

Observations on scale structures and spectroscopic properties of Polyommatus lycaenid butterflies (Lepidoptera: Lycaenidae)

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Abstract – The morphology of scale micro- and nanostructures were investigated in three species of the monophyletic *Polyommatus eros* species group and in two closely related outgroup species *P. ariana* (MOORE, 1865) and *P. icarus* (DENIS et SCHIFFERMÜLLER, 1775) by Scanning Electron Microscopy. The optical properties of the blue wing dorsa were studied by reflection spectroscopy in the ultraviolet-visible range. We found ingroup correlations between nanomorphology of the scales and the spectral position of the reflectivity maxima. The closely related species of the *P. eros* group showed qualitatively similar spectra but quantitatively they revealed characteristic features. The outgroup species *P. ariana* and *P. icarus* underlined these observations as they possessed quantitatively distinct scale nanomorphology and their reflectivity maxima were clearly different as compared with those of the *P. eros* group. We discuss that the optical values of the structural colouration of the species we examined play an important role in pre-mate signaling, which creates a successive barrier between taxa otherwise similar in wing pattern and genital structures. We conclude that reflectance measurements may turn into a cheap and handy tool to probe relationships within a given monophyly, in which taxa are otherwise uniform when traditional taxonomic characters are studied. With 15 figures.

Key-words – Discolouration, microstructures, nanostructures, *Polyommatus*, reflectance, scales, signaling, spectrum.

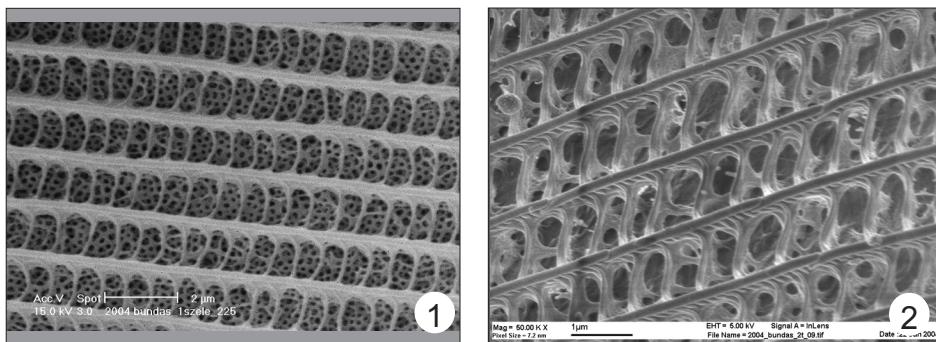
INTRODUCTION

Year by year we learn that more and more insects, aquatic organisms, birds (PARKER 2005) and more recently even plants (VIGNERON *et al.* 2005) rely on so-called photonic crystal type materials to achieve certain biological functions. Photonic crystals, or photonic band gaps (PBG), are materials in

which the optical properties (refractive indices) change in a systematic way according to a three dimensional (3D), regular, or quasi-regular pattern.

One of the most important functions for which 3D PBG materials are used in the natural world is signaling (PARKER 1998). Day flying Lepidoptera use PBG materials most frequently for colour generation: whilst monitoring or perching in their habitats male individuals of dorsally blue polyommatine lycaenid butterflies (Lepidoptera: Lycaenidae: Polyommatusini), in common English name “the Blues”, widely use their vivid blue wing colours generated by PBG materials built in the body of their wing scales for visual communication (LUNDGREN 1977).

The Blues evolved special PBG materials in their dorsal wing scales, called *Urania*-type with “pepper-pot” structures (ELIOT 1973) (Figs 1–2). If these structures are missing from the body of the scales, the wing dura of the individuals are getting to be discoloured exposing the chemical colour (BIRÓ *et al.* 2003), which is brown due to melanin (BÁLINT & JOHNSON 1997) or carotene (BÁLINT, VÉRTESY & BIRÓ 2005). This phenomenon was termed as discolouration (BÁLINT & JOHNSON 1997).



