising: the general appearance of the genital structures of *amymone* males best resembles that of *graeca* by eyes (Fig. 7D,E). Despite the obviously high similarity between *amymone* and *graeca*, this clear separation of *amymone* on the phenogram may arise from the fact that this species showed extremely deviant values of two measured traits (tegumen-uncus angle/180° and uncus-tegumen length ratio), which were sharply different from the group-averages.

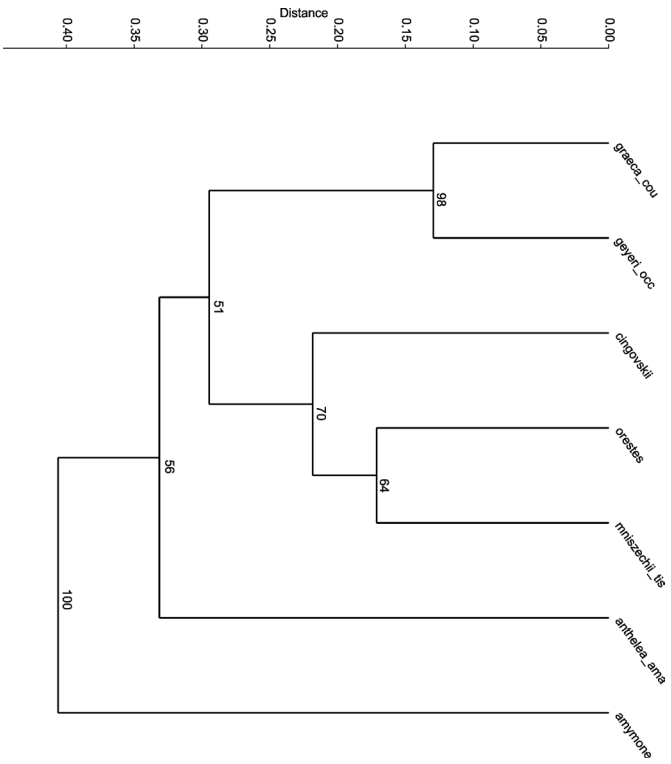


Fig. 8. UPGMA phenogram generated from the Euclidean distances of male genital variables. Bootsrap-values are shown at the nodes of phenogram.

Eventually, this could lead to the increased distances against other taxa in the multivariable space.

In summary, the topology of the two trees correspond well – except for the position of *amymone* –, therefore we can conclude that the similarities of genital structures greatly support our phylogenetic reconstruction based on mtCOI-sequences.

## 5. The case of *P. amymone* and *P. graeca*: is *amymone* a separate species or subspecies of *graeca* only?

The shortest genetic distance has been found between the *amymone* and *graeca*. The barcode region of their mtCOI differs only in 1 nucleotide position which only represents a difference of 0,15%. This minimal difference can raise the question whether these two taxa are separate species or only subspecies of a given species? Interestingly, the Kazakh (ssp. *hippolyte*) and the Spanish subspecies (ssp. *augustini*) of the extremely wide distributed *P. hippolyte* are different in 3 positions for the same mtCOI-region, however, they are often regarded as conspecific taxa (Tshikolovets, 2011; Dincă *et al*., 2015). This difference of 3 nucleotide-positions may not seem significantly larger, than in the case of *amymone* and *graeca*, but the geographical distance between the two areas is immense, ~7000 km, and the Iberian populations are isolated. If they can be treated as conspecific taxa with 3-fold difference in their mtCOI barcode regions, then our question mentioned above is not devoid of any claim.

Additionally, when morphologic data are also included to prove the 'two subspecies theory', we can see that there are specimens of *amymone* and *graeca coutsisi*, primarily females, that can be externally considered as intermediate and are very similar to each other (Fig. 9).

Gascoigne-Pees *et al*. (2014) describe the lifecycle of *amymone* and the morphology of the larva. In the summer of 2015, we have also been provided with 49 *amymone* eggs, thanks to Gábor Simonics. A total of 23 small larvae have hatched from the eggs, these were fed with *Festuca rubra.* The larvae grew very slowly over the first 1-1.5 months (late August - mid September) with high mortality prevailing among them. Keeping them at room temperature, using a supplementary light source, eventually 2 larvae were grown to pupation, which buried themselves on 12-XII-2015 and 24-XII-2015. By their external appearance, the L5 stadium larvae of *amymone* (Fig. 10A) and *graeca* (Fig. 10B) are very similar, contrary for example to larvae of *anthelea* (Fig. 10C), as these latter differ greatly from the larvae of *amymone* and *graeca* with their zigzagging dorso-lateral longitudinal stripes and the alternant light and dark parts in their dorsal band. This latter alternation is also visible in *graeca*, but the proportion of the lighter yellowish parts in the segments is smaller. The longitudinal stripes of the *amymone* larvae are little darker than those of *graeca* specimens, the darker colour of spiracular bands of *amymone* is more pronounced.

