Species delimitation in the Grayling genus *Pseudochazara* (Lepidoptera, Nymphalidae, Satyrinae) supported by DNA barcodes

Rudi Verovnik¹, Martin Wiemers²

¹ Department of Biology, Biotechnical Faculty, University of Ljubljana, Jamnikarjev 101, Ljubljana, Slovenia
² UFZ – Helmholtz-Centre for Environmental Research, Department of Community Ecology, Theodor-Lieser-Str. 4, 06120 Halle, Germany

Corresponding author: Martin Wiemers (martin.wiemers@ufz.de)

Academic editor: A. Hausmann | Received 15 January 2016 | Accepted 9 June 2016 | Published 22 June 2016

http://zoobank.org/CF247405-E63C-4315-AF0B-AC16B6B25156

Citation: Verovnik R, Wiemers M (2016) Species delimitation in the Grayling genus *Pseudochazara* (Lepidoptera, Nymphalidae, Satyrinae) supported by DNA barcodes. ZooKeys 600: 131–154. doi: 10.3897/zookeys.600.7798

Abstract

The Palaearctic Grayling genus *Pseudochazara* encompasses a number of petrophilous butterfly species, most of which are local endemics especially in their centre of radiation in SW Asia and the Balkans. Due to a lack of consistent morphological characters, coupled with habitat induced variability, their taxonomy is poorly understood and species delimitation is hampered. We employed a DNA barcoding approach to address the question of separate species status for several European taxa and provide first insight into the phylogeny of the genus. Unexpectedly we found conflicting patterns with deep divergences between presumably conspecific taxa and lack of divergence among well-defined species. We propose separate species status for *P. tisiphone*, *P. amalthea*, *P. amymone*, and *P. kermana* all of which have separate well supported clades, with the majority of them becoming local endemics. Lack of resolution in the ‘Mamurra’ species group with well-defined species (in terms of wing pattern and coloration) such as *P. geyeri*, *P. daghestana* and *P. alpina* should be further explored using nuclear molecular markers with higher genetic resolution.

Keywords

Papilionoidea, Satyrinae, butterflies, phylogeny, barcoding, taxonomy
Introduction

Depending on which systematic order of classification is adhered to, the genus *Pseudochazara* comprises 27–32 species of Graylings (Gross 1978, Lukhtanov 2007, Savela 2015). It has a wide distribution in the Palaeartic region from North Africa to the Himalayas and Mongolia (Tennent 1996, Tshikolovets 2005, Yakovlev 2012). In addition to vague species delimitation, large intraspecific variation has resulted in the description of over 100 subspecific taxa (Lukhtanov 2007) in this intensively studied taxon.

The main reason for the extensive variation in phenotype can be linked with the specific ecological requirements of these butterflies. They are mostly petrophilous and limited to specific rock substrate to which they are perfectly adapted with their camouflaged underside wing pattern and cryptic coloration. Local adaptation to mimic the coloration of the rock substrate is, therefore, one of the main drivers for such large scale diversification (Lorković 1974, Weiss 1980, Hesselbarth et al. 1995, Tennent 1996, but see Anastassiu et al. 2009).

Trying to resolve the systematics of this genus and its species delimitation has been thwarted by the fact that the genitalia of many *Pseudochazara* species are virtually identical and their wing shape and coloration, both being partially dependant on environmental conditions (Gross 1978, Hesselbarth et al. 1995), is inconsistent. The last comprehensive taxonomic review which was published by Gross (1978) is already outdated. He recognised 24 species, among which *P. obscura* (Staudinger, 1878) is now considered a subspecies of *P. lydia* (Staudinger, 1878) (see Eckweiler and Rose 1988), *P. aurantiaca* (Staudinger, 1878) and *P. xerxes* Gross & Ebert, 1975 have been reclassified as subspecies of *P. beroe* (Herrich-Schäffer, 1844) (see Lukhtanov 2007), *P. schahrudensis* (Staudinger, 1881) is now considered conspecific with *P. mamurra* (Herrich-Schäffer, 1844) (see Eckweiler 2004) and *P. pakistana* Gross, 1978 is conspecific with either *P. gilgitica* (Tytler, 1926) (see Lukhtanov 2007) or *P. baldiva* (Moore, 1865) (see Wakeham-Dawson et al. 2007). Several members of the *Pseudochazara* genus from Central Asia that are currently recognised as separate species were considered subspecific taxa in the revision (e.g. *P. droshica* (Tytler, 1926), *P. gilgitica* (Tytler, 1926), *P. lehana* (Moore, 1878)) while *P. euxina* (Kuznetsov, 1909) from Crimea was entirely neglected. Two additional species were described after the revision, *P. kanishka* (Aussem 1980a) and *P. annieae* (Pagès 2007). Following Gross’ revision (1978) the shape of the androconial scales of several *Pseudochazara* species has proven to be constant, enabling species delimitation (Weiss 1980, Eckweiler and Rose 1989, Wakeham-Dawson and Kudrna 2000, Wakeham-Dawson et al. 2003, Wakeham-Dawson and Kudrna 2005, Wakeham-Dawson 2006, Wakeham-Dawson and Kudrna 2006, Pages 2007, Wakeham-Dawson et al. 2007).

There has been no attempt to reconstruct the phylogeny of the genus or validate species status using molecular markers. Only the taxonomic position within subtribe Satyrina and a sister relationship to *Chazara* has been established (Peña et al. 2011).
In order to resolve the relationship among *Pseudochazara* species and re-evaluate their species status, in particular of some European taxa, we employed DNA barcoding – using a standardized gene region (5’ segment of the mitochondrial gene cytochrome *c* oxidase subunit I = COI) which enabled us to utilize additional *Pseudochazara* sequences available in the Barcode of Life Database (BOLD 2015). DNA barcodes have been widely and successfully used in Lepidoptera taxonomy and species delimitation as an additional set of characters which are independent of habitat conditions (Hebert et al. 2004, Nazari and Sperling 2007, Nazari et al. 2010, Dinca et al. 2011, Yang et al. 2012, Lukhtanov and Novikova 2015, Pazhenkova et al. 2015). However, there are several limitations of this method (see e.g. Wiemers and Fiedler 2004, Brower 2006, Ritter et al. 2013, Song et al. 2008, Toews and Brelsford 2012) which should be taken into account in the interpretation of the gene tree.

**Material and methods**

**Sample collection, DNA extraction, amplification, sequencing, and alignment**

With the aim of achieving consistency, we adopt the nomenclature of the most recent list of *Pseudochazara* species by Lukhtanov (2007). Following the discovery of *Pseudochazara mamurra amymone* in Albania (Eckweiler 2012), we initially sampled all the *Pseudochazara* taxa from the Balkan Peninsula, a hotspot of *Pseudochazara* diversity in Europe (Verovnik et al. 2014, Gascoigne-Pees et al. 2014). We then broadened the range of our sampling adding additional species from Turkey and the Middle East, the main areas of *Pseudochazara* diversification. Altogether 27 specimens belonging to 10 species of *Pseudochazara*, for which the barcoding gene COI was successfully amplified, were included in the study (see Appendix 1). All specimens were dried prior to DNA extraction. In addition, we included COI sequences from 81 individuals belonging to 14 species from the BOLD database (BOLD 2015). Only specimens that could be unambiguously identified by the voucher photos were selected. Following the nomenclature guidelines proposed by Lukhtanov (2007) a total of 34 taxa belonging to 20 species were included in the analysis. As outgroups, we added several sequences of the closely related Satyrine genus *Chazara* from GenBank, based on the results of the phylogenetic study of Satyrinae by Peña et al. (2011).

