SHILAP Revista de lepidopterología, 53 (212) 30 diciembre 2025: 825-834 eISSN: 2340-4078 ISSN: 0300-5267
https://doi.org/10.57065/shilap.1108 https://shilap.org
https://zoobank.org/7842D0A6-AC51-43DF-8733-43E55E351FF2

Confirmation of the subspecific status of *Polyommatus huberti arpaensis* Charmeux, 2018 and description of a new subspecies of *Polyommatus pseudactis* (Forster, 1960) from the Sevan region (Gegharkunik, Armenia) based on evidence from DNA barcodes (Lepidoptera: Lycaenidae)

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Abstract

Based on evidence from mitochondrial DNA barcodes, the subspecific rank of the taxon *Polyommatus huberti arpaensis* Charmeux, 2018 is confirmed. Even though the populations of this species from Vayots Dzor and Gegharkunik show small morphological differences, they are here considered as local variants of *P. huberti arpaensis* due to lack of differentiation in DNA barcodes. Morphological observations supported by DNA barcode data also revealed a new subspecies, described here as *P. pseudactis sevanensis* subsp. nov. from the Sevan Lake region (Armenia, Gegharkunik Province).
Keywords: Lepidoptera, Lycaenidae, Polyommatinae, Polyommatus, *P. pseudactis sevanensis*, DNA barcode, taxonomic, ranking, Palaearctic region.

Confirmación del estatus subespecífico de *Polyommatus huberti arpaensis* Charmeux, 2018 y descripción de una nueva subespecie de *Polyommatus pseudactis* (Forster, 1960) de la región de Sevan (Gegharkunik, Armenia) basada en pruebas de códigos de barras de ADN (Lepidoptera: Lycaenidae)

Resumen

A partir de las pruebas de los códigos de barras del ADN mitocondrial, se confirma el rango subespecífico del taxón *Polyommatus huberti arpaensis* Charmeux, 2018. Aunque las poblaciones de esta especie de Vayots Dzor y Gegharkunik muestran pequeñas diferencias morfológicas, aquí se consideran variantes locales de *P. huberti arpaensis* debido a la falta de diferenciación en los códigos de barras de ADN. Las observaciones morfológicas respaldadas por los datos del código de barras de ADN también revelaron una nueva subespecie, descrita aquí como *P. pseudactis sevanensis* subsp. nov. de la región del lago Sevan (Armenia, provincia de Gegharkunik).
Palabras clave: Lepidoptera, Lycaenidae, Polyommatinae, *Polyommatus*, *P. pseudactis sevanensis*, código de barras de ADN, taxonómico, clasificación, región Paleártica.

Introduction

With 120 species recognized so far, the members of subgenus *Polyommatus (Agrodiaetus)* are among the most diverse groups of butterflies in the palearctic region (Eckweiler & Bozano, 2016). Beginning in the 1950s, karyological studies led by Hubert de Lesse (1914-1972) and others revealed a wide range of hidden complexity within the members of this group that are often externally indistinguishable (de Lesse, 1960; Lukhtanov, 1989; Vershinina & Lukhtanov, 2017). More recently, DNA barcoding has been shown to be a useful tool for resolving the difficult taxonomic and phylogenetic questions in this group (Lukhtanov et al. 2023). Sources list 32 species for Turkey (Hesselbarth et al. 1995), 23 for the Caucasus and Transcaucasia region (Tshikolovets & Nekrutenko, 2012), and 18 species for Armenia (Aghababyan & Khanamirian, 2025).

The taxon *Polyommatus* (*Agrodiaetus*) *huberti* Carbonell, 1993 was described from Turkey, Ağrı Province. It was differentiated from the sympatric taxon *ninae* Forster, 1956 by various morphological characters such as pattern, antennae and genitalia (Carbonell, 1993). The taxon *Polyommatus arpaensis* Charmeux, 2018 from Vayots Dzor Province, Armenia was originally described as a subspecies of *P. huberti*. Unlike the nominotypical population from Ağrı, this population is mainly characterised by the accented markings on the underside of the wings (Charmeux, 2018).