Actually, we do not yet have any information about the sympatric occurrence of the two taxa. It could be expected possibly in Greece and in this case it should refuse the 'two subspecies theory'. Our knowledges in context with the Greek localities of *amymone* are rather deficient, but similar coexistence was not observed also in Greece (Pamperis, pers. commuication, 2016).

In spite of the above similarities, we do not recommend to consider *amymone* as a subspecies of *graeca*, for the reasons below:

1. The limits of the ecological requirements of the two species are almost certainly completely different; to our present knowledge, the occurrence of *amymone* is restricted to a very small area in the Balkan Peninsula, indicating adaptation to a very special habitat with extremely sparse vegetation. In contrast to this, *graeca*'s larger range extending into

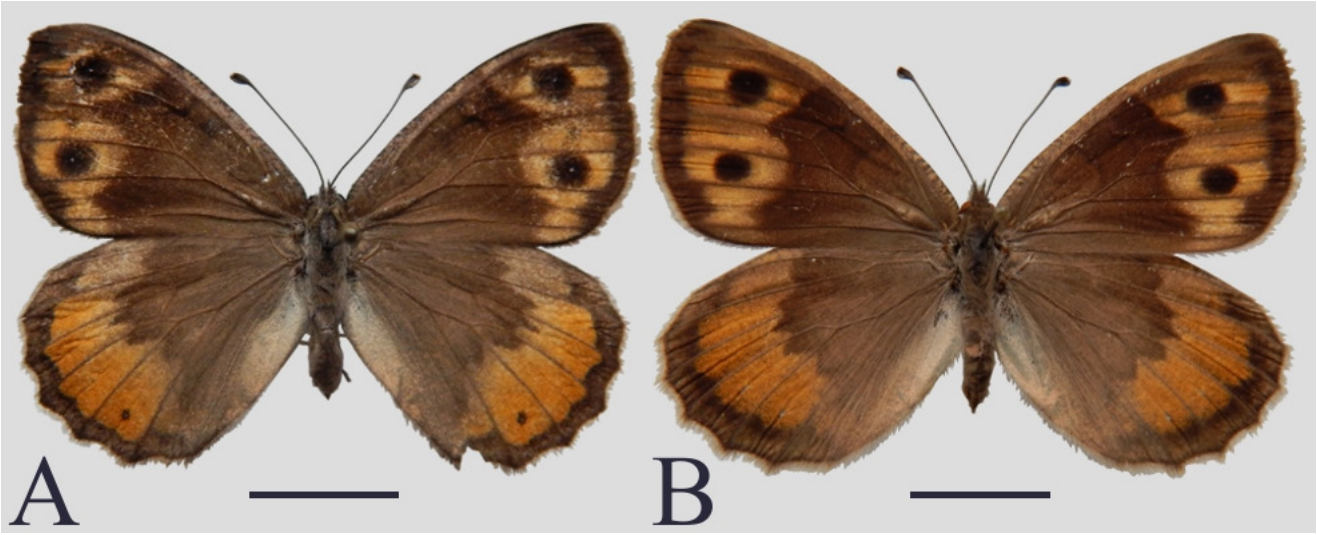


Fig. 9. Specimens of *P. amymone* (A) and *P. graeca coutsisi* (B) that can be considered as individuals with intermediate habit. The scale bars are 10 mm.