Total genomic DNA was extracted from single legs, following the Mammalian tissue preparation protocol (GenElute Mammalian Genomic DNA miniprep kit from Sigma-Aldrich). For each sample a 657 bp fragment of the first subunit of the mitochondrial gene cytochrome *c* oxidase (COI) was amplified using primers LCO1490 and HCO2198 (Folmer et al. 1994). Amplification followed a standard protocol described in Verovnik et al. (2004). PCR products were visualized on an agarose gel to verify amplification success and sequenced by Macrogen in both directions on an Applied Biosystems 3730xl sequencer.
Phylogenetic analysis

We used Bayesian inference to reconstruct a phylogenetic tree. To achieve more clarity the tree was constructed on a subset of samples including only unique haplotypes belonging to the same taxon. A hierarchical likelihood test was employed in order to test alternative models of evolution, using JModeltest v.0.1.1 (Posada 2008). A GTR (Generalised time reversible) model of nucleotide substitution with gamma distributed rate heterogeneity and a significant proportion of invariable sites was selected in accordance with the Akaike Information Criterion. Bayesian analysis was performed with MrBayes v.3.1.2 implementing the best fit substitution model (Huelsenbeck and Ronquist 2001). Markov chain Monte Carlo search was run with four chains for $4 \times 10^6$ generations, taking samples every 100 generations. The approximate number of generations needed to obtain stationarity of the likelihood values (“burn-in”) of the sampled trees was estimated graphically to 2000 trees. From the remaining trees posterior probabilities were assessed for individual clades based on their observed frequencies. Trees were visualised using Figtree v.1.4.2 (Rambaut 2014). Genetic distances (p-) were calculated with MEGA 6.0 (Tamura et al. 2013). In addition, a statistical parsimony network analysis was performed with TCS 1.21 (Clement et al. 2000).

Results

No insertions or deletions were observed in the mitochondrial COI gene and therefore the alignment was unambiguous. For the COI dataset 63 unique haplotypes among 108 Pseudochazara sequences were detected. 114 (17.5%) sites were variable and 95 (14.6%) were parsimony informative. The average interspecific genetic distance was 4.9%, but in the case of P. mniszechii the intraspecific diversity ranged from 0 to 6.7% with highly distinct divergent sequences of P. mniszechii tisiphone. No evident bar-coding gap was observed separating intraspecific from interspecific pairwise genetic distances (Fig. 1). On the contrary, sharing of identical haplotypes was observed in the following taxa: P. graeca / P. mamurra amymone, P. mamurra mamurra / P. daghestana, and P. beroe aurantiaca / P. alpina. On the other hand, 82% of species comparisons showed high (≥2%) interspecific distances.

The calculated maximum connection for parsimony networks at the default 95% limit was 11 steps, and resulted in 9 separate networks within Pseudochazara. 6 of them contain only single species (P. atlantis, P. turkestana, P. thelephassa, P. lehana, P. kanishka, and P. anthelea), whereas the remaining 3 comprise several closely related species (Figs 2–4). Outgroups were contained in 2 distinct networks (Chazara enervata and Chazara briseis/C. heydenreichi).

The topology of the Bayesian Inference tree of all Pseudochazara samples, including the selected outgroup species (Fig. 5), confirms the monophy of the genus. High posterior probability values support a basal position of P. atlantis, the only species of the genus present in (and confined to) North Africa. This is somewhat surprising as
Species delimitation in the Grayling genus *Pseudochazara*

Figure 1. Frequency distribution of pairwise intra- and interspecific p-distances of the COI sequences in the genus *Pseudochazara*. No “barcoding gap” exists between these two data series.

*P. anthelea* and *P. thelephassa* are considered to be morphologically the most distinct and separate species within the genus (Gross 1978). *P. atlantis* has tentatively been placed into two groups, the ‘mamurra’ species group (Brown 1976), based on androconia shape, and the ‘pelopea’ species group (Wakeham-Dawson and Dennis 2001), on account of the shape of male genitalia. *P. atlantis* is also distinctive according to the TCS analysis and forms a separate network. In addition, the second basal split within *Pseudochazara* is well supported, and, apart from some single species clades, three species groups tentatively named as the ‘pelopea’, ‘hippolyte’ and ‘mamurra’ clades received high support. We present the results for these clades separately:

‘Pelopea’ group

This group, which forms a distinct network in the TCS analysis (Fig. 2), includes two species, *P. pelopea* and *P. mniszechii*. However, there is no genetic differentiation between them, with *P. pelopea persica* and *P. pelopea caucasica* intermixed with *P. mniszechii*...
Two well supported clades pertain to geographically isolated subspecies of *P. pelo-pea*, the Levant region (nominotypic *P. pelo-pea pelo-pea*) and Kopet Dhag in NE Iran (*P. pelopea tekkensis*). Both subspecies are morphologically distinct from *P. pelopea persica*, in particular the latter, with much wider and more pronounced orange submarginal bands on their forewings. *P. pelopea tekkensis* is considered a separate species by Nazari (2003). *P. mniszechii* is also polyphyletic due to the separate position of the subspecies *tisiphone* from the southern Balkans, which is clearly not closely related, and belongs to the ‘hippolyte’ group.

**‘Hippolyte’ group**

The ‘hippolyte’ clade sensu stricto includes the widely distributed *P. hippolyte* complex which has a vast range from southern Spain to central China (Tshikolovets 2011) together with a number of local endemics from the southern Balkan Peninsula: *P. cingovskii* in the Republic of Macedonia, *P. orestes* from north-eastern Greece and the neighbouring part of Bulgaria, *P. mniszechii tisiphone* from north-western Greece and southern Albania and *P. euxina* from the Crimean Peninsula. Both, the haplotype net-
Species delimitation in the Grayling genus Pseudochazara...

work analysis (Fig. 3) and the phylogeny (Fig. 5) show that *P. mniszechii tisiphone* is not a subspecies of *P. mniszechii* despite superficial resemblance in wing patterns and coloration. In fact, it is closely related to two other local endemics from the Balkan Peninsula, *P. cingovskii* and *P. orestes*. The presence of *P. mniszechii tisiphone* in the western part of Turkey, near Bursa (Hesselbarth et al. 1995) remains to be verified. The single haplotype of *P. euxina* is nestled among samples of *P. hippolyte*, so our preliminary results do not support its current status as a separate species. Within this clade *P. hippolyte williamsi* from southern Spain appears basally, however with low posterior probability and it is not monophyletic. All other described subspecies (*P. hippolyte pallida*, *P. hippolyte doerriesi*, *P. hippolyte mercurius*) are less distinct from the nominotypical subspecies, with two Central Asiatic subspecies (*P. hippolyte pallida*, *P. hippolyte mercurius*) sharing haplotypes.

**Figure 3.** Statistical Parsimony network of the ‘hippolyte’ species group. Coloured circles represent COI haplotypes and their size corresponds to the number of samples per haplotype. Small white circles represent unsampled haplotypes.
Figure 4. Statistical Parsimony network of the ‘mamurra’ species group. Coloured circles represent COI haplotypes and their size corresponds to the number of samples per haplotype. Small white circles represent unsampled haplotypes.

