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Status and conservation of genetic diversity in the disjunct populations of *Pseudochazara tisiphone* Brown, [1981], in Albania.

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Abstract

Preserving broad genetic diversity is more crucial than ever in the face of the current biodiversity crisis, enabling species to adapt to changing conditions and habitats. Efforts to protect genetic diversity through frameworks like the Kunming-Montreal Global Biodiversity Framework and the EU Nature Restoration Law are gaining increasing attention. While these initiatives hold great promise, they are still in the early stages of practical implementation, and many challenges and questions remain to be addressed. Given that butterflies are commonly used as umbrella species for conservation, a comprehensive understanding of their species delimitation and evolutionary history is cucial for the successful preservation of their genetic diversity.
The "grey zones" in the systematics of Lepidoptera, arising from differing species concepts, are assessed for their potential impact on new initiatives aimed at protecting important genetic diversity. How will limited urgency among policymakers for nature conservation evolve, especially in the context of scarce resources and the potential opportunity for exploiting a substantial hydrogen reservoir? To better understand the potential challenges, an analysis is conducted on *Pseudochazara tisiphone* Brown, [1981], a butterfly species with a limited and fragmented distribution in the Balkan Peninsula, focusing on Albania, a non-EU country. Considering the morphological variability of *P. tisiphone*, limited mtDNA data that does not allow species-level determination, the disjunct distribution, industrial threats in the Bulqizë area, and the need to support a positive conservation decision by policymakers, several challenges must be addressed. However, the disjunct population in Dibër County justifies the description of a new subspecies, *Pseudochazara tisiphone dibra* ssp. nov. (Lepidoptera: Nymphalidae). Comprehensive data from a larger genomic study of Balkan *Pseudochazara* populations are still needed to definitively resolve its taxonomic status.

Key words

Albania, Dibër county, Kunming-Montreal Global Biodiversity Framework, EU Nature Restoration Law, Papilionoidea, genetic diversity, DNA barcoding, morphological variability, reproductive isolation, speciation, subspecies, *ssp. nov., Pseudochazara tisiphone dibra*.