Figs 1–2. Scanning Electron Microscopy images showing *Polyommatus* genus group lycaenid scale micro- and nanostructures: 1 = blue colour generating cover scale of *Polyommatus poseidon* (HERRICH-SCHÄFFER, 1851) with pepper-pot structure, 2 = discoloured cover scale of *Polyommatus admetus* (ESPER, 1785) without pepper-pot structure

In the case of brown dorsal polyommatine wings the melanin granulates are directly facing sunlight in the body of the scale and are able to absorb more energy than the blue coloured species (BIRÓ *et al.* 2003). Discolouration results different thermal regulation in brown and in blue individuals. This change

causes a basic effect in the ecology of polyommatine lycaenid butterflies: generally patrolling and perching blue males in discoloured state becomes sedentary and territorial, and their population density turns to be higher with one magnitude (BÁLINT, unpublished data collected *in situ* in the case of *Polyommatus admetus* (ESPER, 1789) and *P. thersites* (CANTENER, 1834)). Consequently we regard scale micro- and nanostructures to be important factors in how the species and its lineage (species group or genus) fits in time and space, prior to any phenomena including sexual interaction (BÁLINT, VÉRTESY & BIRÓ 2005, BÁLINT, KERTÉSZ, VÉRTESY & BIRÓ 2005 and LUKHTANOV *et al.* 2005).

PBG 3D “pepper-pot” lattices are complex structures. Their development should have been a “cost”-intensive task. Published results, which are in accordance with our observations based on more than 70 investigated lycaenid species from all the subfamilies, suggest that micro- and nanostructures are rather constant in closely related species or genera (TILLEY & ELIOT 2002). Therefore we hypothesise that: (1) allopatric species of a given monophyletic group (with immediate common hypothetical ancestor) possess qualitatively identical optical properties, but they may differ quantitatively as they have a relatively close or immediate common ancestor. Therefore they generate similar colour but always with different hue; (2) lineages branched off but still in the same monophyly (species groups or genera) possess qualitatively different optical characteristics, as they do not share an immediate common ancestor in their lineage.

The present paper is addressed to test these two hypotheses by describing and analysing the PBG scale micro- and nanostructures of the target taxa using Scanning Electron Microscopy (SEM) and by measuring the reflectance of the PBG generated colours using experimental spectroscopic techniques.

MATERIALS AND METHODS

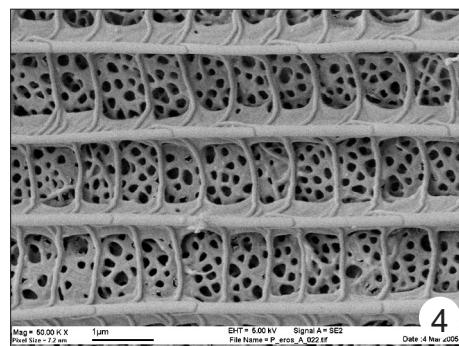
Our target organisms were certain endemic taxa of the *P. eros* species group (*sensu* BÁLINT & JOHNSON, 1997) of the Old World genus *Polyommatus* LATREILLE, 1804 (type species: *Papilio icarus* DENIS et SCHIFFERMÜLLER, 1775). We investigated three representatives of the group, all isolated from each other. They inhabit tundral or oreal steppes (1) in the Alps: *P. eros* (OCHSENHEIMER, 1808) (Figs 3–4), (2) in the Balkans: *P. eroides* (FRIVALDSZKY, 1835) (Figs 5–6) and (3) in the Altai regions: *P. erotides* (STAUDINGER, 1894) (Figs 7–8). As an outgroup, for testing the second hypothesis, we selected two congeneric taxa,

but belonging to different species groups of the same genus (1) *P. ariana* (MOORE, 1865) (*stoliczkanus* group) (Figs 9–10), which lives in high altitude habitats in the Western Himalayas and (2) the Common Blue, *P. icarus* (*icarus* group), which has a Transpalaearctic distribution (Figs 11–12).

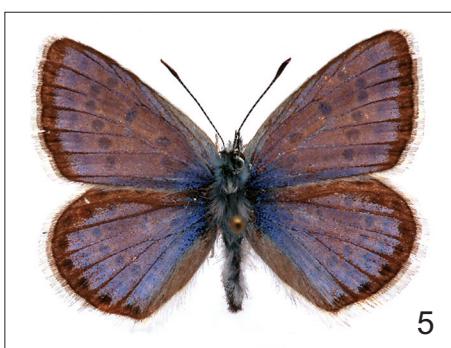
For studies by microscopy and for physical measurements we used museum material. The specimens examined are deposited in the Lepidoptera collection of the Hungarian Natural History Museum. For scale nanostructure investigations half of the forewing was cut as dry sample, fixed with double-sided carbon tape and gold coated for scanning electron microscopy. High-resolution observations were carried out using a LEO FESEM scanning electron microscope.