The sister relationship of *P. thelephassa* and *P. anthelea*, which is indicated by genital morphology (the presence of a distinct costal process on the dorsal side of the valve) and wing pattern (the presence of a well-defined black area in the forewing discal cell)
Species delimitation in the Grayling genus Pseudochazara...

Figure 5. Phylogeny of Pseudochazara species derived from the barcoding gene COI using Bayesian inference analysis. Values on major branches are Bayesian posterior probabilities. Branches with support lower than 50% were collapsed manually. Branch names combine taxon name and sample ID (see Appendix 1). Nomenclature follows Lukhtanov (2007).
(Aussem 1980b, Hesselbarth et al. 1995, Wakeham-Dawson and Dennis 2001), could not be corroborated as *P. anthelea* appears to be a sister clade to the ‘*hippolyte*’ group sensu strictu with high posterior probability. *P. kanishka* from Tajikistan is a sister species of the *anthelea-hippolyte* clade, while *P. thelephassa* is sister taxon to the *anthelea-hippolyte-kanishka* clade, however, with low support. These results concur with wing pattern, i.e. a well-defined black area in the forewing discal cell, also present in specimens of *P. kanishka*.

It is important to note that the average genetic distance between two geographically separated subspecies, *P. anthelea anthelea* from Asia Minor and neighbouring islands, and *P. anthelea amalthea* from the Balkan Peninsula was 1.5%. This result is indicative for differentiation into distinct species as predicted by Kudrna et al. (2011).

In the TCS analysis, this group is split into 3 networks: a) the *hippolyte* clade sensu stricto (Fig. 3), b) *P. anthelea*, and c) *P. thelephassa*.

### ‘*Mamurra*’ group

The only two entirely Central Asian species available for analysis, *P. turkestana* and *P. lehana*, form a well-supported clade together with the ‘*mamurra*’ group, indicating their close relationship, but with a separate network for each in the TCS analysis. All other sequences form a single network (Fig. 4). Although the species sampling in Central Asia is incomplete, there is no evidence of a deep split between Asiatic and European/African taxa as predicted by Wakeham-Dawson and Dennis (2001). The ‘*mamurra*’ group is monophyletic, and includes several well-defined species (in terms of wing patterns, androconia and genitalia) with identical or very similar haplotypes. The following taxa could not be distinguished based on COI haplotypes as they do not form separate monophyletic clades: *P. mamurra*, *P. beroe*, *P. geyeri*, *P. daghestana*, *P. alpina*, and *P. lydia*. Only a single sequence was obtained for *P. geyeri* and *P. lydia*, so their position within this group is tentative. However, it is clear that *P. lydia* is closely related to *P. mamurra* with which it shares similarities e.g. the shape of the androconia (Wakeham-Dawson 2005). *P. alpina* shares the haplotype with *P. beroe* and they appear closely related, however, this is again based on the inclusion of a single sequence.

Within the ‘*mamurra*’ group the only well supported clade includes the taxa *P. schahkuhensis*, *P. mamurra kermana*, *P. graeca* and *P. mamurra amymone*. While *P. schahkuhensis* is sympatric in part of its range with *P. mamurra*, all other taxa have geographically isolated ranges. *P. graeca* and *P. mamurra amymone* are present in the southern part of the Balkan Peninsula with partial range overlap (Pamperis 2009). Both species are clearly morphologically distinct, but genetically not identifiable in COI haplotypes. Clearly this relationship puts in question the status of *P. mamurra amymone* as a subspecies of *P. mamurra*. The same conclusion can be drawn for *P. mamurra kermana* from Iran (Kerman province), which is also well placed within this clade as a sister species to both southern Balkan Peninsula taxa.
Discussion

Our study supports the monophyly of the genus *Pseudochazara* with high posterior probability values of the COI gene tree. Within the genus, however, two conflicting patterns appear with, unexpectedly, deep divergences between presumably conspecific taxa on the one hand and lack of divergence among well-defined species on the other. This is to some extent concordant with similar studies in related genera in the subfamily Satyrinae (Kodandaramaiah and Wahlberg 2009, Nazari et al. 2010, Kreuzinger et al. 2014). The basal position of *P. atlantis* from North-western Africa as sister group to all remaining *Pseudochazara* species falls into the first category. Based on distinct male genitalia morphology and wing shape/patterns *P. anthelea* and *P. thelephassa* were considered to form the basal split within the genus (Gross 1978, Aussem 1980b, Hesselbarth et al. 1995, Wakeham-Dawson and Dennis 2001). The basal position of *P. atlantis* is difficult to explain in terms of biogeography, as it indicates a North African origin of the genus, which has its centre of divergence much further eastwards in the Middle East (Hesselbarth et al. 1995, Tshikolovets 2011). *P. atlantis* is an alpine species distributed only in the Atlas Mountains of Morocco (Tennent 1996), therefore its isolation from the main distribution of the genus could possibly have preceded the last land bridge connections with Europe at the end of the Miocene (Garcia-Castellanos et al. 2009). Hence, its basal position could be an artefact of long-branch attraction (Bergsten 2005) and/or incomplete sampling of the entirely Asiatic species. Therefore, confirmation with additional genetic markers and additional sampling is required.

Another unexpected result is a deep split between *P. mniszechii* and *P. mniszechii tisiphone*, species which are very similar in wing patterns/coloration and considered conspecific in current literature (Hesselbarth et al. 1995, Kudrna et al. 2011, Tshikolovets 2011, Eckweiler 2012) and databases (Lukhtanov 2007, Savela 2015, Fauna Europaea 2016). Based on the COI gene tree *P. tisiphone* Brown, 1980 (stat. n.) is a separate species closely related to two local endemics from the southern part of the Balkan Peninsula, *P. orestes* and *P. cingovskii*. Actually *P. tisiphone* was originally described as a subspecies of *P. cingovskii* (Brown 1980) and its close relationship was hypothesised also by Wakeham-Dawson and Dennis (2001) based on the similarity of the male genitalia. The low level of genetic differentiation between *P. tisiphone*, *P. orestes*, and *P. cingovskii* indicates a relatively recent speciation, however, we are inclined towards supporting their separate species status based on constant differences in wing patterns/coloration and also their ecological specialization (Pamperis 2009, Verovnik et al. 2013).

A split between *P. anthelea anthelea* from Asia Minor and *P. anthelea amalthea* from the Balkan Peninsula has been suggested based on minor differences in male genitalia and consistent differences in female wing coloration between both taxa (Olivier 1996, Wakeham-Dawson and Dennis 2001). They are considered separate morphospecies by Kudrna et al. (2011). We can agree with separate species status as the split between the two taxa is much older compared to almost no differentiation in three morphologically and ecologically well defined species: *P. tisiphone*, *P. orestes*, and *P. cingovskii*. Following
this reasoning, *P. pelopea tekkensis* from NE Iran could also be considered a distinct species, however, inclusion of more samples is needed to confirm this status.

Given the high resolution of the basal clades within the COI gene tree, the lack of differentiation between taxa within the ‘*mamurra*’ and ‘*pelopea*’ group was unexpected. In particular, species like *P. geyeri* and *P. daghestana* are among the most easily recognisable species in the genus with uniform and very distinct wing patterns/coloration. There are several possible hypotheses to explain this lack of differentiation:

- **Incomplete lineage sorting:** recent speciation could result in unresolved relationships among these closely related species; however, well-defined species borders in terms of constant wing pattern differentiation coupled with broad overlaps in species ranges challenges this hypothesis.