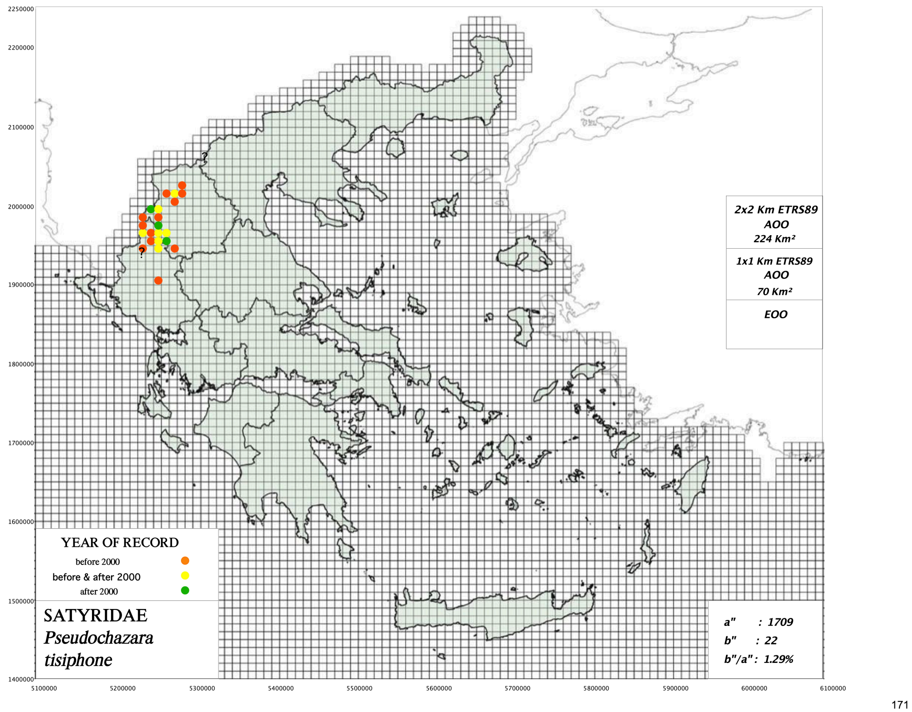
Introduction

In recent years, the conservation of genetic diversity has gained significant attention through initiatives like the Kunming-Montreal Global Biodiversity Framework (url) and the EU Nature Restoration Law (url), both of which aim to ensure that populations retain the potential for evolutionary adaptation to ongoing environmental challenges.
The objective to ensure that biodiversity will be sustainably conserved, focuses on halting and reversing biodiversity loss by protecting ecosystems, species, and genetic diversity. It also seeks to promote the responsible use of biodiversity resources to ensure that human activities do not degrade ecosystems and species diversity, ensuring long-term sustainability.
As there are quite some different species concepts that often result in "gray zones" with different conclusions about speciation, it looks needed to have a concensus how to compare genetic diversity. Within the Lepidoptera, the most charismatic taxa, such as *Parnassius apollo* (Linnaeus, 1758) have attracted a disproportionate attention from taxonomists, leading to an inflated number of subspecies compared to many other Lepidoptera species. Without standardisation the selection of key cases of genetic diversity will be highly subjective.
The Biological Species Concept (BSC) is the most widely used and intuitively appealing, yet it becomes challenging to apply in cases of allopatry. A central tenet of the BSC is the ability of populations to interbreed and produce fertile offspring, which serves as a major criterion for defining species. The Phylogenetic Species Concept (PSC) relies on genetic data to trace evolutionary history, making it valuable in cases where reproductive isolation is not readily apparent. The Morphological Species Concept (MSC) is based on shared physical characteristics. The selection of which morphological traits to prioritize can be highly subjective. Different taxonomists may focus on different characteristics, leading to varying interpretations of what constitutes a species. For example, one taxonomist may

emphasize wing shape in butterflies, while another might prioritize coloration, genital structures or early stages, even if the populations exhibit a broad range of these traits.
Despite such bottle necks, integrating genetic diversity data into conservation efforts offers promise for reversing biodiversity decline. However it faces also a number of conceptual challenges and a key issue is how to implement this approach in practice, especially in countries with high genetic diversity, where there is limited urgency among policymakers and where resources are scarce.
Currently, the short fragment of the mitochondrial cytochrome c oxidase subunit I (COI) gene is likely the most readily available genetic data for obtaining a global view of the distribution of genetic diversity across species, as it is the most frequently used genetic marker. But the use of mtDNA in connection with biodiversity conservation assessment should be interpreted with caution as it only represents a miniscule portion of the full genome and may not capture the complexity of genetic diversity. Its maternal inheritance is a bias of genetic diversity, certainly if males contribute significantly to the gene pool of the taxon. In small populations mtDNA can become (nearly) fixed. Introgression can lead to challenges in accurately assessing genetic diversity.
All these factors do not reflect current levels of genetic diversity. The incorporation of mtDNA should be considered only as part of a comprehensive, multifaceted genetic approach.
Owing to recent technological advancements, high-throughput sequencing, a larger view of the genome becomes accessible and is much less expensive than whole genome sequencing. Using such a novel approach, Joshi *et al.* tested species delimitation both within and across continents in selected Lepidoptera cases, discovering mitonuclear discordance in half of the instances. This confirms that the taxonomic delimitation of individual populations by only mtDNA often does not reflect the true genealogy of the species. Describing new species based solely on mitochondrial DNA (mtDNA) is a potential pitfall that can be avoided by ensuring decisions are supported by broader nuclear data. When only mtDNA data are available, it therefore seems useful to refrain from making a taxonomic choice at species level, thinking as well on the validity of the choice as on the potential harmful effects of inflating the lists of populations for conservation efforts as well as protecting such areas.
At a national level, the results from Chiocchio *et al.* (2024) revealed that protected areas in Italy cover only a small fraction of the regions holding the highest levels of intraspecific genetic diversity for terrestrial non-volant vertebrates. They also highlighted a significant discordance between the locations of genetic hotspots based on mitochondrial and nuclear data, especially at biologically relevant spatial scales. It would not be surprising if a similar model applied to Lepidoptera yielded comparable findings.
This study examines two disjunct populations of *Pseudochazara tisiphone* Brown, [1981] from Albania, within the charismatic genus *Pseudochazara* Lesse, 1951, which exhibit significant morphological variability and a debated classification. These populations are considered within the framework of emerging conservation initiatives aimed at preserving genetic diversity with a particular focus on Albania, a European country that is not a member of the European Union.