The areas to the North of the Sevan Lake region were first visited in 2018 by Y. Diringer and later by J. F. Charmeux and X. Mérit in 2022. Many *Polyommatus* species were observed in this region, among them two populations of *P. huberti* and *P. pseudactis* (Figures 2-3) showing different patterns from their counterparts in other regions. Even though some sources have considered *pseudactis* only as a subspecies of *Polyommatus actis* (Herrich-Schäffer, 1851) (e.g. Eckweiler & Bozano, 2016), recent molecular studies list it as a separate species (e.g. Lukhtanov et al. 2023).

We aimed to find answers to these taxonomic uncertainties, and test for the presence of additional undetected taxonomic diversity in this region, by application of DNA barcoding to the material collected in those expeditions as well as samples from populations in neighbouring areas. More specifically, we tested whether sequence data from DNA barcodes can support the observed morphological differences among various populations of *P. pseudactis* and *P. huberti.*

Materials and Methods

Taxon sampling: Fieldwork was conducted in Armenia between 2018-2022. All collected samples are held at the private collections of JFC, YD and XM. Legs from 17 specimens representing 9 *P. huberti* and 8 *P. pseudactis* were sampled from across their range, of which 14 yielded usable barcode sequences (Table 1). In addition, 16 public barcode records from BOLD and GenBank of *Polyommatus* from previous studies were retrieved and added to our dataset. We also included sequences for several closely related species of *Polyommatus (Agrodiaetus)* (i.e. *P. sigberti, P. actis, P. artvinensis, P. ernesti, P. haigi, P. ninae, P. turcicolus,* and *P. elbursicus*) as putative outgroups (Table 1). The voucher data are publicly available through the BOLD dataset "DS-PSDACTIS", accessible at https://doi.org/10.5883/DS-PSDACTIS. We excluded one public sample identified as *A. pseudactis* (AY556845), as it likely represents an undescribed lineage of *P. firdussii*.

Molecular analysis: The extraction of total genomic DNA, amplification and sequencing were performed in the Centre for Biodiversity Genomics (Guelph, Ontario, Canada) using mini-primers (LepF/mLepR and mLepF/LepR) (Hajibabaei et al. 2006). Many samples produced full length barcodes (658 bp). Fourteen new barcode sequences were submitted to GenBank (Accessions PQ884470 - PQ884484), and all sequences are also publicly available in the BOLD dataset "DS-PSDACTIS" (dx. https://doi.org/10.5883/DS-PSDACTIS).

The combined dataset was assembled using MEGA 11.0.8 (Tamura et al. 2021). Alignment of sequences was carried out using MUSCLE modules implemented in AliView 1.28 (Larsson, 2014) and double-checked visually. The final dataset file was analyzed using the IQtree web server (https://iqtree.cibiv.univie.ac.at) (Trifinopoulos et al. 2016) with 1000 Ultrafast bootstrap replicates. The resulting tree was edited using FIGTREE 1.4.4 (Rambaut, 2018). Uncorrected *p*-distances were calculated using MEGA 11.0.8 (Tamura et al. 2021).

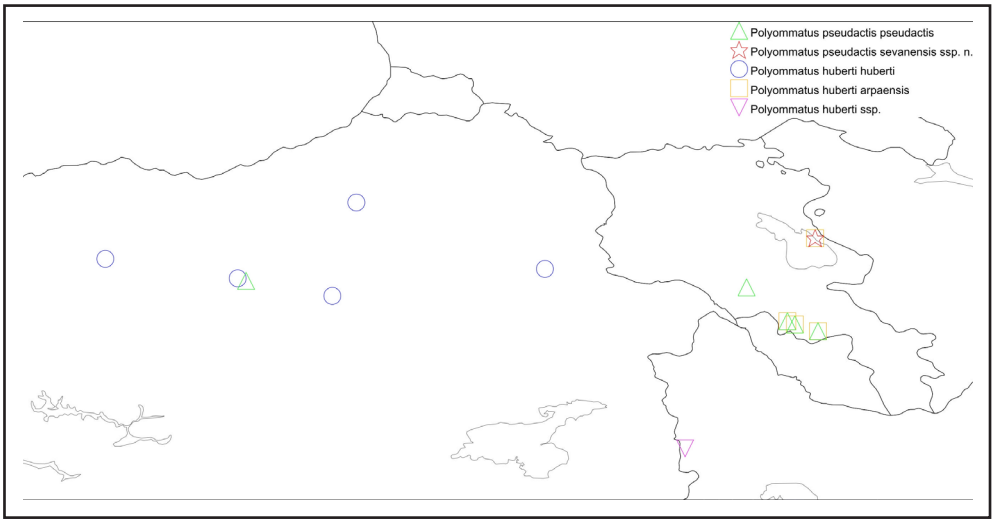
Table 1. Names, sample ID, GenBank accession numbers, locality, and geographic coordinates of the samples used for this study.