- **Recent gene flow:** gene flow between closely related taxa is a known phenomenon (Descimon and Mallet 2009) and masks relationships among species especially with mitochondrial DNA (Gompert et al. 2008). The species involved have broadly overlapping ranges and could sometimes be found syntopic (Aussem 1980c, Hesselbarth et al. 1995), so hybridization is possible. Actually hybridization is documented even among the most distantly related species such as *P. anthelea* and *P. geyeri* (Aussem, 1980c). Nuclear markers with higher genetic resolution (e.g. microsatellites, SNPs) would be required to study the contact zones between these taxa to confirm ongoing gene flow. It must be noted that partial exclusion is evident when two or more *Pseudodochazara* species are syntopic, as one is always dominant, while the others appear in very low frequencies (Hesselbarth et al. 1995, Verovnik et al. 2014).

- **Pseudogenes or *Wolbachia* infections:** both are common in invertebrates, particularly in arthropods (Bensasson et al. 2011, Gerth et al. 2014, Leite 2012, Ritter et al. 2013). As the vast majority of the haplotypes in the ‘*mamurra*’ and ‘*pelopea*’ clades originate from the BOLD database it is impossible to check or correct for this potential error.

The most enigmatic taxon among the ‘*mamurra*’ group is *P. mamurra amymone* from northern Greece and Albania (Eckweiler 2012, Verovnik et al. 2014). Apart from the author’s original description (Brown 1976) little has been published regarding this elusive taxon for a long time. Failed attempts to locate the vaguely described type locality (Cuvelier 2010) have led to several misleading hypotheses, resulting in speculation that it may even be a rare hybrid between *P. tisiphone* and *P. anthelea* (Wakeham-Dawson and Dennis 2001, Kudrna et al. 2011). Somewhat surprisingly, the COI gene tree suggests it has a close relationship with *P. graeca*, another species from the southern Balkan Peninsula. These two taxa have distinct and constant wing patterns and differ in their habitat requirements, with *P. mamurra amymone* inhabiting steep and hot rocky gorges at lower elevations (Gascoigne-Pees et al. 2014) while *P. graeca* is predominantly a montane (high elevation) species endemic to Greece (Anastassiu et al. 2009). Thus, despite paraphyly of *P. amymone* Brown, 1976 (stat. n.) in relation to *P. graeca*, we believe they both represent valid species within the ‘*mamurra*’ group. Consequently *P. kermana* Eckweiler, 2004
Species delimitation in the Grayling genus *Pseudochazara*...

143

... (stat. n.), sister species to *P. amymone* and *P. graeca* combined, should also be elevated to species rank, although additional populations of *P. mamurra* in Iran should be examined to confirm this status. Alternatively, all the taxa within the ‘*mamurra*’ group, including the monophyletic *P. schakuhensis*, a sister species to the *amymone-graecca-kermana* clade, should be treated as a single very polymorphic species, a rather more destructive approach given the current taxonomy.

Although we are aware of the pitfalls of using single gene trees in the interpretation of phylogenetic patterns (Nichols 2001), we believe that strongly supported basal branching and splits between taxa, considered conspecific, represent valid insights into speciation in the *Pseudochazara* genus and together with distinct morphology and ecology allows species delimitation. Hence, we propose separate species status for the following taxa: *P. tisiphone*, *P. amalthea*, *P. amymone*, and *P. kermana*. This has important conservation implications, as most of these species are local endemics and therefore potentially threatened (Verovnik et al. 2014). Wider taxon sampling and inclusion of nuclear markers would undoubtedly help to a better understanding of the taxonomy of this fascinating butterfly genus.

**Acknowledgments**

We would like to express our gratitude to Wolfgang Eckweiler for his identification of several specimens from voucher photos housed in the BOLD database and we thank Evgeny V. Zakharov, Vlad Dinca and Axel Hausmann for their agreement to use unpublished DNA sequences from their projects in the BOLD database. We are thankful to our colleagues Tarkan Soyhan, Filip Franeta, Dubi Benyamini and Joseph Verhulst for providing additional samples of *Pseudochazara* for DNA analysis and Martin Gascoigne-Pees for checking the English. We also thank Niklas Wahlberg and an anonymous reviewer for helpful comments to improve the manuscript.

**References**

Anastassiu HT, Coutsis JG, 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 lycan* (Lepidoptera: Nymphalidae, Satyrinae). Phegea 37: 135–145. http://www.phegea.org/Phegea/2009/Phegea37-4_135-145.pdf

Aussem B (1980a) Eine neue Satyride der Gattung *Pseudochazara* de Lesse, 1951 aus Afghanistan (Satyrinae). Nota lepidopterologica 3: 5–15.

Aussem B (1980b) Zur Kenntnis der Androkonienfelder von *Pseudochazara thelephassa* (Geyer, 1827) und *Pseudochazara anthelea* (Hübner, 1824) (Lepidoptera, Satyrinae). Entomofauna – Zeitschrift für Entomologie 17: 354–358.
Aussem B (1980c) Ein Freiland-Hybrid der Gattung *Pseudochazara* (Lep., Satyridae). Entomologische Zeitschrift mit Insektenbörse 90: 161–165.

Bensasson D, Zhang X, Hartl DL, Hewitt GM (2011) Mitochondrial pseudogenes: evolution’s misplaced witnesses. Trends in Ecology and Evolution 16: 314–321. doi: 10.1016/S0169-5347(01)02151-6

Bergsten J (2005) A review of long-branch attraction. Cladistics 21: 163–193. doi: 10.1111/j.1096-0031.2005.00059.x

BOLD (2015) Barcoding Life. http://www.barcodinglife.com [accessed 15.10.2015]

Brower AVZ (2006) Problems with DNA barcodes for species delimitation: ‘ten species’ of *Astraptes fulgerator* reassessed (Lepidoptera: Hesperiidae). Systematics and Biodiversity 4: 127–132. doi: 10.1017/S147720000500191X

Brown J (1976) A review of the genus *Pseudochazara* de Lesse, 1951 (Lepidoptera, Satyridae) in Greece. Entomologist’s Gazette 27: 85–90.

Brown J (1980) On the status of a little known satyrid butterfly from Greece. Entomologist’s Record and Journal of Variation 92: 280–281.

Clement M, Posada D, Crandall KA (2000) TCS: a computer program to estimate genealogies. Molecular Ecology 9: 1657–1660. doi: 10.1046/j.1365-294x.2000.01020.x

Descimon H, Mallet J (2009) Bad species. In: Settele J, Shreeve TG, Konvicka M, Van Dyck H (Eds) Ecology of butterflies in Europe. Cambridge University Press, Cambridge, 219–249.

Dinca V, Zakharov EV, Hebert PDN, Vila R (2011) Complete DNA barcode reference library for a country’s butterfly fauna reveals high performance for temperate Europe. Philosophical Transactions of the Royal Society B: Biological Sciences 278: 347–355. doi: 10.1098/rspb.2010.1089

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

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

Eckweiler W, Rose K (1988) Identität, Verbreitung und subspezifische Gliederung von *Pseudochazara lydia* (Staudinger, 1878) (Lepidoptera, Satyridae). Nachrichten des Entomologischen Vereins Apollo, N.F. 9: 213–223.