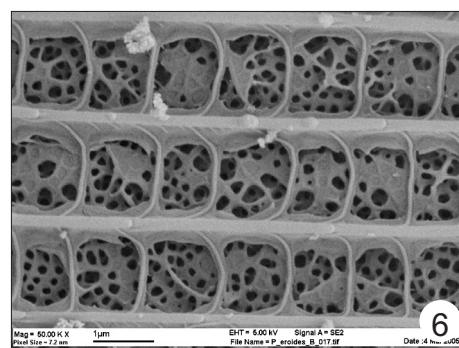
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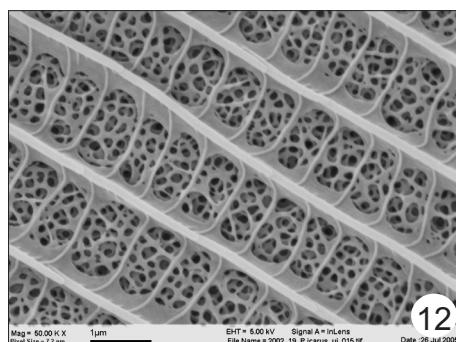
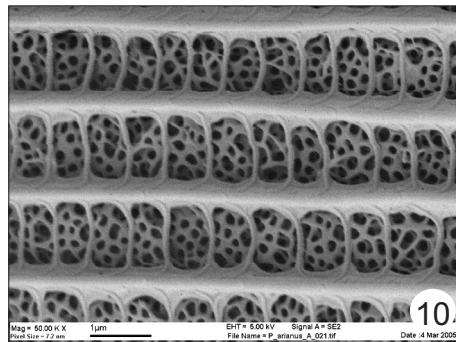
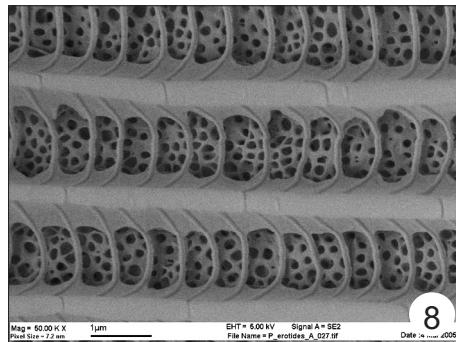
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Figs 3–6. Male individuals of *Polyommatus* taxa investigated by spectroscopy, showing dorsal surfaces scanning electron micrographs showing structural blue colour generating cover scales with pepper-pot structures: 3 = *P. eros* (OCHSENHEIMER, 1808) individual, 4 = *P. eros* scale,

5 = *P. eroides* (FRIVALDSZKY, 1835) individual, 6 = *P. eroides* scale



Figs 7–12. Male individuals of *Polyommatus* taxa investigated by spectroscopy, showing dorsal surfaces scanning electron micrographs showing structural blue colour generating cover scales with pepper-pot structures: 7 = *P. erotides* (STAUDINGER, 1892) individual, 8 = *P. erotides* scale, 9 = *P. ariana* (MOORE, 1865) individual, 10 = *P. ariana* scale, 11 = *P. icarus* (ROTTEMBURG, 1775) individual, 12 = *P. icarus* scale

Sizes and ratios of scale structures were measured and calculated with relevant commands of the program BioPhot Analyzer developed jointly by our home institutes in the frame of the project BioPhot (see Acknowledgements). Length and distance of the longitudinal ribs (LR) and the cross ribs (CR) were measured, and the filling factors (FF) of the pepper-pot structures can be seen in the windows (microcells) of the scales have been calculated. The 3D lattices (pepper-pot structures *sensu* ELIOT) are divided into irregular and distinct domains of complex chitinous material having different rates between closed (chitinised) and open (empty) spaces, this ratio is called the FF. The FF and the complex 3D arrangement of the filled and empty volumes determine the hue of the blue colour generated by the wing dorsal surfaces.