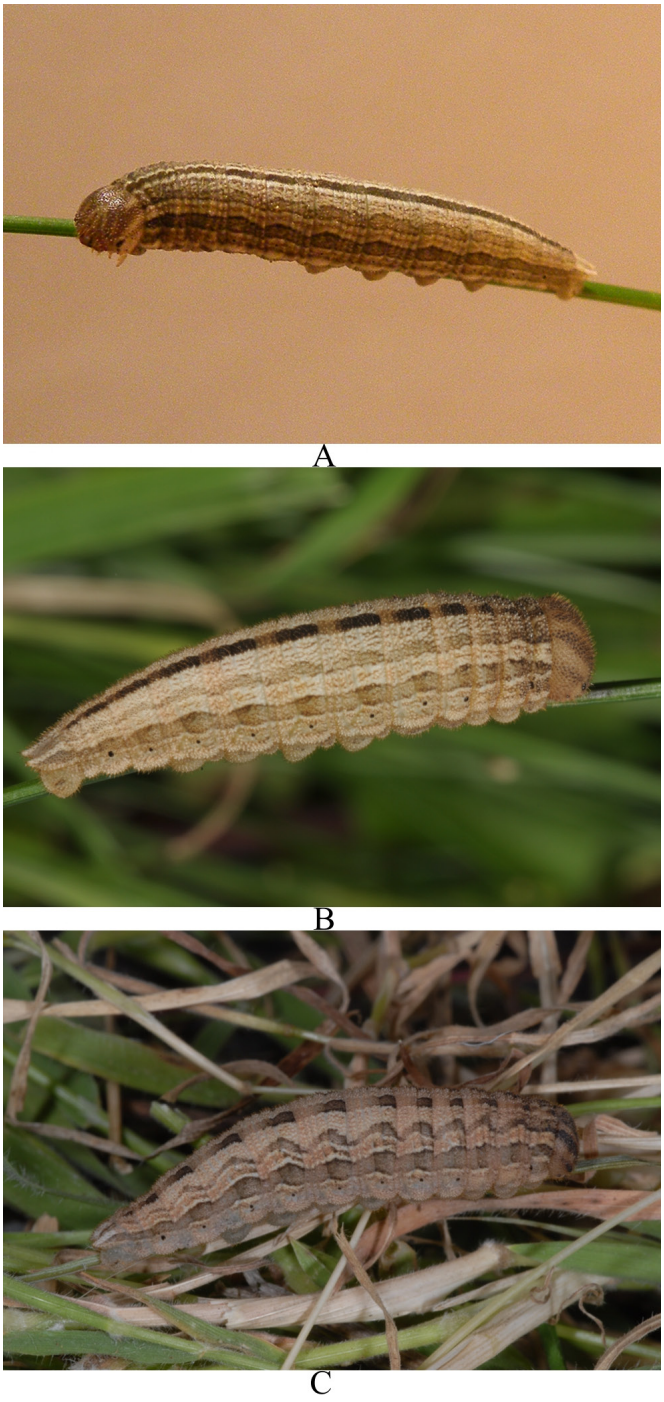

Fig. 10. Larva of *P. amymone* (A), *P. graeca* (B) and *P. anthelea* (C). The pictures B and C were taken by Wolfgang Wagner.

the Peloponnese is indicative of a less specialised species.

2. There are species, that can be distinguished by external morphological characters with perfectly identical mitochondrial COI barcode regions (Hausmann *et al*., 2011; Hausmann *et al*., 2011a).

3. There are certain elements of wing pattern (white spots in the S3-S4 inter-venal spaces, the fringe, the colour of the submarginal band, the appearance of ocelli, etc.), which are quite reliable differential characters. The number of specimens that can be considered as externally intermediate is insignificant, most of the specimens can be identifed without any problem and the list of differential characters is so extensive that it supports two clearly separated species.

## 6. The role of substrate in the divergence of the wing colouration

A number of authors report of a correlation existing between the colouration of the wing pattern – especially the hindwing underside – of the *Pseudochazara* species occurring in the Balkan Peninsula and the colour of the substrate of their habitat (Gross, 1978; Anastassiu *et al*., 2009; Gascoigne-Pees *et al*., 2014), especially in context with *P. graeca* and *P. amymone*. The underside of the wings of *P. cingovskii,* found almost exclusively on the slopes of the Pletvar Massif consisting of whitish light grey marble and gneiss (Fig. 11B), has a pale yellowish light grey colour (Fig. 11A). Furthermore, while the underside of the forewings of the *P. orestes* living in the greyish coloured limestone mountains around Drama is rather yellowish or yellowish-brownish, the underside of its hindwings are predominantly light grey (Fig. 11E,F). The third member of the '*mniszechii*-group', *P. mniszechii tisiphone* is on the other hand found in the mountains belonging to Western Vardar ophiolite belt (the South Albanian area also known as „Mirdita ophiolite"; Schmid *et al*.). This is in essence what remains of the crust of the Tethys Ocean that existed in the Mesozoic era. The ophiolite is the rock mass of the oceanic crust, consisting mostly of mafic (gabbro and basalt) and ultramafic rocks (e.g. harzburgite, herzolite and dunite). The latter differ mostly by the ratio of their content of olivine ($(Mg,Fe)_2SiO_4$), the main ingredient of their mineral assemblage, giving a greenish or yellowish hue to these characteristically dark coloured rocks. Olivine-containing ultramafic rocks are relatively easily transformed to serpentinite (Coleman, 1977). In South Albania, such serpentinite mountains form the habitat of populations of *P. amymone* and *P. mniszechii tisiphone* (Fig. 11D) (Verovnik *et al*., 2014; Gascoigne-Pees *et al*., 2014). The underside of both species is dominated by brown and yellowish-brown colours (Fig. 11C,H).