Fauna Europaea (2016) Fauna Europaea version 2.5. Web Service available online at http://www.faunaeur.org [accessed on 6.3.2016]

Folmer OM, Black M, Hoeh R, Lutz R, Vrijehoek R (1994) DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Molecular Marine Biology and Biotechnology 5: 304–313. http://www.mbari.org/staff/vrijen/PDFS/Folmer_94MMBB.pdf

Garcia-Castellanos D, Estrada F, Jiménez-Munt I, Gorini C, Fernández M, Vergés J, De Vicente R (2009) Catastrophic flood of the Mediterranean after the Messinian salinity crisis. Nature 462: 778–781. doi: 10.1038/nature08555
Gascoigne-Pees M, Verovnik R, Franeta F, Popović M (2014) The lifecycle and ecology of *Pseudochazara amymone* (Brown, 1976) (Lepidoptera: Nymphalidae, Satyrinae). Nachrichten des Entomologischen Vereins Apollo, N.F. 35: 129–138.

Gerth M, Gansauge MT, Weigert A, Bleidorn C (2014) Phylogenomic analyses uncover origin and spread of the *Wolbachia* pandemic. Nature Communications 5: 5117. doi: 10.1038/ncomms6117

Gompert Z, Forister ML, Fordyce JA, Nice CC (2008) Widespread mito-nuclear discordance with evidence for introgressive hybridization and selective sweeps in *Lycaeides*. Molecular Ecology 17: 5231–5244. doi: 10.1111/j.1365-294X.2008.03988.x

Gross FJ (1978) Beitrag zur Systematik von *Pseudochazara*-Arten (Lep., Satyrinae). Atalanta 9: 41–103. http://www.zobodat.at/pdf/Atalanta_9_0041-0103.pdf

Hebert PDN, Penton EH, Burns JM, Janzen DH, 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. doi: 10.1073/pnas.0406166101

Hesselbarth G, van Oorschot H, Wagener S (1995) Die Tagfalter der Türkei unter Berücksichtigung der angrenzenden Länder. Selbstverlag Sigbert Wagener, Bocholt, Germany, 2201 pp.

Huelsenbeck JP, Ronquist F (2001) MRBAYES: Bayesian inference of phylogenetic trees. Bioinformatics 17: 754–755. doi: 10.1093/bioinformatics/17.8.754

Kodandaramaiah U, Wahlberg N (2009) Phylogeny and biogeography of *Coenonympha* butterflies (Nymphalidae: Satyrinae) – patterns of colonization in the Holarctic. Systematic Entomology 34: 315–323. doi: 10.1111/j.1365-3113.2008.00453.x

Kreuzinger AJ, Fiedler K, Letsch H, Grill A (2014) Tracing the radiation of *Maniola* (Nymphalidae) butterflies: new insights from phylogeography hint at one single incompletely differentiated species complex. Ecology and Evolution 18: 1153–1161. doi: 10.1002/ece3.1338

Kudrna O, Harpke A, Lux K, Pennerstorfer J, Schweiger O, Settele J, Wiemers M (2011) Distribution atlas of butterflies in Europe. Gesellschaft für Schmetterlingsschutz, Halle, 576 pp.

Lorković Z (1974) Die Verteilung der Varibilität von *Hipparchia statilinus* Hufn. (Lepid., Satyrinae) in Beziehung zum Karstboden des ostadratischen Küstenlandes. Acta entomologica Jugoslavica 10: 41–53.

Leite LAR (2012) Mitochondrial pseudogenes in insect DNA barcoding: differing points of view on the same issue. Biota Neotropica 12: 301–308. doi: 10.1590/S1676-06032012000300029

Lukhtanov VA (2007) Nymphalidae: Satyrinae. In: Global Butterfly Names Project. Global Butterfly Names – http://www.ucl.ac.uk/taxome/gbn/ [accessed 16.1.2015]

Lukhtanov VA, Novikova AV (2015) Interpretation of mitochondrial diversity in terms of taxonomy: a case study of *Hyponephele lycaon* species complex in Israel (Lepidoptera, Nymphalidae, Satyrinae). ZooKeys 538: 21–34. doi: 10.3897/zookeys.538.6689

Nazari V (2003) Butterflies of Iran. Dayereye Sabz publications, Tehran, 564 pp.
Nazari V, Sperling FAH (2007) Mitochondrial DNA divergence and phylogeography in western Palaearctic Parnassiinae (Lepidoptera: Papilionidae): How many species are there? Insect Systematics & Evolution 38: 121–138. doi: 10.1163/187631207788783996

Nazari V, Ten Hagen W, Bozano GC (2010) Molecular systematics and phylogeny of the ‘Marbled Whites’ (Lepidoptera: Nymphalidae, Satyrinae, Melanargia Meigen). Systematic Entomology 35: 132–147. doi: 10.1111/j.1365-3113.2009.00493.x

Nichols R (2001) Gene trees and species trees are not the same. Trends in Ecology & Evolution 16: 358–364. doi: 10.1016/S0169-5347(01)02203-0

Olivier A (1996) Notes on the taxonomic status and supposed biogeographic affinity of the Pseudochazara anthelea (Hübner, [1824]) populations from Kípros (Cyprus) and from the Greek island of Kós (Lepidoptera: Nymphalidae Satyrinae). Phegea 24: 5–12. http://ua-host.uantwerpen.be/vve/Phegea/1996/Phegea24-1_5-12.pdf

Pagès J (2007) Une nouvelle espèce de Pseudochazara du Pakistan (Nymphalidae, Satyrinae). Nota lepidopterologica 30: 361–365. http://www.soceurlep.eu/uploads/nota/bd30_2/08_Pag%E8s.pdf

Pazhenkova EA, Zakharov EV, Lukhtanov VA (2015) DNA barcoding reveals twelve lineages with properties of phylogenetic and biological species within Melitaea didyma sensu lato (Lepidoptera, Nymphalidae). ZooKeys 538: 35–46. doi: 10.3897/zookeys.538.6605

Peña C, Nylin S, Wahlberg N (2011) The radiation of Satyrini butterflies (Nymphalidae: Satyrinae): a challenge for phylogenetic methods. Zoological Journal of the Linnean Society 161: 64–87. doi: 10.1111/j.1096-3642.2009.00627.x

Pamperis LN (2009) The Butterflies of Greece. Editions Pamperis, Athens, 768 pp.

Posada D (2008) jModelTest: phylogenetic model averaging. Molecular Biology and Evolution 25: 1253–1256. doi: 10.1093/molbev/msn083

Rambaut A (2014) Figtree v1.4.2. Computer program and documentation distributed by the author. http://tree.bio.ed.ac.uk/software [accessed 15.10.2014]

Ritter S, Michalski SG, Settele J, Wiemers M, Fric ZF, Sielezniew M, Šašić M, Rozier Y, Durka W (2013) Wolbachia infections mimic cryptic speciation in two parasitic butterfly species, Phengaris teleius and P. nausithous (Lepidoptera: Lycaenidae). PLoS ONE 8: 1–13. doi: 10.1371/journal.pone.0078107

Savela M (2015) Lepidoptera and some other life forms. FUNET database – http://www.nic.funet.fi/pub/sci/bio/life/insecta/lepidoptera/ [accessed 6.1.2015]

Song H, Buhay JE, Whiting MF, Crandall KA (2008) Many species in one: DNA barcoding overestimates the number of species when nuclear mitochondrial pseudo-genes are coamplified. Proceedings of the National Academy of Sciences of the United States of America 105: 13486–13491. doi: 10.1073/pnas.0803076105

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S (2013) MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0. Molecular Biology and Evolution 30: 2725–2729. doi: 10.1093/molbev/msr197

Tennent J (1996) The Butterflies of Morocco, Algeria and Tunisia. Gem Publishing Company, Oxfordshire, 217 pp.