Discussion

The original description (url) of *Pseudochazara tisiphone* Brown, [1981] underscores the taxonomic challenges associated with the genus *Pseudochazara.*
Brown proposed that the populations on Mount Smolikas (Greece) represented an undescribed subspecies of *Pseudochazara cingovskii* (Gross, 1973), which he classified as *ssp. tisiphone* (url).
However, since Wakeham-Dawson's (1997) discriminant analysis by androconia in the genus *Pseudochazara*, the taxon has frequently been referred to as *ssp. tisiphone* of the Turkish *P. mniszechii*.
Takáts & Mølgaard (2016) and Verovnik & Wiemers (2016) employed DNA barcoding to address the taxonomic status of the described *Pseudochazara* species and to provide insights into the phylogeny of the genus. Based on their findings, Verovnik & Wiemers (2016) proposed recognizing *P. tisiphone* as a distinct species.
Although the publication date is frequently cited as 1980, evidence suggests that the publication was not released to the public until 1981, indicating a pattern of repeated miscitation and adapted in Taymans & Cuvelier (2024). The work was referenced in the *Entomologist's Record and Journal of Variation* 92(11/12): 280–281 (url) in 1980, but it was not distributed to readers until 1981, which is the actual publication date according to the ICZN code (url).
Pamperis L. kindly provided the most recent distribution map of *P. tisiphone* (Fig. 1), which highlights its restricted range in Greece and emphasizes the reported declines in populations within the Greek portion of its distribution.
In the decades following its discovery, *P. tisiphone* was considered a Greek endemic, primarily due to the limited access to Albanian sources, which made references such as Misja & Kurrizi (1984), Misja (1993), and Misja (2005) unknown and nearly impossible to obtain. For Albania, *P. mamurra* (Herrich-Schäffer, [1846]) was first reported by Misja & Kurrizi (1984). In 1993, Misja documented two new *Pseudochazara* species for Albania, identifying them as *P. cingovskii* and *P. graeca* based on the androconial scales and genitalia. However, the list by van Swaay & Warren (1999) and the Atlas by Misja (2005) retained only one species: *P. cingovskii*. A comparison of the androconial scale drawings of the two taxa in Misja (1993) with photographs in Cuvelier & Mølgaard (2015) and figures in Gross (1978) suggests that these populations likely correspond to *P. tisiphone* and *P. amymone*.
In Cuvelier *et al.* (2018) and Supplementary Material 2, the discovery of a new, isolated population of *P. tisiphone* in Dibër county was discussed and detailled.
On 13.vii.2017, the first observations were made near Bulqizë (Fig. 3-4) by Cuvelier S., where *P. tisiphone* was found to be widespread across 4 visited localities. On 18.vii.2017, Parmentier L. confirmed the occurence of *P. tisiphone* in the Bulqizë area and also observed the species at a few kilometers near Krastë. In 2022, Qirinxhi X. informed us of her discovery of the species in the Lurë-Dejë National Park, located more than 30 km north of Bulqizë (url).
All observations known to us until the end of 2024 are shown in the distribution map (Fig. 2) which highlights a disjunct distribution acrosss various Albanian ophiolite zones.