If, in the '*mniszechii*-group', the appearance of the underside colour is compared to the diversity of the barcode regions, then we see that while these species hardly differ at all in their mtCOI-sequences (a difference of 658 bp (=0.30-0.45%) in 2 and 3 nucleotide positions), it is not challenging to distinguish them visually, especially by their undersides. It seems convenient to assume that the colour of the underside could have diverged more rapidly over time than the COI sequences. We think that the colour of the substrate may have greatly advanced the formation of the underside colours of the populations extant today, contributing as a factor of natural selection; the mutations that have produced phenotypes more similar in colour to the rocks of certain habitats may have rapidly spread in the population in question, as a better camouflage obviously improved the survival chances of such specimens.

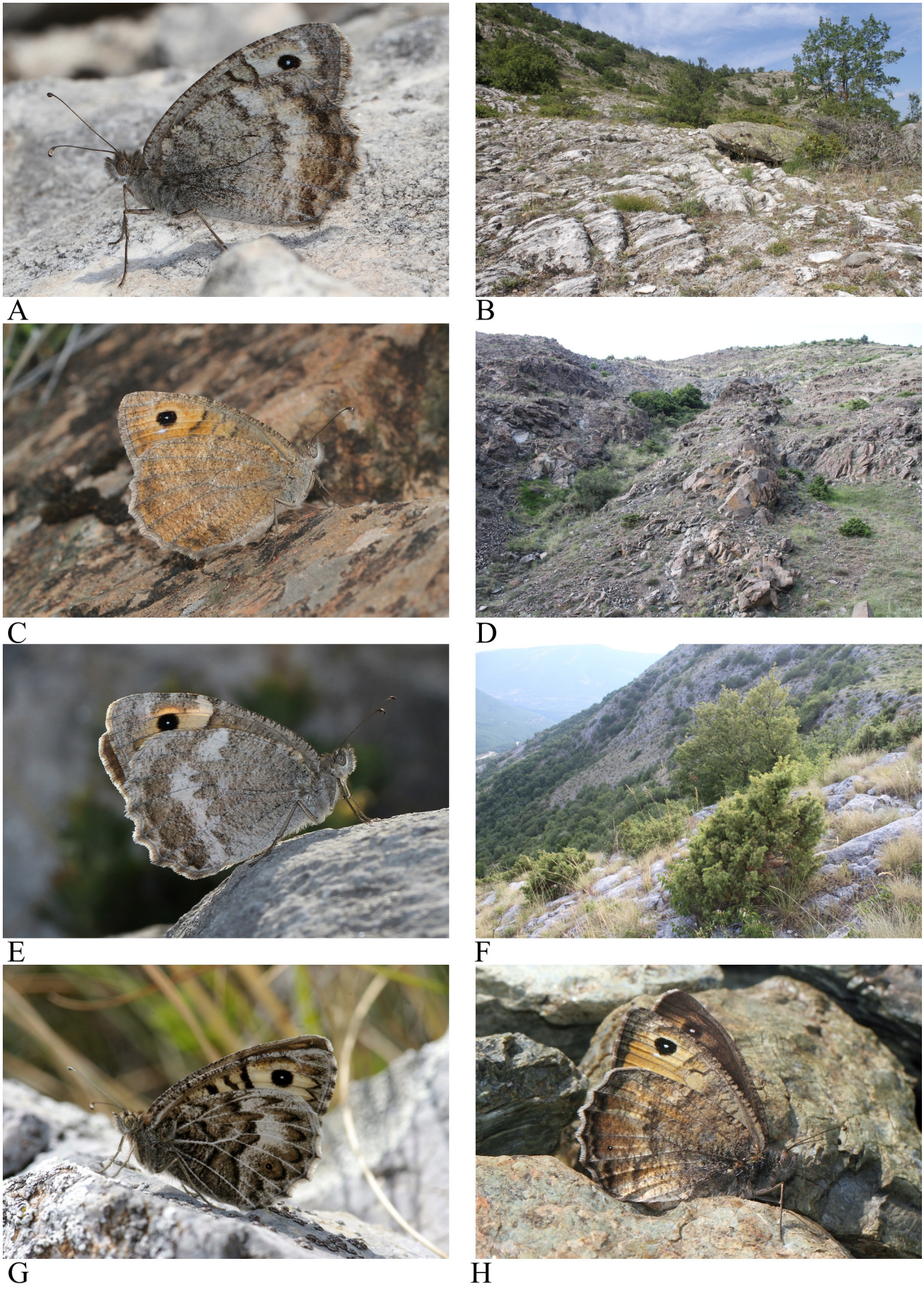


Fig. 11. Underside camouflage of some Balkan *Pseudochazara*-spp. and their habitats. A: *P. cingovskii*, B: the habitat of *P. cingovskii* on the metamorphic rocks of the Pletvar Massif. C: *P. mniszechii tisiphone*, D: the habitat of *P. mniszechii tisiphone* and *P. amymone* on ophiolitic substrate. E: *P. orestes*, F: the habitat of *P. orestes* on limestone in the Falakro Massif. G: *P. geyeri* and H: *P. amymone*. The photos were taken by Tamás Hapka.