Toews DPL, Brelsford A (2012) The biogeography of mitochondrial and nuclear discordance in animals. Molecular Ecology 21: 3907–3930. doi: 10.1111/j.1365-294X.2012.05664.x
Tshikolovets VV (2005) The Butterflies of Ladak (N.-W. India) (Lepidoptera, Rhopalocera). Tshikolovets publications, Brno–Kyiv, 176 pp.
Tshikolovets VV (2011) Butterflies of Europe & the Mediterranean area. Tshikolovets publications, Pardubice, Czech Republic, 544 pp.
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. doi: 10.1007/s10841-013-9576-6
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: 675–682. doi: 10.1007/s10841-014-9674-0
Verovnik R, Sket B, Trontelj P (2004) Phylogeography of subterranean and surface populations of water lice Asellus aquaticus (Crustacea: Isopoda). Molecular Ecology 13: 1519–1532. doi: 10.1111/j.1365-294X.2004.02171.x
Wakeham-Dawson A (2005) Further descriptions of androconia from Staudinger’s Pseudochazara de Lesse, 1951 (Lepidoptera: Nymphalidae, Satyrinae) type specimens in the Zoologisches Museum der Humboldt-Universität zu Berlin. Entomologist’s Gazette 56: 139–146.
Wakeham-Dawson A (2006) Descriptions of wing androconia from some Pseudochazara de Lesse, 1951 (Lepidoptera: Nymphalidae, Satyrinae) type specimens in The Natural History Museum, London. Entomologist’s Gazette 57: 99–107.
Wakeham-Dawson A, Kudrna O (2000) A quantitative description of androconia from Staudinger’s Pseudochazara de Lesse, 1951 (Lepidoptera: Nymphalidae, Satyrinae) type specimens in the Zoological Museum of the Humboldt University of Berlin. Entomologist’s Gazette 51: 75–81.
Wakeham-Dawson A, Dennis RLH (2001) A quantitative description of the male genitalia of 23 taxa of Pseudochazara de Lesse, 1951 (Lepidoptera: Nymphalidae, Satyrinae). Entomologist’s Gazette 52: 227–250.
Wakeham-Dawson A, Parker R, John E, Dennis RLH (2003) Comparison of the male genitalia and androconia of Pseudochazara anthelea acamanthis (Rebel, 1916) from Cyprus, Pseudochazara antheleae anthelea (Hübner, 1924) from mainland Turkey and Pseudochazara anthelea amathea (Frivaldsky, 1845) from mainland Greece (Lepidoptera: Nymphalidae, Satyrinae). Nota lepidopterologica 25: 251–263.
Wakeham-Dawson A, Kudrna O (2005) Further descriptions of androconia from Staudinger’s Pseudochazara de Lesse, 1951 (Lepidoptera: Nymphalidae, Satyrinae) type specimens in the Zoologisches Museum der Humboldt-Universität zu Berlin. Entomologist’s Gazette 56: 139–146.
Wakeham-Dawson A, Kudrna O (2006) Description of wing androconia from the lectotype of Pseudochazara caucasica (Lederer, 1864) (Lepidoptera: Nymphalidae, Satyrinae), with notes on the toptype wing androconia of related taxa. Entomologist’s Gazette 57: 137–141.
Wakeham-Dawson A, Kudrna O, Dennis RLH (2007) Description of androconia in the Palearctic Asian Pseudochazara baldiva (Moore, 1865) butterfly species-group (Nymphalidae: Satyrinae) with designation of two lectotypes and reference to type and other material in the Natural History Museum, London. Nota lepidopterologica 30: 211–223. http://www.soceurllep.eu/uploads/nota/bd30_2/01_Wakeham-Dawson.pdf
Weiss JC (1980) Le genre *Pseudochazara* de Lesse en Europe et en Afrique du Nord. Description d’une sous-espèce nouvelle de *Ps. hippolyte* Esper (Lep.: Satyridae). Linneana Belgica 8: 98–108.

Wiemers M, Fiedler K (2007) Does the DNA barcoding gap exist? – a case study in blue butterflies (Lepidoptera: Lycaenidae). Frontiers in Zoology 4: 8. doi: 10.1186/1742-9994-4-8

Yakovlev RV (2012) Checklist of Butterflies (Papilionoidea) of the Mongolian Altai Mountains, including descriptions of new taxa. Nota lepidopterologica 35: 51–96. http://www.soceurllep.eu/uploads/nota/bd35_1/07_Yakovlev.pdf

Yang Z, Landry J-F, Handfield L, Zhang Y, Solis MA, Handfield D, Scholtens BG, Mustanen M, Nuss M, Hebert PDN (2012) DNA barcoding and morphology reveal three cryptic species of *Anania* (Lepidoptera: Crambidae: Pyraustinae) in North America, all distinct from their European counterpart. Systematic Entomology 37: 686–705. doi: 10.1111/j.1365-3113.2012.00637.x
### Table 1

List of samples of the genus *Pseudochazara* included in the barcoding analysis (either own samples with “LA” ID or from BOLD).