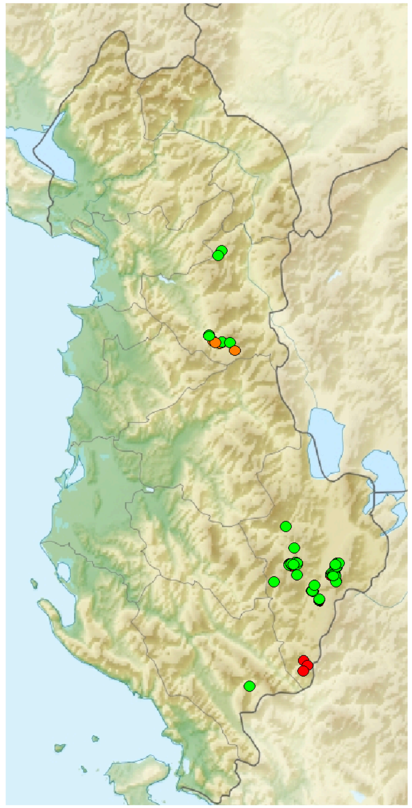


Fig. 1. ETRS89 *Pseudochazara tisiphone*-Greece-distribution map (before and after 2000) version 01.i.2025 (Source: Lazaros N. Pamperis). **Fig. 2.** *Pseudochazara tisiphone*, Albania distribution map version 09.i.2025, (Source: Fluturat e Shqipërisë) 🔴 Historical data ; 🟠 Additional data from the 2018 update ; 🟢 New observations since the 2018 update.

On average, both ♂♂ as ♀♀ of *P. tisiphone* from Dibër county appear smaller and paler, though without clear outliers, than specimens from Korçë county, and exhibit notable variability in the dimensions of the orange submarginal bands on the upperside of the hindwings.

The marked variability of *P. tisiphone* in Korçë county was documented by Cuvelier & Mølgaard (2015) and was also observed in the population from Dibër county. As with many Satyrinae, the butterflies exhibit variable external characteristics. The ground colour of the wings, the presence of ocelli, and the dimensions of the pale bands of *Pseudochazara* species are influenced by factors such as annual mean temperatures and substrate type, with notable variation across different localities. This variation can be particularly pronounced in areas located just 10 km apart, yet distinguished by distinct geological features, such as the limestone and ophiolite localities near Metsovo (Greece) where *Pseudochazara graeca* (Staudinger, 1870) exhibits significant morphological differences. This variation is an example of phenotypic plasticity, where environmental factors shape the phenotype of the butterfly, enhancing its camouflage and aiding in predator avoidance.

The populations of *P. tisiphone* in Bulqizë (Dibër county) and Boboshtiçë (Korçë county) inhabit similar ophiolite habitats. Yet they display significant differences in annual average maximum and minimum temperatures, as well as precipitation (Meteoblue History & Climate, accessed on 15.i.2025). Bulqizë experiences higher annual average maximum temperatures (16.8°C vs. 15.3°C) and minimum temperatures (6.8°C vs. 4.6°C), along with nearly double the annual precipitation (1501 mm vs. 727 mm) compared to Boboshtiçë.

In Wakeham-Dawson (1997) the basal shape (flat, convex, concave), length, and breadth of androconia were examined across 21 taxa, revealing differences within the genus *Pseudochazara.* However, androconia proved unreliable for identifying 10 out of the 23 taxa studied. For these 10 taxa, the probability of correctly identifying a specimen was 0.50 or less, including *cingovskii* and Greek *tisiphone.* Furthermore, the discovery of two distinct types of androconia, with different basal shape and variation within the examined specimens, in *P. tisiphone* further limits the use of androconia as a reliable morphological criterion for speciation within these populations.

