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ORIGINAL ARTICLE

Improving knowledge of the subgenus *Agrodiaetus* (Lepidoptera: Lycaenidae: *Polyommatus*) in Eastern Europe: Overview of the Romanian fauna

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Abstract. The butterfly subgenus *Agrodiaetus* of the genus *Polyommatus* (Lepidoptera: Lycaenidae) is distributed in the western and central Palaearctic and represents a taxonomically challenging group due to its rapid diversification coupled, in many cases, with very limited availability of morphological diagnostic characters. In this study we provide a detailed overview of this subgenus in the Romanian fauna, a country where scattered, poorly documented records suggest the presence of three species: *Polyommatus* (*Agrodiaetus*) *damon*, *P.* (*A.*) *admetus* and *P.* (*A.*) *ripartii*. By analyzing material from museum collections and published records, and combining them with new faunistic findings and DNA data, we show that only *P. admetus* is currently undoubtedly present in the Romanian fauna (northern Dobrogea), where it reaches its north-eastern range limit in the Balkans. Historical records and DNA data suggest that the occurrence of *P. admetus* in northern Dobrogea is not likely to reflect a recent range expansion caused by climate or other environmental changes. Several historical records of this species that suggest its much wider distribution in Romania represent confusion with *P. damon* and *Phengaris alcon*. *Polyommatus damon*, reported mainly from western Romania, lacks records after 1938, while *P. ripartii* is only known from a single male labelled as originating from the Danube Delta, and requiring confirmation. There is a great need for directed studies to clarify the status of *P. damon* and *P. ripartii*, which are scarce and declining north of the Balkans and may represent taxa of conservation concern.

INTRODUCTION

The lycaenid butterfly subgenus *Agrodiaetus* Hübner, 1822 (of the genus *Polyommatus* Latreille, 1804), distributed throughout the western and central Palaearctic, is one of the most striking examples of explosive diversification in Lepidoptera, with over 120 species appeared in the last three million years (Kandul et al., 2004, 2007; Talavera et al., 2013). This subgenus is also characterized by remarkable interspecific variability in chromosome numbers, ranging from n = 10 to n = 134 (de Lesse 1960; Lukhtanov & Dantchenko, 2002). Sexes are often dimorphic, females have brown dorsal wings while those of males vary in colour (greenish, blue, violet or brown), which has been shown to likely represent a consequence of reinforcement of pre-zygotic reproductive isolation (Lukhtanov et al., 2005). Despite this variation in male wing colour, the high number of species in this subgenus, coupled with the limited number of other diagnostic characters, has resulted in many morphologically very similar species that can often be reliably differentiated with karyological and/or genetic data. These difficulties have led to a poor understanding of the distribution and composition of species even in areas such as Europe where butterflies have been intensively studied.

Most species of *Agrodiaetus* in Europe are restricted to the southern, warmer, parts of the continent. Only *Polyommatus* (*Agrodiaetus*) *admetus* (Esper, 1783), *P.* (*A.*) *ripartii* (Freyer, 1830), and *P.* (*A.*) *damon* (Denis & Schiffermüller, 1775) reach areas of Central Europe and the European part of Russia (e.g., Anikin et al., 1993; Bálint et al., 2006;

Beneš et al., 2002; Tshikolovets, 2011; Przybyłowicz et al., 2014; Kudrna et al., 2015). *P. damon* even occurs very locally as far north as the Luga district in the Leningrad region of Russia (Matov & Ivanov, 1999). Recent studies (e.g., Vila et al., 2010; Dincă et al., 2013) have improved knowledge of taxon composition, distribution and genetic structure for several *Agrodiaetus* species in Europe, but the subgenus remains insufficiently documented in the Balkans, despite this being apparently one of the richest European regions for *Agrodiaetus* taxa (Vila et al., 2010; Kudrna et al., 2015; Vishnevskaya et al., 2016).

Species of *Agrodiaetus* are poorly known in Romania where scattered, poorly documented records suggest the presence of as many as three species: *P. admetus*, *P. damon* and *P. ripartii* (Székely, 2008; Rákosy, 2013; Rákosy & Török, 2013). In the red list of Romanian butterflies (Rákosy, 2002), *P. admetus* and *P. ripartii* were listed as data deficient, while *P. damon* was placed as critically endangered. In fact, a recent study (Rákosy & Török, 2013) noted that *P. ripartii* and *P. admetus* are only represented in museum collections by a single and four specimens from Romania respectively, none collected after 1980. Improving knowledge of *Agrodiaetus* in Romania would provide data of potential conservation value because its component taxa are very local north of the Balkans, the region for which southern Romania represents the northern limit.

In this study, we combine historical data from museum collections with new faunistic findings and DNA sequences to provide an overview regarding *Agrodiaetus* in Romania.

MATERIAL AND METHODS

Faunistic data

Historical data for *P. damon*, *P. ripartii* and *P. admetus* were gathered by screening all published records and by checking material in collections stored at the Museum of Natural History "Grigore Antipa" Bucharest (MGAB), the Brukenthal National Museum Sibiu (MBSR), the Hungarian Natural History Museum Budapest (HNHM), the Municipal Museum Mediaş (MMM), and the Székely National Museum Sfântu Gheorghe (MOSG).