Taxon	SampleID	Accession	Locality	Lat.	Long.
Polyommatus sigberti	MW98284	AY557020	Turkey, Kayseri, Ala Daglar	37.916667	35.216667
Polyommatus actis	MW98162	AY556999	Turkey, Sivas, Gokpinar	38.708931	37.336226
Polyommatus artvinensis	MW99058	AY557038	Turkey, Artvin, Kilickaya	40.719749	41.475189
Polyommatus ernesti	MW98097	AY556989	Turkey, Isparta, Dedegol Gecidi	37.667222	31.302778
Polyommatus haigi	VL-01-L340	AY496750	Turkey, Van, Catak	38.027364	43.039766
Polyommatus pseudactis pseudactis	AD-00-P263	AY496777	Armenia, Aiodzor Mts, Gnishyk	39.656828	45.297763
Polyommatus pseudactis pseudactis	JFC23034	PQ884484	Armenia, Vayots Dzor, env. Martiros	39.597673	45.499464
Polyommatus pseudactis pseudactis	JFC23035	PQ884480	Armenia, Vayots Dzor, env. Martiros	39.597673	45.499464
Polyommatus pseudactis sevanensis ssp. nov.	JFC23036	PQ884476	Armenia, Gegharkunik, Sevan region, Tsapatagh	40.409004	45.474675
Polyommatus pseudactis sevanensis ssp. nov.	JFC23037	PQ884477	Armenia, Gegharkunik, Sevan region, Tsapatagh	40.409004	45.474675
Polyommatus pseudactis?	JFC23038	-	Turkey, Erzurum, Kopdagi Gecidi	40.036389	40.513611
Polyommatus pseudactis	JFC24026	PQ884479	Armenia, Vayots Dzor, Noravank	39.684769	45.232724
Polyommatus pseudactis	JFC24027	PQ884473	Armenia, Vayots Dzor, Gnishyk	39.656828	45.297763
Polyommatus pseudactis	JFC24028	-	Armenia, Ararat, Ararat, Vedi river valley	39.980391	44.876892
Polyommatus pseudactis	JFC24032	-	Turkey, Erzurum, Kopdagi Gecidi	40.036389	40.513611
Polyommatus ninae	MW99508	AY557113	Turkey, Agri	39.719074	43.050591
Polyommatus turcicolus	MW99479	AY557110	Turkey, Van, Erek Dagi	38.468611	43.518056
Polyommatus elbursicus	AD02W269	AY953986	Iran, Mazandaran, Veresk	35.907136	52.982781
Polyommatus huberti huberti	VL-01-L123	AY496753	Turkey, Gumushane, Dilekyolu	40.2285	39.286671
Polyommatus huberti huberti	VL-01-L315	AY496754	Turkey, Erzurum	39.905499	41.265824
Polyommatus huberti huberti	MW99053	AY557036	Turkey, Artvin, Kilickaya	40.719749	41.475189
Polyommatus huberti huberti	MW99095	AY557046	Turkey, Kars, Kagizman	40.140648	43.119118
Polyommatus huberti huberti	MW99552	AY557123	Turkey, Bayburt, Kop Gecidi	40.058889	40.440556
Polyommatus huberti arpaensis	JFC23010	PQ884470	Armenia, Gegharkunik, Sevan region, Tsapatagh	40.409004	45.474675
Polyommatus huberti arpaensis	JFC23011	PQ884478	Armenia, Gegharkunik, Sevan region, Tsapatagh	40.409004	45.474675
Polyommatus huberti arpaensis	JFC23012	PQ884483	Armenia, Gegharkunik, Sevan region, Tsapatagh	40.409004	45.474675
Polyommatus huberti arpaensis	JFC23006	PQ884474	Armenia, Vayots Dzor, env. Martiros	39.597673	45.499464
Polyommatus huberti arpaensis	JFC23009	PQ884471	Armenia, Vayots Dzor, Noravank	39.684769	45.232724
Polyommatus huberti arpaensis	AD-00-P260	AY496752	Armenia, Aiodzor Mts, Gnishyk	39.656828	45.297763
Polyommatus huberti arpaensis	AD98024	AY556848	Armenia, Gnyshik	39.656828	45.297763
Polyommatus huberti arpaensis	JFC23007	PQ884475	Armenia, Vayots Dzor, env. Martiros	39.597673	45.499464
Polyommatus huberti arpaensis	JFC23008	PQ884482	Armenia, Vayots Dzor, Noravank	39.684769	45.232724
Polyommatus huberti	ARLY-146b-002	PQ884481	Iran, West Azerbaijan, Khoy, Mohamad Agha Mt	38.577	44.341