Similar differences can be observed in the '*mamurra*-group'; apart from the aforementioned *amymone*, the occurrence of *P. geyeri occidentalis* is almost completely restricted to the mountainous regions surrounding the Ohrid and Prespa lakes, where the substrate is whitish-grey coloured limestone, generally. The larger share of light grey colours can also be observed on the underside of the *geyeri* as well, though accompanied by pale yellowish and brownish colours (Fig. 11G).

The case of *P. graeca* is slightly more complicated: specimens living in the northern part of the range are darker, with yellowish-brown undersides, while the undersides of specimens found on the Mt. Parnassos and the Peloponnese have lighter, greyish undersides. The correlation between the colour of the substrate and of the undersides is less unambiguous here, because for example the surrounding area of Mt. Chelmos belongs to the Budva-Cukali-Pindos Zone consisting of formations with mostly reddish-yellowish coloured stones.

Anastassiu *et al*. (2009) reported the discovery of new populations with intermediate appearance between the areas of ssp. *graeca* in the south and the areas of ssp. *coutsisi* in the north. Thus, they also questioned the validity of the ssp. *coutsisi* taxon, as the different wing colouration seems to appear only as a result of clinal variation. Indeed, this fact argues against delimiting subspecies; however, considering practical reasons and the fact that both northern an southern populations show strong homogenity in wing colouration that are significantly different between the two groups, we treated them as separate subspecies in this paper. At the same time, it is unclear which environmental factors could be responsible for the approximately SE-NW gradient axis that appears in the wing colouration of *P. graeca*. It is possible that the higher annual mean temperatures also influenced the formation of the lighter colouration of the southern specimens. It is a known phenomenon that the butterfly populations of colder habitats at higher altitudes can be darker in appearance as a part of their adaptation, since the higher albedo of the darker colours permits more efficient heat absorption (Karl *et al*., 2009).

Of the *Pseudochazara* species extant in the Balkan Peninsula, five can be considered local or extremely local. It appears that several species are linked to habitats of special geological characteristics and their wing colours have adapted well to this. As they are local, it would be important to find new populations and involve geological parameters in the predictions for the new localities, as Verovnik *et al*. did in the case of *P. amymone* (Verovnik *et al*., 2014). By comparing detailed geological maps with Google Earth satellite images, for example the presence of *P. cingovskii* cannot be excluded at the marble mountain areas near Vir and Devich in Macedonia.

**Acknowledgements**

First of all, we wish to say thank you to Tamás Hapka for his living butterfly and habitat pictures, to Gábor Simonics for his cooperation in the collecting of a living female of *Pseudochazara amymone* that made it possible to observe the development of the next generation. The first author expresses his thanks to both of them for the positive emotional environment during the visiting trips. Many thanks to László Ronkay and Zsolt Bálint for their help in genitalia dissections and photographing. We would like to thank Zsolt Pénzes for his help in the phylogenetic reconstruction. The *Pseudochazara geyeri* mtCOI barcode sequence was provided by Martin Gascoigne-Pees and Vlad Dincă for which we are also very grateful. The photo of the *Pseudochazara cingovskii* male genitalia was provided by John Chainey and Geoff Martin, associates of the NHM Lepidoptera Collection, London. The pictures of the larvae of *Pseudochazara graeca* and *Pseudochazara anthelea* were provided by Wolfgang Wagner. Many thanks for the advisements of Filip Franeta, Csaba Gáspár and Jonathan Agius regarding the rearing questions, to Stoyan Beshkov for Bulgarian data of *Pseudochazara*-spp., to Lazaros Pamperis for his observations, to Péter Ozsvárt for detailed geological maps and informations, to Jonathan Agius for the *Pseudochazara geyeri* leg-samples and to Zoltán Barina for the identification of the applied *Festuca* hostplant of *Pseudochazara amymone*. Last, we would like to express our gratefulness to Rita Sipos, Beáta Brozsó, Anita Mohr and Adrienn Micsinai, associates of BIOMI Kft., for their precise and willing cooperation during our research.

## References

Abu Al-Soud, W. and Radstrom, P. (2000) Effects of amplification facilitators on diagnostic PCR in the presence of blood, feces, and meat. *Journal of Clinical Microbiology* 38: 4463–4470.

Anastassiu, H.T., Coutsis, J.G. and Ghavalas, N. (2009) New data regarding the geographical distribution of *Pseudochazara graeca* in Greece, with notes about its wing coloration, the status of its ssp. *coutsisi* (= *zagoriensis*), as well as the supposed correlation between the HW underside ground colour and the geological character of the habitat in both *P. graeca* and *Hyponephele lycaon* (Lepidoptera: Nymphalidae, Satyrinae). *Phegea* 37(4): 135–145.