Field work directed towards the rediscovery of *P. admetus* in south-eastern Romania (Dobrogea) was done by some of the authors between the end of June and the end of July 2006, 2007, 2014 and 2015 (Table 1).

DNA sequencing

The presence in museum collections of two Romanian specimens requiring confirmation (one *P. admetus* and one *P. ripartii*), prompted us to analyse DNA data of several specimens of the above mentioned species. To place the genetic data obtained from Romanian specimens in a broader context, we used sequence records for 114 *Polyommatus* specimens including 74 mined from GenBank which included sequences for *P. ripartii* and *P. admetus* that overlap at least 400 base pairs (bp) of the mitochondrial DNA fragment sequenced in this study. More precisely, we used a part of the cytochrome *c* oxidase subunit I (COI) and subunit II (COII) dataset from Dincă et al. (2013) and references therein: we selected only sequences representative for each unique haplotype of *P. ripartii* and therefore retained 72 of the 112 sequences in the original dataset. To this we added two COI sequences of *P. admetus* from Lukhtanov et al. (2015), as well as 39 COI sequences of *P. admetus* and one of *P. ripartii* obtained in this study (Table 2).

Following Lukhtanov et al. (2015), sample VL03-F816 (GenBank accession number AY954019) was renamed from *Polyommatus rjabovi* to *P. rjabovianus rjabovianus*, and sample VL02-X474 (GenBank accession number AY954006) was renamed from *Polyommatus rjabovi* to *P. rjabovianus masul*. Following Vishnevskaya et al. (2016), five specimens listed as *Polyommatus admetus* in GenBank have been renamed to *P. yeranyani* (Dantchenko & Lukhtanov, 2005) (Table 2).

We sequenced 655 bp of COI for 39 *Polyommatus* (*Agrodiaetus*) specimens, seven of which originated from Romania, as well as a 164 bp COI fragment for one Romanian *P. admetus* (Table 2). For 38 specimens, total genomic DNA was extracted using Chelex 100 resin, 100–200 mesh, sodium form (Biorad), under the following protocol: one leg was removed and introduced into 100 µl of Chelex 10% and 5 µl of Proteinase K (20 mg/ml) were added. The samples were incubated overnight at 55°C and were subsequently incubated at 100°C for 15 min. Samples were then centrifuged for 10 s at 3000 rpm. A 655-bp fragment near the 5′ end of COI was amplified by polymerase chain reaction using the primers LepF1b (a slightly modified version of LepF1) and LepR1 (Table 3). Double-stranded DNA was amplified in 25-µl volume reactions containing: 14.4 µl autoclaved Milli-Q water, 5 µl 5 × buffer, 2 µl 25 mM $MgCl_2$, 0.5 µl 10 mM dNTPs, 0.5 µl of each primer (10 µM), 0.1 µl Taq DNA Polymerase (Promega, 5U/µl) and 2 µl of extracted DNA. The typical thermal cycling profile followed this protocol: first denaturation at 92°C for 60 s, followed by five cycles of 92°C for 15 s, 48°C for 45 s and 62°C for 150 s, and then by 35 cycles of 92°C for 15 s, 52°C for 45 s and 62°C for 150 s and a final extension at 62°C for 420 s. PCR products were purified and sequenced by Macrogen Inc.

Two of the sequences obtained in this study were recovered from specimens that were over 30 years old and were analyzed at the Biodiversity Institute of Ontario, Canada. In one case (sample RVcoll14U820), a full DNA barcode (658-bp) was obtained combining amplicons obtained using the primers [LepF1 + MLepR2] + [MLepF1 + LepR1]. In the other case (sample RVcoll14U819), a 164-bp amplicon was obtained using the primer set C_microLepF1_t1 + C_TypeR1 (Table 3). For these two samples, standard DNA extraction procedures were used (Ivanova et al., 2006), and DNA sequencing was performed on an ABI 3730xL capillary sequencer (Applied Biosystems).

Sequences were edited and aligned using GENEIOUS PRO 6.1.8 created by Biomatters (http://www.geneious.com/).

The new sequences over 200 bp obtained in this study have been submitted to GenBank (see Table 2 for accession numbers) and all sequences are also publicly available in the dataset DS-ADMRIP (dx.doi.org/10.5883/DS-ADMRIP) from the Barcode of Life Data Systems (http://www.boldsystems.org/).

Analyses of DNA sequences

Prior to phylogenetic analysis, we used TCS 1.21 (Clement et al., 2000) to remove duplicated COI haplotypes of *P. admetus* present in the 114 sequence alignment. The short COI sequence (164 bp) from sample RVcoll14U819 was not subjected to this filtering. The 42 COI sequences available for *P. admetus* were collapsed to 23 unique haplotypes that, in addition to phylogenetic analysis, were also used to construct a maximum parsimony haplotype network using TCS 1.21, with a 95% connection limit. The network presented one loop, which was broken according to frequency and geographic criteria (Excoffier & Langaney, 1989). The five sequences of *P. yeranyani*, formerly attributed to *P. admetus*, have also been collapsed to four unique haplotypes.