Results

The distribution of our barcodes samples are shown in Figure 1. We found an average of 0.6% (0.58±0.22%) difference between populations of *P. huberti* from Armenia (ssp. *arpaensis*, Gnyshik, Vayots Dzor, Gegharkunik, n=9) and those from Turkey (ssp. *huberti*, Bayburt, Erzurum, Gumushane, Kars, n=5). The two subspecies also show clear morphological differences (Figure 2-3), with individuals form ssp. *huberti* often having smaller spots on the underside, obsolete submarginal patterns on the underside of the forewings (brownish lunules in S2 and S3 in ssp. *arpaensis*), and weak submarginal chevrons on the underside of the hindwings. In our phylogenetic reconstruction, these two populations formed two independent clades that always stayed together with moderate support (bootstrap=75) confirming their sister-group relationship at species-level (Figure 4). We noted that individuals of the *P. huberti* population in Sevan were even smaller in size and often showed a stronger reduction of markings on the underside of the hindwings; however, they showed no differentiation in DNA barcodes from other Armenian populations.

Figure 1. Distribution of specimens examined in this study.



We also found an average of 0.3% (0.29±0.03%) distance between populations of *P. pseudactis* from Gegharkunik (n=2) and those from the type locality ("Armenia, mts. Daralagez, pag. Martiros" = Vayots Dzor; Forster, 1960) (n=5). Morphologically they showed a more pointed tip of the forewings, ground-colour of the upperside brighter blue-violet, and all markings strongly reduced on the underside of hindwings. Despite the limited number of barcoded samples in our study, which was largely due to the limited number of available specimens, we consider a lack of genetic differentiation within each of these two groups, and presence of a consistent and notable genetic gap between the two, in addition to clear morphological differences between them, as sufficient evidence to describe the population of *P. pseudactis* from Gegharkunik as a new subspecies.

***Polyommatus pseudactis sevanensis* Charmeux, Diringer, Mérit & Nazari, subsp. nov.**
(Figures 2 D-E, 3B)
https://zoobank.org/A7230E8D-2BD8-4AAE-8148-07CCBEB356AA

Holotype ♂. SampleID JFC23036, GenBank accession PQ884476. Armenia, Gegharkunik, N Sevan Lake, Tsapatagh, 2000 m, 01-VII-2022, J.-F. Charmeux leg., deposited in Muséum National d'Histoire Naturelle (MNHN), Paris, France.

Paratypes: Same data as holotype, 1 ♂, SampleID JFC23037 (MNHN); same locality, 2000 m, 3 ♂,