| ID   | GenBank   | Species                                      | Location                        | Lat       | Long        | Date     | Legit            |
|------|-----------|----------------------------------------------|---------------------------------|-----------|-------------|----------|------------------|
| LA16 | KU499958  | *Pseudochazara mamurra amymone*              | Baboshtice, Korce, Albania     | 40°31.038'N | 20°47.647'E | 11.vii.2012 | Rudi Verovnik    |
| LA17 | KU499959  | *Pseudochazara mniszechii tisiphone*         | Baboshtice, Korce, Albania     | 40°31.038'N | 20°47.647'E | 11.vii.2012 | Rudi Verovnik    |
| LA19 | KU499960  | *Pseudochazara mamurra amymone*              | Devoll Gorge, Korce, Albania   | 40°42.576'N | 20°31.446'E | 10.vii.2012 | Rudi Verovnik    |
| LA24 | KU499961  | *Pseudochazara cingovskii*                   | Pletvar Pass, Prilep, Macedonia| 41°22.456’N | 21°38.805'E | 14.vii.2010 | Rudi Verovnik    |
| LA28 | KU499962  | *Pseudochazara mniszechii*                   | Sivas, Turkey                  | 39°41.519’N | 36°59.877'E | 22.vii.2009 | Tarkan Soyhan    |
| LA29 | KU499963  | *Pseudochazara mniszechii*                   | Eskişehir, Turkey              | 39°43.801’N | 30°31.428'E | 16.vi.2007  | Tarkan Soyhan    |
| LA75 | KU499964  | *Pseudochazara geyeri occidentalis*          | Galičica Pass, Macedonia       | 40°57.379’N | 20°48.961'E | 30.vii.2013 | Filip Franeta    |
| LA76 | KU499965  | *Pseudochazara oreste*                       | Katar Pass, Metsova, Greece    | 39°47.580’N | 21°12.272'E | 22.vii.2012 | Filip Franeta    |
| LA78 | KU499967  | *Pseudochazara oreste*                       | Granitis, Drama,Greece         | 41°18.533’N | 23°54.862'E | 27.vii.2013 | Rudi Verovnik    |
| LA79 | KU499968  | *Pseudochazara gnea*                        | Katar Pass, Metsova, Greece    | 39°47.580’N | 21°12.272'E | 26.vii.2013 | Rudi Verovnik    |
| LA80 | KU499969  | *Pseudochazara mniszechii tisiphone*         | Drenovë, Korcë, Albania       | 40°35.352’N | 20°48.508'E | 21.vii.2013 | Rudi Verovnik    |
| LA81 | KU499970  | *Pseudochazara mniszechii tisiphone*         | Drenovë, Korcë, Albania       | 40°35.352’N | 20°48.508'E | 21.vii.2013 | Rudi Verovnik    |
| LA82 | KU499971  | *Pseudochazara pelopea*                      | Mt. Hermon, Israel             | 33°19.766’N | 35°47.243'E | 2013      | Dubi Benyamini   |
| LA83 | KU499972  | *Pseudochazara pelopea*                      | Mt. Hermon, Israel             | 33°19.766’N | 35°47.243'E | 2013      | Dubi Benyamini   |
| LA84 | KU499973  | *Pseudochazara cingovskii*                   | Pletvar Pass, Prilep, Macedonia| 41°22.456’N | 21°38.805'E | 2013      | Filip Franeta    |
| LA85 | KU499974  | *Pseudochazara cingovskii*                   | Pletvar Pass, Prilep, Macedonia| 41°22.456’N | 21°38.805'E | 2013      | Filip Franeta    |
| LA86 | KU499975  | *Pseudochazara anthbeia amalthea*            | Veles, Topolka, Macedonia      | 41°41.915’N | 21°46.927'E | 2010      | Filip Franeta    |
| LA87 | KU499976  | *Pseudochazara anthbeia amalthea*            | Mt. Parnassos, Greece          | 38°31.233’N | 22°36.566'E | 2010      | Filip Franeta    |
| LA88 | KU499977  | *Pseudochazara anthbeia amalthea*            | Drenovë, Korcë, Albania       | 40°35.352’N | 20°48.508'E | 2013      | Filip Franeta    |
| LA89 | KU499978  | *Pseudochazara mamurra birgit*               | Mt. Aladaglar, Turkey          | 37°47.568’N | 35°9.242'E  | 2006      | Filip Franeta    |
| LA90 | KU499979  | *Pseudochazara mniszechii*                   | Mt. Aladaglar, Turkey          | 37°47.568’N | 35°9.242'E  | 2006      | Filip Franeta    |
| LA92 | KU499980  | *Pseudochazara gnea*                        | Mt. Iti, Greece               | 38°49.333’N | 22°16.635'E | 1999      | Filip Franeta    |
| LA94 | KU499981  | *Pseudochazara mamurra amymone*              | Drenovë, Korcë, Albania       | 40°35.352’N | 20°48.508'E | 2013      | Filip Franeta    |
| LA95 | KU499982  | *Pseudochazara mamurra amymone*              | Devoll Gorge, Korce, Albania  | 40°42.576’N | 20°31.446'E | 2013      | Filip Franeta    |
| LA97 | KU499983  | *Pseudochazara lydia obscura*                | Mersin, Turkey                | 36°57.017’N | 34°23.019'E | 12.vii.2010 | Tarkan Soyhan    |
| LA124| KU499984 | *Pseudochazara lehane*                       | Saibo Digur La, Ladakh, India  | 34°10.554’N | 77°39.529'E | 15.vii.2013 | Joseph Verhulst  |
| ID          | GenBank   | Species                                      | Location                                           | Lat       | Long       | Date       | Legit       |
|-------------|-----------|----------------------------------------------|----------------------------------------------------|-----------|------------|------------|-------------|
| BPAL1699–12 |           | *Pseudochazara mamurra*                      | Azerbaijan: near Shamkir, 1300 m                   | 40.6989   | 45.8697    | 31.vii.2011| Tikhonov V. |
| BPAL1700–12 |           |                                               | Azerbaijan: near Shamkir, 1300 m                   | 40.6989   | 45.8697    | 31.vii.2011| Tikhonov V. |
| BPAL2136–13 |           | *Pseudochazara alpina*                       | Russia: North Ossetia-Alania, rv. Ardon, Skasan, 1050 m | 42.6956   | 43.9989    | 12.viii.2011| Petrov A.   |
| BPAL1703–12 |           | *Pseudochazara alpina*                       | Azerbaijan: near Shamkir, 1300 m                   | 40.6989   | 45.8697    | 31.vii.2011| Tikhonov V. |
| BPAL2137–13 |           | *Pseudochazara thelephassa*                  | Tajikistan: Khodra-Mumin Mnt.                     | 42.6956   | 43.9989    | 12.viii.2011| Petrov A.   |
| BPAL2139–13 |           |                                               | Tajikistan: Khodra-Mumin Mnt.                     | 42.6956   | 43.9989    | 12.viii.2011| Petrov A.   |
| BPAL2140–13 |           | *Pseudochazara thelephassa*                  | Russia: North Ossetia-Alania, rv. Ardon, Skasan, 1050 m | 42.6956   | 43.9989    | 12.viii.2011| Petrov A.   |
| BPAL2141–13 |           |                                               | Iran: Ker. Kuh-e-Madvar, 5 km S Jowzan, 2400–2600 m | 42.6956   | 43.9989    | 12.viii.2011| Hofmann P.  |
| BPAL2145–13 |           | *Pseudochazara dagestana savalanica*         | Iran: Azarbayjan-e-Sharqi, N’Iran, Kuh-e-Sabalan, 2900–3000 m | 42.6956   | 43.9989    | 12.viii.2011| Hofmann P.  |
| BPAL2152–13 |           | *Pseudochazara dagestana savalanica*         | China: Xinjiang, Tian Shan, Bororo Shan, 40 km SSW Kyren, 1850–2050 m | 42.6956   | 43.9989    | 12.viii.2011| Hofmann P.  |
| BPAL2153–13 |           | *Pseudochazara dagestana savalanica*         | Iran: Kherasan, Kopet Dagh, 18 km E Ermans, 2300–2600 m | 42.6956   | 43.9989    | 12.viii.2011| Hofmann P.  |
| BPAL2154–13 |           | *Pseudochazara dagestana savalanica*         | Iran: Kherasan, Kopet Dagh, Qoran, 1800 m          | 42.6956   | 43.9989    | 12.viii.2011| Hofmann P.  |
| BPAL2155–13 |           | *Pseudochazara mamurra schahrudensis*        | Iran: Kherasan, Kopet Dagh, Qoran, 1800 m          | 42.6956   | 43.9989    | 12.viii.2011| Hofmann P.  |
| BPAL2156–13 |           |                                               | Iran: Kherasan, Kopet Dagh, Qoran, 1800 m          | 42.6956   | 43.9989    | 12.viii.2011| Hofmann P.  |
| BPAL2157–13 |           |                                               | Iran: Kherasan, Kopet Dagh, Qoran, 1800 m          | 42.6956   | 43.9989    | 12.viii.2011| Hofmann P.  |
| ID | GenBank | Species                                      | Location                                      | Lat     | Long     | Date       | Legit         |
|----|---------|----------------------------------------------|-----------------------------------------------|---------|----------|------------|---------------|
|    |         | *Pseudochazara nanuza nanuza*               | Turkey: Arvın, Dilekçe, SW Çandır, 1300 m     |         |          | 1. vi. 1998| P. Hofmann    |
|    |         | *Pseudochazara nanuza nanuza*               | Turkey: Erzurum, 5 km N Bayburt, 1500 m      |         |          | 10. vii. 1998| P. Hofmann    |
|    |         | *Pseudochazara nanuza nanuza*               | Turkey: Gumushane, Demlik, 5 km NW, 1400 m   |         |          | 08. vi. 1998| P. Hofmann    |
|    |         | *Pseudochazara nanuza nanuza*               | Turkey: Erzurum, 5 km SE Çaglayan, 1400 m    |         |          | 06. vi. 1998| P. Hofmann    |
|    |         | *Pseudochazara nanuza nanuza*               | Turkey: Erzurum, road Bayburt-Ispir, Laleli, 1300-1400 m |         |          | 11. vii. 1998| P. Hofmann    |
|    |         | *Pseudochazara nanuza nanuza*               | Turkey: Erzurum, 5 km S Bayburt, 1500 m      |         |          | 28. v. 2002 | P. Hofmann    |
|    |         | *Pseudochazara nanuza nanuza*               | Turkey: Erzurum, 5 km S Çaglayan, 1400 m     |         |          | 24. v. 2002 | P. Hofmann    |
|    |         | *Pseudochazara nanuza nanuza*               | Turkey: Erzurum, 5 km S Bayburt, 1500 m      |         |          | 19. vi. 2001| P. Hofmann    |
|    |         | *Pseudochazara nanuza nanuza*               | Turkey: Erzurum, 5 km S Çaglayan, 1400 m     |         |          | 24. v. 2002 | P. Hofmann    |
|    |         | *Pseudochazara nanuza nanuza*               | Turkey: Erzurum, 5 km S Bayburt, 1500 m      |         |          | 23. v. 2013 | P. Hofmann    |
|    |         | *Pseudochazara nanuza nanuza*               | Turkey: Erzurum, 5 km S Çaglayan, 1400 m     |         |          | 22. vi. 2013| P. Hofmann    |
|    |         | *Pseudochazara nanuza nanuza*               | Turkey: Erzurum, 5 km S Bayburt, 1500 m      |         |          | 22. vi. 2013| P. Hofmann    |
|    |         | *Pseudochazara nanuza nanuza*               | Turkey: Erzurum, 5 km S Çaglayan, 1400 m     |         |          | 16. vi. 1999| A. Salk       |