Male individuals of each species have been studied and measured; to determine the variability of spectral characteristics we selected five individuals of each species. The data of the specimens are listed in the Appendix I. The reflectance spectra were always measured under normal incidence. According to our observations, this was reliable as the individuals revealed the same spectral characteristics. When deviations from the reflectance typical for the taxon were observed, this could be associated with a damaged wing. Micro- and nano-structures can be damaged by the relaxing procedure before the specimen is placed on the setting board in the museum, but usually such alteration can be identified by examination with the naked eye. Normal incidence reflectance spectra of the butterfly wings were recorded using an Avantes 2048 fiber optic spectrometer in the ultraviolet-visible-near-infra-red (UV-VIS-NIR) range. A diffuse white standard (Avantes) was used to calibrate the spectrometer over the examined spectral range.

RESULTS

Micro- and nanomorphology – The wing scales of all the species we investigated show qualitatively the same micro- and nanostructures discussed as *Urania*-type with pepper-pot structures of ELIOT (1973) and TILLEY & ELIOT (2002). These 3D structures were classified as Type III Scale by VUKUSIC *et al.* (2000) stressing that these type of structures display physical colouration that does not arise from multi-layering but from scattering within and on the body of the scale. The measurements we carried out on the LR and the CR reveal that all the taxa investigated are in the same nano domain but differing in quantitative terms (PARKER 2006). The 3D lattices reveal quantitative and qualitative differences (Table 1).

Spectroscopy – The reflectance spectra (Fig. 13) show that all the investigated species have two reflectance peaks: (1) the main peak is in the visible range between 400 and 500 nm in accordance to the displayed blue colours and their hue, whilst (2) the secondary peak is below 300 nm that suggests all the species possess a pattern in ultraviolet.

Table 1. Measurements in nm and filling factors taken on the scales of five *Polyommatus* species investigated. FF = filling factor of the microcells, LR = longitudinal ridges, CR = cross ribs, d = distance, w = width

Taxon name	FF	LR	CR
<i>Eros</i> species group			
<i>Polyommatus erooides</i>	5.77	w = 183.4; d = 1599.5	W = 174.6; d = 1080.9
<i>Polyommatus eros</i>	4.83	w = 181.9; d = 1401.6	W = 85.8; d = 688.3
<i>Polyommatus erotides</i>	2.53	w = 349.2; d = 1760.0	W = 95.5; d = 560.3
Outgroup taxa			
<i>Polyommatus ariana</i>	2.56	w = 196.3; d = 1404.4	W = 92.3; d = 616.5
<i>Polyommatus icarus</i>	1.96	w = 151.2; d = 1678.5	W = 69.6; d = 673.7

Species belonging to the *P. eros* species group display similar spectral characteristics: their reflectivity is low between 10 and 30% in the visible with the main peak around 450 nm. The secondary peaks can be observed at 250 nm. The spectral properties of the species pair *P. erooides* and *P. eros* are almost identical, *P. erotides* displays a characteristic plateau between 500 and 600 nm with relatively high (30%) reflectivity compared to its sisters.

The outgroup species *P. ariana* and *P. icarus* display strikingly different spectral characteristics both in qualitative and quantitative terms. They show strikingly higher main peaks of reflectance maxima (over 60%) between 400 and 450 nm. The secondary peaks they display are also higher in terms of reflectivity and shifted to 260 nm (Table 2).

DISCUSSION

Measurements, spectral distinctness and similarities – According to the results we presented the monophyly of *eros-erooides-erotides* reveals similar micro- and nanostructures plus spectral properties, but quantitative differences can

be expressed well by measurements. Their low level of distinctness is not a surprise as the taxa are presumably quite young, originating from glacial isolations (*eros*: Alpine, *eroides*: Balkan and *erotides*: Altai) of a hypothetical ancestor widely distributed on cold steppe regions of the Old World. The rather similar reflectance spectra of *P. ariana* and *P. icarus* can indicate that they have a relatively close common ancestor, and probably it would be correct to place them in the same species group, which is distinct from the *eros* species group. This scenario can be easily tested not only by modern molecular studies but also via studying certain closely related taxa formerly placed in the *ariana* and *icarus* species groups.

Importance of light signaling – The importance of light manipulation as pre-mating signal of the polyommatus lycaenids is stressed by the observation of the senior author with more than 30 years of field experience: He carried out field works in habitats where often more than five *Polyommatus* species with qualitatively similar genital structures were in flight syntopically and synchronically (BÁLINT, *in prep.*, see Appendix II), but never found any interspecific copulation in the field.