Arnscheid, W. and Arnscheid, W. (1981) Vom Ohrid-See zur Akropolis, entomologische Reiseeindrücke aus Griechenland. *Mitteilungen der westfälischen Entomologen*. Bochum 5(4): 36–42.

Aussem, B. (1980) Satyriden-Studie I. Zur Kenntnis der Androkonienfelder von *Pseudochazara telephassa* (Geyer, 1827) und *Pseudochazara anthelea* (Hübner,1824). *Entomofauna* 1: 354–356.

Bader, M. (2012) MB-Ruler v.5.0 – http://www.markus-bader.de/MB-Ruler/index.d.php

Brown, J. (1980) On the status of a little known satyrid butterfly from Greece. *Entomologist's Record and Journal of Variation*, West Wickham, 92(11/12): 280–281.

Braby, M.F., Vila, R. and Pierce, N.E. (2006) Molecular phylogeny and systematics of the Pieridae (Lepidoptera: Papilionoidea): higher classification and biogeography. *Zoological Journal of the Linnean Society* 147: 239–275.

Brown, T.A. (2002) *Molecular phylogenetics*. In: Genomes, 2nd ed. Wiley-Liss, Oxford (Chapter 16).

Chen, W.-J. and Mayden, R.L. (2010) A phylogenomic perspective on the new era of ichthyology. *Bioscience* 60(6): 421–432.

Chimpanzee Sequencing and Analysis Consortium (CSAAC) (2005) Initial sequence of the chimpanzee genome and comparison with the human genome. *Nature* 437(7055): 69–87.

Coleman, R.G. (1977) *Ophiolites. Ancient Oceanic Lithosphere*? Springer-Verlag, Berlin.

Cox, M., Nelson, D.R. and Lehninger, A.L. (2005) *Lehninger Principles of Biochemistry*. San Francisco: W.H. Freeman. ISBN 0-7167-4339-6.

Cuvelier, S. and Mølgaard, M. (2015) *Pseudochazara amymone* (Lepidoptera, Nymphalidae) in Albania: Variability analysis, androconial scales and new distributional data. *Nota Lepidopterologica* 38(1): 1–22.

De Prins, W. and van der Poorten, D. (1981) Een nieuwe *Pseudochazara*-soort voor de wetenschap uit Noordoost-Griekenland (Lepidoptera: Satyridae). *Phegea* 10(1): 7–21.

Dincă, V., Montagud, S., Talavera, G., Hernández-Roldán, H., Munguira, M.L., García-Barros, E., Hebert, P.D.N. and Vila, R. (2015) DNA barcode reference library for Iberian butterflies enables a continental-scale preview of potential cryptic diversity. *Scientific Reports* 5: 12395.

Eckweiler, W. (2004) Die Verbreitung und subspezifische Gliederung von *Pseudochazara mamurra* (Herrich-Schäffer, [1846]) (Lepidoptera: Nymphalidae, Satyrinae). *Nachrichten des Entomologischen Vereins Apollo*, Frankfurt am Main, N.F. 25(1/2): 9–14.

Eckweiler, W. (2012) New discoveries of *Pseudochazara mamurra amymone* Brown, 1976 (Lepidoptera: Nymphalidae, Satyrinae). *Nachrichten des Entomologischen Vereins Apollo*, N. F. 33(1): 1–4.

Frivaldszky, I. (1845) Rövid áttekintése egy természetrajzi utazásnak, az európai Törökbirodalomban, egyszersmind néhány a közben újdonnat fölfedezett állatnak leírása. *A Királyi Magyar Természettudományi Társulat Évkönyvei*, Pest 1: 163-187, 3 pls.

Gascoigne-Pees, M., Verovnik, R., Franeta, F. and Popović M. (2014) The lifecycle and ecology of *Pseudochazara amymone* (Brown, 1976) (Lepidoptera: Nymphalidae, Satyrinae). *Nachrichten des Entomologischen Vereins Apollo*, N. F. 35(3): 129–138.

Gross, F.J. (1973) *Satyrus sintenisi* auch in Europa, nebst Beschreibung einer neuen Unterart (Lep., Satyridae). *Entomologische Zeitschrift*, Stuttgart, 83(18): 211–214.

Gross, F.J. (1978) Beitrag zur Systematik von *Pseudochazara*-Arten (Lep., Satyridae). *Atalanta*, 9(1): 41–103.

Hammer, Ø., Harper, D.A.T. and Ryan, P. D. (2001) PAST: Paleontological Statistics Software Package for Education and Data Analysis. *Palaeontologia Electronica* 4(1): 9pp.