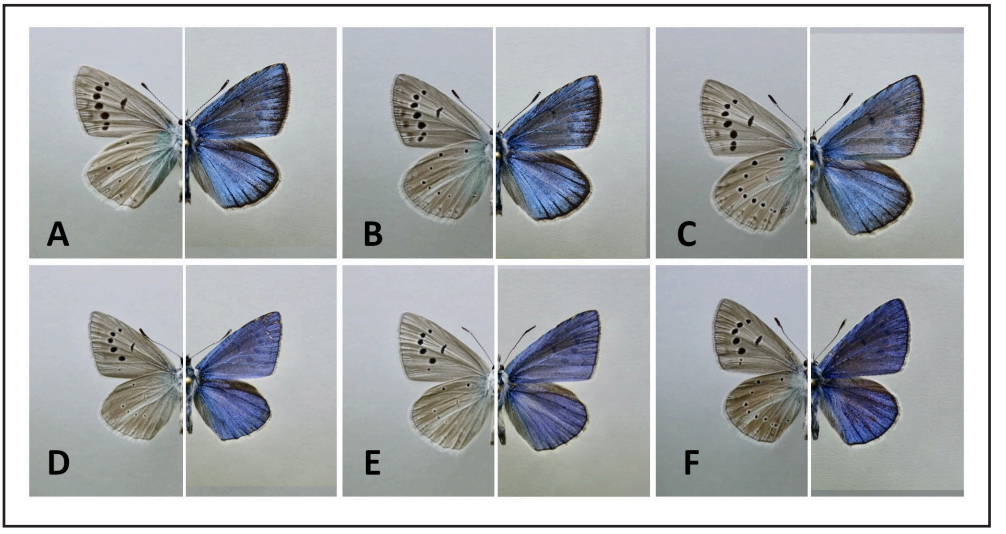
10-11-VII-2018, Y. Diringer leg.; same data as holotype, 1 ♂, J.-F. Charmeux leg. (in coll. Y. Diringer); same locality, 1 ♂, 11-VII-2022, X. Mérit leg. (in coll Mérit); same data as holotype, 1 ♂, J.-F. Charmeux leg. (in coll. X. Mérit).

Description male (Figures 2, D-E): Forewing length in Holotype 15.2 mm, in Paratypes 14.4-17 mm (n=7). Wingspan between apex in Holotype 26.5 mm, in Paratypes 24-27 mm. On the forewings, outer edge practically straight, pointed tip. Upperside. Ground-color luminous violet-blue, veins very slightly lined distally with black. Underside. Ground-color light grey, very thin marginal line. Hindwings with a conspicuous white band along M3, series of white triangular markings, weak greenish blue basal suffusion and all markings strongly reduced: the postdiscal black dots are very small and incomplete, more often obsolete.

Female: unknown

Diagnosis: Compared to the nominotypical subspecies (see Figure 2F), the new subspecies is on average slightly smaller in size (25.5 vs. 26 mm) with a straighter outer edge of the forewings (more rounded in *pseudactis*); the ground-color on the upperside of the wings is luminous violet-blue (dark purple in ssp. *pseudactis*) and on the underside it is lighter gray (dark grey in *pseudactis*); postdiscal black markings small and incomplete (larger and present in complete series in *pseudactis*) and marginal line is very thin (nearly double the width in *pseudactis*).

Figure 2. Adults of *Polyommatus*. Left: Underside, Right: Upperside of the wings. **A-C** *P. huberti arpaensis* **A.** Armenia, Gegharkunik, N Sevan Lake, Tsapatagh, 2000 m, 18-VII-2018, J.-F. Charmeux leg. & coll. **B.** Armenia, Gegharkunik, N Sevan Lake, Tsapatagh, 2000 m, 01-VII-2022, J.-F. Charmeux leg. & coll. **C.** Armenia, Vayots Dzor, Noravank, 1550 m, 23-VI-2015, J.-F. Charmeux leg. & coll. **D-E** *P. pseudactis sevanensis* **subsp. nov. D.** Paratype, Armenia, Gegharkunik, N Sevan Lake, Tsapatagh, 2000 m, 01-VII-2022, J.-F. Charmeux leg., MNHN. **E.** Holotype, Armenia, Gegharkunik, N Sevan Lake, Tsapatagh, 2000 m, 01-VII-2022, J.-F. Charmeux leg. & coll. **F.** *P. pseudactis pseudactis,* Armenia, Vayots Dzor, env. Martiros, 2000 m, 15-VI-2019, J.-F. Charmeux leg. & coll. (Photos: JFC).



Etymology: Toponymic, from the Sevan region in Central-Eastern Armenia.