Table 2. Spectral characteristics of the species investigated. All spectra are composed from five individuals (see Appendix I)

Taxon name	Spectral position of the reflectance maximum: reflectance (%)/wavelength (nm)	Spectral position of the secondary reflectance peak: reflectance (%)/wavelength (nm)
<i>Eros</i> species group		
<i>Polyommatus eroides</i>	22/445	12/250
<i>Polyommatus eros</i>	24/445	11/250
<i>Polyommatus erotides</i>	28/500	10/250
Outgroup taxa		
<i>Polyommatus arinaus</i>	62/450	20/255
<i>Polyommatus icarus</i>	70/400	22/255

Hence butterfly scale micro- and nanostructures should play an important role as they create pre-mating barrier on individual as well as on population level in the life of butterflies. This barrier plays an important role in sexual communication more promptly than any other contact based on physical or quasi-physical approaches of the individuals. Signaling is not only important to male-male interactions, but also in male-female communication.

Presumable females first identify their possible mate from larger distances, via light signals, and males do the same *vice versa*. Brown females have conspicuously different spectral properties from those of the males (Fig. 14), resembling discoloured polyommataine lycaenid males (cf. BIRÓ *et al.* 2003).

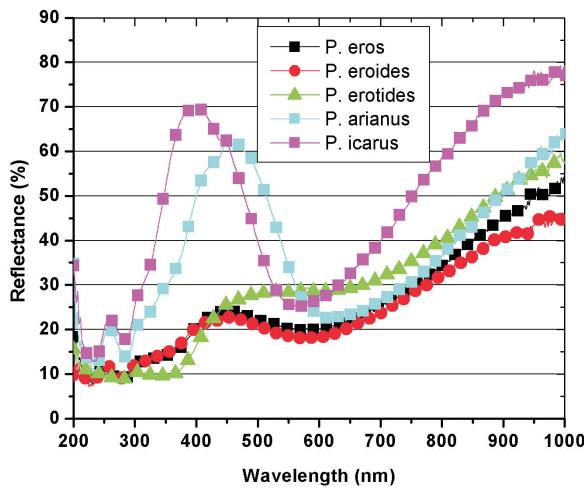


Fig. 13. Reflectance spectra of five *Polyommatus* species measured on dorsal forewing surfaces in the area of the discal cell apex, composed from five male individuals of each species

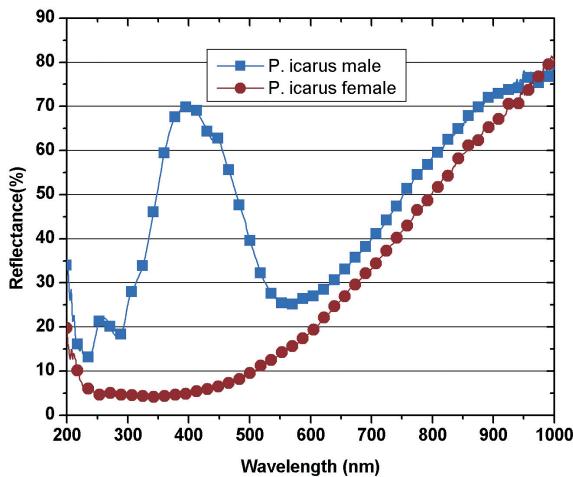


Fig. 14. Reflectance spectra of *Polyommatus icarus* (ROTTEMBURG, 1775) male and brown female phenotypes measured in the area of the discal cell apex, composed from five male individuals; the female spectrum is based on a single specimen

Then during courtship, when sexes are physically getting closer the male scent activates the female for copulation by pheromones, which are in the special androconial scales covered by the piliform scales (Fig. 15). Therefore most probably the function of the piliform scales, the male “fur” is to keep the scent in the male wing as long as possible (BÁLINT *et al.*, *in prep.*). However the scents can work only when light signal allows conspecific individuals of proper sexes to communicate.