Hausmann, A., Haszprunar, G., Segerer, A.H., Speidel, W., Behounek, G. and Hebert, P.D.N. (2011) Now DNA-barcoded: the butterflies and larger moths of Germany (Lepidoptera: Rhopalocera, Macroheterocera). *Spixiana* 34(1): 47–58.

Hausmann, A., Haszprunar, G. and Hebert, P.D.N. (2011a) DNAbarcoding the Geometrid fauna of Bavaria (Lepidoptera):successes, surprises, and questions. *PloS ONE* 6(2): e17134.

Hebert, P.D.N., Cywinska, A., Ball, S. L. and deWaard, J.R. (2003) Biological identifications through DNA barcodes. *Proceedings of the Royal Society London* B 270(1512): 313–322.

Hebert, P. .N., Penton, E.H., Burns, J.M., Janzen, D. H. and Hallwachs, W. (2004) Ten species in one: DNA barcoding reveals cryptic species in the neotropical skipper butterfly *Astraptes fulgerator*. *Proceedings of the National Academy of Sciences of the United States of America*, 101:14812-14817.

Higgins, L.G. (1975) *The Classification of European Butterflies*. William Collins Sons & Co. Ltd., Glasgow. ISBN 0-00-219624-7.

Ivanova, N. and Grainger, C. (2006) *Primer Sets. Canadian Centre for DNA Barcoding* (CCDB) – http://www.ccdb.ca/docs/CCDB_PrimerSets.pdf

Karl, I., Geister, T.L. and Fischer, K. (2009) Intraspecific variation in wing and pupal melanization in copper butterflies (Lepidoptera: Lycaenidae). *Biological Journal of the Linnean Society* 98(2): 301–312.

Kawahara, A.Y., Mignault, A.A., Regier, J.C., Kitching, I.J. and Mitter, C. (2009) Phylogeny and biogeography of hawkmoths (Lepidoptera: Sphingidae): Evidence from five nuclear genes. *PLoS ONE* 4(5): e5719.

Kimura, M. (1980) A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. *Journal of Molecular Evolution* 16: 111–120.

Knölke, S., Erlacher, S., Hausmann, A., Miller, M.A. and Segerer, A. (2005) A procedure for combined genitalia dissection and DNA extraction in Lepidoptera. *Insect Systematics and Evolution* 35: 401–409.

Misja, K., Kurrizi, A. (1984) Resultats des recherche des papillons diurnes (Rhopalocera, Grypocera) de notre pays. *Buletini i Shkencave të Natyrës* 12: 105–111.

Nazari, V., Hagen, W.T., Bozano, G.C. (2009) Molecular systematics and phylogeny of the 'Marbled Whites' (Lepidoptera: Nymphalidae, Satyrinae, *Melanargia* Meigen). *Systematic Entomology* 35: 132–47.

Pamperis, L.N. (1997) *The butterflies of Greece.* 1th Edition. A. Bastas, D. Plessas Graphic Arts, Athens.

Pamperis, L.N. (2009) The butterflies of Greece. 2nd Edition. Editions Pamperis, Athens.

Parr, C.S., Guralnick, R., Cellinese, N., Page, R.D. (2012) Evolutionary informatics: unifying knowledge about the diversity of life. *Trends in Ecology & Evolution* 27(2): 94–103.

Patthy, L. (1999) *Protein Evolution*. Oxford: Blackwell Science. ISBN 0-6320-4774-7.

Rebel, H., and Zerny, H. (1931) Die Lepidopterenfauna Albaniens (mit Berücksichtigung der Nachbargebiete). *Denkschriften der Kaiserlichen Akademie der Wissenschaften*, Wien, Mathematisch-Naturwissenschaftliche Klasse, 103: 37–161.

Regier, J.C., Brown, J.W., Mitter, C., Baixeras, J., Cho, S., Cummings, M.P., *et al*. (2012) A molecular phylogeny for the leaf-roller moths (Lepidoptera: Tortricidae) and its implications for classification and life history evolution. *PLoS ONE* 7(4): e35574.

Regier, J.C., Mitter, C., Zwick, A., Bazinet, A.L., Cummings, M.P., *et al.* (2013) A Large-Scale, Higher-Level, Molecular Phylogenetic Study of the Insect Order Lepidoptera (Moths and Butterflies). *PLoS ONE* 8(3): e58568. doi:10.1371/journal.pone.0058568

Ridley, M. (2006) Genome. New York, NY: Harper Perennial. ISBN 0-06-019497-9.

Ronquist, F., Huelsenbeck, J.P. (2003) MRBAYES 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* 19: 1572–1574.

Saccone, C., DeCarla, G., Gissi, C., Pesole, G. and Reynes, A. (1999) Evolutionary genomics in the Metazoa: the mitochondrial DNA as a model system. *Gene* 238: 195–210.