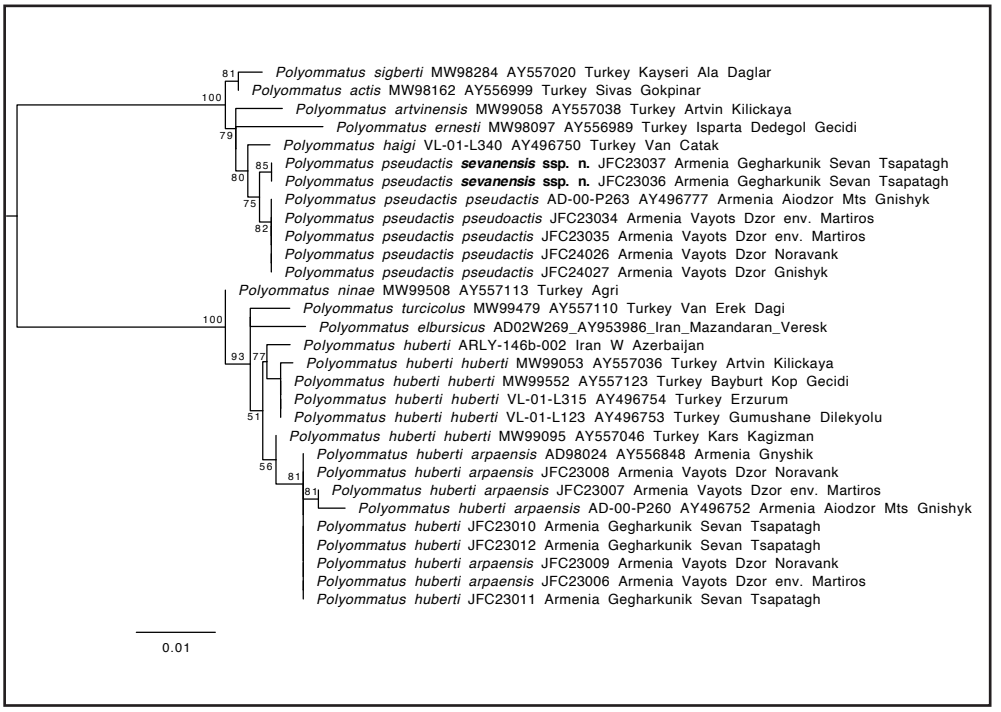
Distribution: Valleys to the north of Sevan Lake area. The new subspecies can be observed flying together with numerous other blue and brown Polyommatinae (e.g. *P. huberti arpaensis* Charmeux, 2018; *P. damon kotshubeji* Sovinsky, 1915; *P. ninae* Forster, 1956; *P. vanensis sheljuzhkoi* Forster, 1960; *P. emmeli* Lukhtanov & Dantchenko, 2024; and *P. demavendi antonius* Lukhtanov & Dantchenko, 2024. Other species that occur in the area include *P. amandus* (Schneider, [1792]); *P. daphnis dugijani* Schurian & Häuser, 1981; *Plebejus idas altarmena* (Forster, 1936); *Plebejus argus aegidion* (Meisner, 1818); *Pyrgus sidae* (Esper, [1784]); *Pyrgus cinarae* (Rambur, [1839]), and *Pieris napi meridionalis* Heyne, 1895.

Figure 3. Adults specimens and habitat. **A.** *P. huberti arpaensis***,** Vayots Dzor, Noravank, 06-VII-2014. **B.** *P. pseudactis sevanensis* subsp. nov. Gegharkunik, Tsapatagh, 06-VII-2024. **C.** *P. pseudactis pseudactis*, Vayots Dzor, Sers, 26-VI-2024. **D.** habitat of *P. pseudactis sevanensis* subsp. nov. and *P. huberti* in the Sevan region. **E.** habitat of *P. pseudactis pseudactis* and *P. huberti arpaensis* in the Vayots Dzor region. **F.** Lycaenidae mud puddling at Gegharkunik, Tsapatagh, 18-07-2018. In the center of the picture a specimen of *P. huberti arpaensis* can be seen, next to *Plebejus idas* and *Polyommatus emmeli* (Photos: JFC).

Ecology: The habitat of *P. pseudactis sevanensis* consists of narrow river valleys on the southern face of the Sevan Mountains, at an altitude between 2,000 and 2,450 m. These valleys extend over a mountainous area that spreads out over a pocket of Mesozoic and Cenozoic sedimentary rocks characterized by outcrops of ophiolitic sequences composed of basaltic rocks and limestone zones. Wedged between the Lesser Caucasus and Lake Sevan, this area is subject to the micro-climate of Lake Sevan, whose temperatures are cooler than those of the surrounding regions. The north-facing slopes of these valleys are covered by relict oak forests (*Quercus* spp.) and century-old junipers (*Juniperus polycarpos* K. Koch. and *J. oblonga* M. Bieb.), while the bottoms of the valleys host retracted riparian vegetation and small semi-humid meadows. The dry slopes and summits are dominated by typical high-altitude Mediterranean plants such as *Onobrychis cornuta* (L.) Desv., *Acantholimon* spp., *Androsace villosa* L., *Alyssum tortuosum* Waldst. & Kit. ex Willd., *Astragalus cornutus* Pall., *Sedum caucasicum, sempervirum* ssp. and *Spiraea* ssp., and endemism's such as *Astragalus schuschaensis* Grossh., and *Acantholimon gabrieljaniae* Mizoeva.