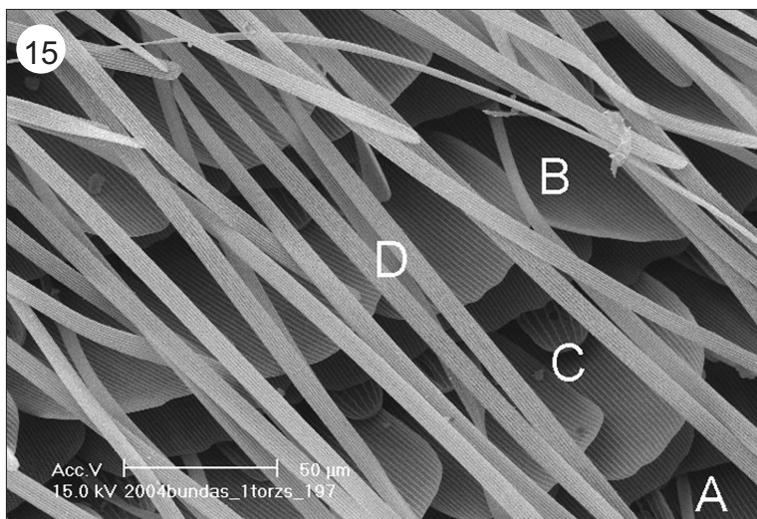


Fig. 15. Scanning electron micrograph of *Polyommatus coridon* (PODA, 1761) showing ground-scales (A) and cover-scales (B), plus specialized androconial (C) and piliform (D) scales

Methodology – Via our calculations, measurements and observations it seems that PBG indeed can characterise and identify small natural groups (= clusters of species with an immediate common ancestor), and in quantitative terms they can be used for species discrimination. Therefore both of our hypotheses were supported by measurable objective data. The spectral characteristics of the targeted taxa reflect the actual position of the species in the phyletic lineage, whether they have a common origin or they are distantly related. Thus spectral data may prove a very simple and handy tool in characterising butterfly

taxa with exceedingly similar structures but having different wing colouration. Hitherto developed and widely used karyologic or DNA studies are costly in every terms including life, money and time and often resulting contradictory results (cf. KANDUL *et al.* 2004, CARBONELL *et al.* 2004, LUKHTANOV *et al.* 2006). Our methods can be applied practically everywhere where spectroscopic instruments and scientifically curated collections are available. A further advantage is that if limited to the measurements of the reflectance spectra, the method is non-destructive, so even unique specimens of very rare species can be examined without having to sacrifice them.

APPENDIX I: MATERIAL EXAMINED FOR SPECTROSCOPIC INVESTIGATIONS

Polyommatus erotides (STAUDINGER, 1892), Nos 30–35 (males) – No. 30: Mongolia, Central aimak, Ulaan chodag 16 km S von Somon Öndörschireet, 1500 m, Exp. Dr. Z. KASZAB, 1966, Nr. 738, 24. VII. 1966.; No. 31: Mongolia, Central aimak, Zuun Chara, Duusch ul, 1100 m, Exp. Dr. Z. KASZAB, 1964, Nr. 283, 8. VIII. 1964.; No. 32: Mongolia, Central aimak, 30 km NW of Ulaan Baator, 1987. VII. 16, leg. L. PEREGOVITS, M. HREBLAY, T. STÉGER; Nos. 33–35: Mongolia, Central aimak, Tosgoni ovoо, 5–10 km N von Ulaan-Baator, 1500–1700 m, Exp. Dr. Z. KASZAB, 1967, Nr. 925, 19–20., 23–24. VIII. 1967.

Polyommatus eroides (FRIVALDSZKY, 1835), Nos 36–39 (males) – No. 36: Bulgaria, Kostenec, 1929.VII.15, leg. PAWLASZ; No. 37: Bulgaria, Kostenec, 1929. VII. 17, leg. PAWLASZ; No. 38: Bulgaria, Rila planina, inter Rilski Monaster, Lenin vrh, ca. 1200–1300 m, 1954.VII.19., leg. dr. SZATALA (moistered during relaxation); No. 39. Bulgarian, Ryla Geb., 1200 bis 1800 m, Juni, 1923, leg. O. BUBACEK.

Polyommatus eros (OCHSENHEIMER, 1808), Nos 40–43 (males), Nos 60–61 (blue morph “semibrunnea” females) – No. 40: Schweiz, Engadin, St. Moritz, 1965.VIII.2., leg. WETTSTEIN J.; No. 41: Schweiz, Wallis, Zermatt, 1968.VII.14., leg. WETTSTEIN J.; Nos 41–42: Schweiz, Wallis, Zermatt, 1968. VII. 18., leg. WETTSTEIN J.; No. 60: Helvetia, Pontresia, Piy Languard, coll. V. V. BARTHA; No. 61: Österreich, Heiligenblut, Altal Pochel, 1964.VII.25, leg. Dr. GY. ÉHIK.