In Cuvelier *et al.* (2018), the androconial scales of a *P. tisiphone* specimen from Dibër county were examined. These scales shared the same global morphological characteristics for basal shape as those of two specimens from Korçë county. However, the scales of the Dibër specimen were slightly shorter, likely reflecting its smaller wingspan and overall dimensions.

Such morphological phenomena likely reflect processes strongly influenced by phenotypic plasticity making the small differences between the two distant populations of *P. tisiphone* inconsistent.

Overemphasizing the statistical significance of phenotypic differences, can involve traits that do not indicate reproductive isolation which can lead to oversplitting of phylogeographic lineages into multiple taxa. This practice often leads to what has been referred to as "taxonomic vandalism" as highlighted by Dufresnes *et al.* (2023).

Fig. 3. Locus typicus of *Pseudochazara tisiphone dibra*. Bulqizë, Dibër county, Albania (© Sylvain Cuvelier). **Fig. 4.** Habitat of *Pseudochazara tisiphone dibra*. West of Bulqizë, Dibër county, Albania (© Sylvain Cuvelier)

While major differences in genitalia could suggest potential reproductive isolation, the genitalia of closely related *Pseudochazara* species are remarkably similar, offering limited diagnostic value.The genitalia of the two *P. tisiphone* populations have not been compared, and the probability of finding distinct differences is minimal. Further research of the ♂♂ and ♀♀ genitalia is therefore recommended.

The mtDNA barcodes, from 4 specimens (RVcoll14U545, RVcoll14U546, RVcoll14U547 and RVcoll17E547) collected in Dibër county shows low divergence, with a maximum p-distance of 0,8% (Dapporto *et al.* 2022).

The only available genetic data come from Sanger sequencing (Takáts & Mølgaard 2016; Verovnik & Wiemers 2016; Dincă *et al.* 2021; Dapporto *et al.* 2022). For the genus *Pseudochazara*, it has been documented that mtDNA barcoding provides low resolution specifically in closely related taxa. It does not provide information on reproductive isolation.

Striking discrepancies between mitochondrial and nuclear variation patterns are commonly found within a single species (Després 2019l) and have also been documented across different Lepidoptera genera (Dincă *et al*. 2019; Hinojosa *et al*. 2019; Ebdon *et al.* 2020). Mitonuclear discordance in Lepidoptera has become a recognized phenomenon, playing a significant role in shaping evolutionary relationships and species delineation within the group. The use of both mitochondrial and nuclear markers across the genome is essential for accurately assessing genetic diversity, species boundaries, and evolutionary histories in Lepidoptera. This approach has the potential to better align the concepts of lineage species and biological species, while also reducing personal subjectivity in decision-making.

The Dibër population may represent a significant, distinct phylogeographic lineage, but comprehensive data from a larger genomic study (e.g. high-throughput sequencing) of Balkan *Pseudochazara* populations are necessary for definitive resolution. This would help to exclude potential mitonuclear discordance and provide valuable insights into gene flow between different populations within the genus, as well as between the closely related populations of *P. tisiphone*.

Due to their sensitivity to environmental changes, butterflies serve as key indicators of ecosystem health, and their conservation can often reflect the success (or failure) of more comprehensive environmental protection measures. Red Lists have undoubtedly drawn attention to the plight of endangered butterfly species, but their true impact on preventing declines and extinction depends on how well conservation measures are implemented, funded, and enforced.

P. tisiphone is listed as Least Concern (LC) in both the European Red List (2010) and the Mediterranean Red List (2016). Given its limited distribution and reported declines in Greece, this species may warrant re-evaluation in the future. In the Albanian Red List (2022) the Vulnerable (VU) status from two previous Red Lists (2006 and 2013) was confirmed.

Given concerns about extensive mining activities and the recent discovery of a substantial hydrogen gas reservoir around Bulqizë (Truche 2024) and in order to promote the conservation of this population, it was already suggested (Cuvelier 2023) that the population in Dibër county, with low divergence in the mtDNA barcode and potential allopatry (in the absence of sufficient research in potential, intermediate ophiolite areas) might represent an Evolutionary Significant Unit.