We noted in these meadows the presence of several species of Leguminosae (*Onobrychis, Medicago*, *Astragalus*) which are hostplants for numerous blue Lycaenidae elsewhere in Armenia (e.g. *Onobrychis transcaucasica* Grossh., *O. altissima* Grossh. and *O. hajastana* Gross., hosts of *Polyommatus turcicus, P. damon* and *P. iphigenia*). It is highly likely that *P. pseudactis sevanensis* larvae use *Onobrychis cornuta* as their host plant, as in the case of the nominotypical populations of the Vayots Dzor Province mountains where we were able to observe oviposition in situ on this plant. However, we were unable to identify the host plant of *P. huberti*. Similarly, the data available to us are still too fragmentary to provide a clear picture of the phenology of the new subspecies. However, it appears that the adults emerge in July, somewhat later than the nominotypical populations in Vayots Dzor.

Figure 4. Maximum Likelihood tree of *Polyommatus pseudactis* and *P. huberti* COI barcodes inferred using IQTree. Other *Polyommatus* species are included as outgroups. Values on the nodes represent bootstrap support for 1000 replicates for supported nodes (Scale bar = 1%).



Discussion

The advent of DNA barcoding in the past two decades has provided the researchers an additional tool that has allowed the discovery of previously undetected diversity in many groups of Lycaenidae, particularly in *Polyommatus* (e.g. Lukhtanov et al. 2020, 2021, 2023; Naderi et al. 2024, Karbalaye & Lukhtanov, 2024). Our findings demonstrate that even in relatively well-studied regions such as Armenia, cryptic diversity may persist in Lepidoptera that need further investigation. Compilation of comprehensive regional barcode libraries can further elucidate taxonomic diversity in those regions and help clarify the true extent of variation and diversity in taxonomically difficult groups such as *Polyommatus* species.

Compared to the nominotypical *P. huberti*, the Armenian population from Vayots Dzor (ssp. *arpaensis*) has larger spots on the underside of the wings, well-developed submarginal patterns on the forewings with brownish lunules in spaces 2 and 3, as well as well-developed submarginal chevrons on the hindwings. The *P. huberti* population from Sevan differs from the nominotypical *P. huberti* as well as *P. huberti arpaensis* by its smaller size and the obsolescence of the postdiscal markings underside of hindwings. Initially we thought that this population potentially represented a separate subspecies, however since they showed no differentiation in their DNA barcodes from other Armenian populations, for now we consider them a local variant of *P. huberti arpaensis* until further evidence proves otherwise. Noteworthy are also specimens of *P. huberti* from Kars (AY557046) and NW Iran (PQ884481), both of which are notably divergent in their DNA barcodes from other populations and may signal additional undetected diversity.

The low but consistent sequence divergence between the populations of *P. pseudactis* from Gegharkunik and those in Vayots Dzor (0.3%) falls well within the spectrum of intra-specific variation in *Polyommatus*. Considering the morphological differentiation between them, we determined that the Gegharkunik population merited a new subspecific name, here described as ssp. *P. pseudactis sevanensis* ssp. nov**.** The molecular characteristics of the Iranian populations of *P. pseudactis* distributed in Western Iran and south to Esfahan (Rajaei et al. 2023) remain unstudied, and indeed they may represent yet another undescribed subspecies. A focused phylogeographic study on the whole complex is needed to further delineate species and subspecies in this complex.

Acknowledgments

We would like to thank Alireza Naderi (Tehran, Iran) for providing us with a sequence of *P. huberti* from NW Iran, and the anonymous SHILAP reviewers who helped improve the earlier versions of this manuscript.

Conflict of interest

The authors declare that there is no known financial interest or personal relationships that could have influenced the work presented in this article.

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(Recibido para publicación / *Received for publication* 24-X-2024)
(Revisado y aceptado / *Revised and accepted* 10-IV-2025)
(Publicado / *Published* 30-XII-2025)