Schmid, M.S., Bernoulli, D., Fügenschuh, B., Georgiev, N., Kounov, A., Matenco, L., Oberhänsli, R., Pleuger, J., Schefer S., Schuster R., Ustaszewski K. and van Hinsbergen, D. (2014) *Tectonic units of the Alpine collision zone between Eastern Alps and Western Turkey.* Unpublished map.

Settele, J., Kudrna, O., Harpke, A., Kühn, I., van Swaay, C., Verovnik, R., Warren, M., Wiemers, M., Hanspach, J., Hickler, T., Kühn, E., van Halder, I., Veling, K., Vliegenthart, A., Wynhoff, I. and Schweiger, O. (2008) *Climatic Risk Atlas of European Butterflies*. Pensoft Moscow-Sofia.

Staudinger, O. (1870) Beitrag zur Lepidopterenfauna Griechenlands. *Horae Societatis Entomologicae Rossicae* 7: 3–304.

Tamura, K., Stecher, G., Peterson, D., Filipski, A. and Kumar, S. (2013) MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0. *Molecular Biology and Evolution* 30(12): 2725–2729.

Tolman, T. and Lewington, R. (2004) *Butterflies of Britain and Europe.* 2nd Edition. Harper Collins.

Tuzov, V.K., Bogdanov, P.V., Devyatkin, A.L., Kaabak, L.V., Korolev, V.A., Murzin, V.S., Samodurov, G.D. and Tarasov, E.A. (1997) *Guide to the Butterflies of Russia and Adjacent territories (Lepidoptera: Rhopalocera).* Volume 1. Pensoft, Moscow, Russia.

Tshikolovets, V.V. (2011) *Butterflies of Europe & the Mediterranean area.* Tshikolovets publications, Pardubice. ISBN 1978-80-904900-0-0.

Vanin, E. F. (1985) Processed pseudogenes: characteristics and evolution. *Annual Review of Genetics* 19: 253–72.

Verovnik R., Micevski B., Maes D., Wynhoff, I., van Swaay, C., Warren, M. (2013) Conserving Europe's most endangered butterfly: the Macedonian Grayling (*Pseudochazara cingovskii*). *Journal of Insect Conservation* 17: 941–947.

Verovnik, R., Popović, M., Šašić, M., Cuvelier, S., Maes, D. (2014) Wanted! Dead or alive: the tale of the Brown's Grayling (*Pseudochazara amymone*). *Journal of Insect Conservation* 18(4): 675–682.

Warren, A. D., Austin, G. T., Llorente-Bousquets, J. E., Luis-Martínez, A., Vargas-Fernández, I. (2008) A new species of *Neominois* from northeastern Mexico (Lepidoptera: Nymphalidae: Satyrinae) *Zootaxa* 1896: 31–44.

Windsor, A.M., Felder, D.L. (2014) Molecular phylogenetics and taxonomic reanalysis of the family Mithracidae MacLeay (Decapoda: Brachyura: Majoidea). *Invertebrate Systematics.* 28: 145–173.

Yuan, M.-L., Zhang, Q.-L., Wang, Z.-F., Guo, Z.-L., Bao, G.-S. (2015) Molecular Phylogeny of Grassland Caterpillars (Lepidoptera: Lymantriinae: *Gynaephora*) Endemic to the Qinghai-Tibetan Plateau. *PLoS ONE* 10(6): e0127257.

Zahiri, R., Holloway, J.D., Kitching, I.J., Lafontaine, D., Mutanen, M., *et al*. (2012) Molecular phylogenetics of Erebidae (Lepidoptera, Noctuoidea). *Systematic Entomology* 37: 102–124.

Zahiri, R., Lafontaine, J.D., Schmidt, B.C., deWaard, J.R., Zakharov, V. et al. (2014) A Transcontinental Challenge — A Test of DNA Barcode Performance for 1,541 Species of Canadian Noctuoidea (Lepidoptera). *PLoS ONE* 9(3): e92797. doi:10.1371/journal.pone.0092797

Zimmermann, J., Hajibabaei, M., Blackburn, D.C., Hanken, J., Cantin, E., *et al*. (2008) DNA damage in preserved specimens and tissue samples: a molecular assessment. *Frontiers of Zoology* 5: 18.

Kornél Takáts
Rókus u. 11,
H-2083
Solymár, Hungary
E-mail: *kornel.takats@gmail.com*

Morten Mølgaard
Gertrud Rasks Vej 86
DK-9210
Aalborg SØ, Denmark
E-mail: *msm2@stofanet.dk*

Received: 22.12.2015
Accepted: 20.01.2016
Published online: 26.02.2016
Published:
Online article number: ER1920141504