Species delimitation in the Grayling genus *Pseudochazara...*
| ID       | GenBank       | Species                  | Location                              | Lat       | Long      | Date       | Legit           |
|----------|---------------|--------------------------|---------------------------------------|-----------|-----------|------------|-----------------|
| BPAL2282–14 |              | *Pseudochazara pelopea pelopea* | Syria: Bloudan, 1500 m               |           |           | 16.vii.1999 | A. Salk         |
| BPAL2692–14 |              | *Pseudochazara pelopea pelopea* | Israel                               |           |           | 03.vii.2014 | V. Lukhtanov & A. Novikova |
| BPAL2701–14 |              | *Pseudochazara pelopea pelopea* | Israel                               |           |           | 03.vii.2014 | V. Lukhtanov & A. Novikova |
| BPAL2702–14 |              | *Pseudochazara pelopea pelopea* | Israel                               |           |           | 03.vii.2014 | V. Lukhtanov & A. Novikova |
| BPAL2728–14 |              | *Pseudochazara pelopea pelopea* | Israel                               |           |           | 04.vii.2014 | V. Lukhtanov    |
| BPAL2731–14 |              | *Pseudochazara pelopea pelopea* | Israel                               |           |           | 04.vii.2014 | V. Lukhtanov    |
| EULEP451–14  |              | *Pseudochazara euxina*     | Ukraine                              |           |           | 11.vii.2007 | local collector |
| EULEP452–14  |              | *Pseudochazara euxina*     | Ukraine                              |           |           | 11.vii.2007 | local collector |
| EULEP453–14  |              | *Pseudochazara euxina*     | Ukraine                              |           |           | 11.vii.2007 | local collector |
| EULEP487–14  |              | *Pseudochazara hippolyte hippolyte* | Russia                              | 52.65     | 59.5667   | 23.vii.1998 | K. Nupponen     |
| EULEP488–14  |              | *Pseudochazara hippolyte hippolyte* | Russia                              | 51.8      | 57.0833   | 14.vii.1998 | K. Nupponen     |
| EZHBA660–07  |              | *Pseudochazara doerriesi*  | Russia                               | 51.717    | 94.4      | 17.vii.2000 | Oleg Kosterin   |
| EZHBA661–07  |              | *Pseudochazara doerriesi*  | Russia                               | 51.717    | 94.4      | 17.vii.2000 | Oleg Kosterin   |
| EZHBA662–07  |              | *Pseudochazara doerriesi*  | Russia                               | 51.717    | 94.4      | 17.vii.2000 | Oleg Kosterin   |
| EZHBA899–07  |              | *Pseudochazara doerriesi*  | Russia                               | 51.7667   | 91.9333   | 30.vi.2004  | Oleg Kosterin   |
| EZHBA900–07  |              | *Pseudochazara doerriesi*  | Russia                               | 51.7667   | 91.9333   | 30.vi.2004  | Oleg Kosterin   |
| EZROM089–08  | HQ004207     | *Chazara briseis*         | Romania: Transylvania: Saturu        | 46.783    | 23.95     | 16.viii.2006 | Dinca Vlad     |
| EZROM848–08  | HQ004205     | *Chazara briseis*         | Romania: Transylvania: Saturu        | 46.799    | 23.959    | 16.viii.2006 | Dinca Vlad     |
| EZSPM470–09  | GU676107     | *Pseudochazara hippolyte* | Spain: Granada: San Juan (Sierra Nevada) | 37.094    | -3.115    | 16.vii.2009 | Dinca V.       |
| EZSPN732–09  | GU676410     | *Pseudochazara hippolyte* | Spain: Granada: Laguna Seca, Hueneja | 37.097    | -2.97     | 18.vii.2008 | S. Montagud, J. A. Garcia-Alama & J. Garcia |
| EZSPN733–09  | GU676411     | *Pseudochazara hippolyte* | Spain: Granada: Laguna Seca, Hueneja | 37.097    | -2.97     | 18.vii.2008 | S. Montagud, J. A. Garcia-Alama & J. Garcia |
| EZSPN735–09  | GU676413     | *Pseudochazara hippolyte* | Spain: Granada: Laguna Seca, Hueneja | 37.097    | -2.97     | 18.vii.2008 | S. Montagud, J. A. Garcia-Alama & J. Garcia |
Species delimitation in the Grayling genus Pseudochazara...
| ID         | GenBank   | Species                      | Location | Lat  | Long  | Date       | Legit                  |
|------------|-----------|------------------------------|----------|------|-------|------------|------------------------|
| LOWAB047–07|           | *Pseudochazara pelepea persica* | Armenia | 40.083 | 44.917 |            | Andrei Sourakov        |
| LOWAB048–07|           | *Pseudochazara pelepea persica* | Armenia | 40.083 | 44.917 |            | Andrei Sourakov        |
| WMB1212–13 |           | *Pseudochazara atlantis*     | Morocco  | 33.025 | -5.071 | 01.vii.2011 | Vila, R., Dinca, V. & Voda, R. |
| WMB1213–13 |           | *Pseudochazara atlantis*     | Morocco  | 33.025 | -5.071 | 01.vii.2011 | Vila, R., Dinca, V. & Voda, R. |
| WMB2163–13 |           | *Pseudochazara atlantis*     | Morocco  | 31.09  | -7.915 | 15.vii.2012 | Tarrier, Michel        |