While there is growing awareness of the importance of preserving key phylogeographic lineages in conservation efforts, this awareness has yet to be effectively translated into practical actions in many countries. The National Agency of Protected Areas of Albania (AKZM) is currently evaluating the Kunming-Montreal Global Biodiversity Framework (url) for potential implementation.

Fortunately, it appears that the population discovered by Qirinxhi X. in the Lurë-Dejë National Park is receiving the necessary protection. However, in the Balkan Peninsula, increasing overgrazing and road construction that converts unpaved roads into large asphalt roads continu to be common practices in protected areas. This continues to pose a threat, and it is crucial to address these issues to ensure the long-term survival of local *P. tisiphone* populations. How will limited urgency among policymakers for nature conservation evolve, especially in the context of scarce resources and the potential opportunity for exploiting a substantial hydrogen reservoir?

Based on all available data and in the absence of a large genomic study, speciation appears unlikely. However, the population still warrants conservation efforts. In such cases, Evolutionary Significant Units (ESUs), clades or lineages often do not receive sufficient attention in checklists and red lists, which can hinder their conservation.

In accordance with Dufresnes *et al.* (2023) the Dibër population is described as ***Pseudochazara tisiphone dibra*** **ssp. nov.**, associating this population with the county where the population was first discovered in 2017. Since the name of the county Dibër ends in a consonant, the suffix -a is used, following the standard pattern for Latinizing geographic names.

Pseudochazara tisiphone dibra

Cuvelier & Marafi, 2025, ZooBank: https://zoobank.org/E8AC9881-7EDE-47A2-B1CF-067152352AAE

Type specimens and repository

Holotype: ♂, Albania, Dibër county, Bulqizë 1400-1500 m (41.44N 20.20E), 13.vii.2017, leg. and coll. Cuvelier S. (Fig 5-6), presently in first author's reference collection.
Allotype: ♀, same locality and date, leg. and coll. Cuvelier S, presently in first author's reference collection.
Paratypes: 1 ♂, 3 ♀♀, same locality and date, leg. and coll. Cuvelier S, presently in first author's reference collection.

Furthermore, 2 complete specimens (RVcoll17F522 and RVcoll17F554) and the legs of 3 specimens (RVcoll14U545 ; RVcoll14U546 and RVcoll14U547) are deposited in the collection of the Butterfly Diversity & Evolution Lab at the Institut de Biologia Evolutiva (CSIC-UPF): Barcelona, Spain.

Fig. 5. Holotype of *Pseudochazara tisiphone dibra* ♂, upperside, Bulqizë 1400-1500 m (41.44N 20.20E), 13.vii.2017, leg. and coll. Cuvelier S (© Sylvain Cuvelier). **Fig. 6.** Holotype of *Pseudochazara tisiphone dibra* ♂, underside, Bulqizë, 1400-1500 m (41.44N 20.20E), 13.vii.2017, leg. and coll. Cuvelier S. (© Sylvain Cuvelier)

Conclusion

Pseudochazara tisiphone displays typical morphological variation, as observed within the genus *Pseudochazara* and across different localities, reflecting the influence of environmental factors on phenotypic plasticity. While mtDNA barcoding reveals low divergence between the populations of Korçë and Dibër counties, nuclear data are needed for a definitive resolution on the taxonomic status and phylogeographic relationships of the Dibër population. Although speciation appears unlikely without further genomic study, the population may still represent a significant evolutionary lineage, warranting conservation attention. The ongoing threats, including overgrazing, road construction, and mining activities, underscore the need for effective conservation strategies to protect this butterfly, especially in light of its declining populations in Greece and its vulnerable status in Albania. Consequently, this population has been described as a new subspecies, *Pseudochazara tisiphone dibra* ssp. nov., with the hope that future research will provide clearer insights into its genetic and ecological significance.

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