Polyommatus ariana (MOORE, 1865) Nos 46–49 (males) – Nos 46–49: Pakistan, Hindukush Mts, 5 km E of Shandur pass, 3250 m, 72°38' E, 38°07' N, 24–25. VI. 2000, leg. Z. VARGA & G. RONKAY.

Polyommatus icarus (ROTTEMBURG, 1775) Nos 50–54 (males), Nos 55–59 females – No. 50: male, [Hungary], Normafa, 1973.VII.24, leg. FÁBIÁN; No. 51: male, [Hungary], Balatonszemes, 1968. VIII. 25, leg. WETTSTEIN J.; No. 52: male, [Hungary], Szécsény, Fehérhegy, 1946. IX. 7., leg. LIPTHAY; No. 53: male, [Hungary], Budapest, Pünkösdfürdő, 1966. VII. 10, leg. WETTSTEIN J.; No. 54: male, [Hungary], Csepel, 1962. VII. 31., leg. WETTSTEIN; No. 55: female, blue morph: [Hungary], Kótpuszta, 1962. IX. 14., leg. WETTSTEIN J.; No. 56: female, blue

morph: [Hungary], Budakeszi, 1956.VIII.5., Coll. Dr. SZABÓ R.; No. 57: female, blue morph: [Hungary, Bükk], Garadna-völgy, 1951. VII. 18., Coll. Dr. SZABÓ R.; No. 58: female, blue morph: [Hungary], Hatvan, Nagytelek, 1957. VIII. 23., leg. FABRICIUS; No. 59: female, brown morph: [Hungary, Budapest], Czinkota, 1938. VIII. 28., leg. GAÁL.

APPENDIX II: POLYOMMATUS-GENUS GROUP DIVERSITY IN TWO LOCALITIES

Polyommatus genus group high summer (June-August) diversities in a natural reserve under high anthropogenic pressure (Hungary: Budapest: Normafa) and in traditionally managed hay fields (Romania: Transylvania: Tordaszentlászló = Săvădisla) in Central Europe are presented here. Taxa are listed in alphabetical order according to their genus then species group names. "M" means that the species is "monomorphic" in regard having both sexes with brown dorsal wing surfaces. Further dorsally blue lycaenids which are also synchronic and syntopic, but possessing qualitatively different genital structures representing different monophyletic groups (eg. *Everes*, *Glauopsyche* and *Lycaenopsis* genus groups) are not listed.

Normafa, 400 m, Budapest, Hungary – *Aricia agestis* (DENIS & SCHIFFERMÜLLER, 1775) (M), *Cyaniris semiargus* (PODA, 1761), *Plebejus argus* (LINNAEUS, 1758), *P. argyrognomon* (BERGSTRÄSSER, 1779), *Polyommatus admetus* (ESPER, 1782) (M), *P. amanda* (SCHNEIDER, 1792), *P. bellargus* (ROTTEMBURG, 1775), *P. coridon* (PODA, 1761), *P. damon* (DENIS & SCHIFFERMÜLLER, 1775), *P. daphnis* (DENIS & SCHIFFERMÜLLER, 1775), *P. dorylas* (DENIS & SCHIFFERMÜLLER, 1775), *P. icarus* (ROTTEMBURG, 1775), *P. thersites* (CANTENER, 1834).

Szénafű, 450 m, Tordaszentlászló, Transylvania, Romania – *Aricia agestis* (DENIS & SCHIFFERMÜLLER, 1775) (M), *A. artaxerxes* (FABRICIUS, 1781) (M), *A. eumedon* (ESPER, 1782) (M), *Cyaniris semiargus* (PODA, 1761), *Plebejus argus* (LINNAEUS, 1758), *P. argyrognomon* (BERGSTRÄSSER, 1779), *P. idas* (LINNAEUS, 1758), *P. sephirus* (FRIVALDSZKY, 1835), *P. bellargus* (ROTTEMBURG, 1775), *P. coridon* (PODA, 1761), *P. daphnis* (DENIS & SCHIFFERMÜLLER, 1775), *P. dorylas* (DENIS & SCHIFFERMÜLLER, 1775), *P. icarus* (ROTTEMBURG, 1775), *P. polonus* (ZELLER, 1848), *P. thersites* (CANTENER, 1834).

*

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