Following the removal of duplicated haplotypes, the alignment used for phylogenetic analysis included 94 COI and COII concatenated sequences, and was 2174-bp long.

Table 1. Synthesis of *Polyommatus damon*, *P. ripartii* and *P. admetus* records from Romania, based on literature surveys and museum collection data, as well as original findings of this study. Abbreviations of museum names follow those indicated in the material and methods section of the study. GPS coordinates are approximate and have been provided where relatively precise locality data were available.

Taxon	Locality	County	Original name on label or in publication	Date	Specimens / Sex	Alt (m)	Lat (DD)	Lon (DD)	Leg. / Coll. / Source	Material examined by the authors	Comments
P. damon	Bihorului Mountains		Biharhegység						Abafi-Aigner (1911)	Only literature data	
P. damon	Băița	Bihor	Rézbánya				46.48	22.58	Horváth & Pável (1876)	Only literature data	
P. damon	Cerna Valley	Caraș-Severin	Csernavölgy, Cernatal						Frivaldszky (1873), also taken over by Rebel (1911)	Only literature data	
P. damon	Domogled	Caraș-Severin		28.vi.–16.vii.1910			44.87	22.44	Keynes et al. (1911)	Only literature data	
P. damon	Mehadia	Caraș-Severin	Mehádia				44.89	22.36	Horváth & Pável (1876)	Only literature data	
P. damon	Mehadia	Caraș-Severin	Mehádia				44.89	22.36	Abafi-Aigner (1911)	Only literature data	
P. damon	Mehadia	Caraș-Severin	Mehádia	10.vii.1896	1 m		44.89	22.36	coll. N. Tomala (HNHM no. 2503)	Specimen examined at the HNHM	
P. damon	Steierdorf	Caraș-Severin	Stájerlak	17.vii.1910	1 m		45.07	21.84	Leg. B. & A. Lipthay, coll. B. Lipthay (HNHM no. 2431)	Specimen examined at the HNHM	
P. damon	Steierdorf	Caraș-Severin	Stájerlak	17.vii.1910	1 f		45.07	21.84	Leg. B. & A. Lipthay, coll. B. Lipthay (HNHM no. 2493)	Specimen examined at the HNHM	
P. damon	Steierdorf	Caraș-Severin	Stájerlak	17.vii.1910	1 f		45.07	21.84	Leg. B. & A. Lipthay, coll. Diószeghy (MOSG)	Specimen examined at the MOSG	Originally published as *P. admetus* (Căpușe & Kovács, 1987)
P. damon	Turnu-Severin	Mehedinți		1853–1855			44.65	22.66	Caradja (1895), Rebel (1903); also taken over by other authors such as Fleck (1900) and Salay (1910)	Only literature data	Data from Jozsef Haberhauer, collected between 1853–1855
P. damon	Saschiz	Mureș	Keisd		1 m		46.19	24.96	Leg. E. Silbernagel, coll. D. Czekelius, MBSR. Czekelius (1917)	Specimen examined at the MBSR	
P. damon	Nădrag	Timiș	Nadrág, Bánát	21.vi.1938	1 m		45.65	22.18	Leg. & coll. Sztankov (HNHM no. 2496)	Specimen examined at the HNHM	
P. damon	Tapia	Timiș	Tapia, Bnt	1911	1 m		45.68	21.96	Leg. Pfalfel, coll. Sztankov (HNHM no. 2502)	Specimen examined at the HNHM	
P. damon	Tapia	Timiș	Tapia, Bnt	1911	1		45.68	21.96	Leg. Pfalfel, coll. Sztankov (HNHM no. 2497)	Specimen examined at the HNHM	
P. damon	Tapia	Timiș	Tapia, Bnt	1911	1 f		45.68	21.96	Leg. Pfalfel, coll. Sztankov (HNHM no. 2498)	Specimen examined at the HNHM	
P. damon	Timișoara	Timiș	Temesvár				45.74	21.29	Abafi-Aigner (1911)	Only literature data	
P. damon	Retezat Mountains		Retyezát		1 m		45.33	22.89	coll. Diószeghy (HNHM no. 2490)	Specimen examined at the HNHM	Imprecise data
P. damon	The valleys of the Criș rivers	Arad-Bihor	Körösvölgy						Abafi-Aigner (1911)	Only literature data	Imprecise data
P. damon	Transylvania, Banat								Abafi-Aigner et al. (1896)	Only literature data	Imprecise data. Mentioned as common in Transylvania and Banat ("In reg. I–VI sat frequens")
P. damon	Maramureș	Maramureș	Mármaros vm.						Abafi-Aigner (1911)	Only literature data	Imprecise data, but not to be completely excluded since *P. damon* was known (now considered extinct) from near Vynohradiv in south-western Ukraine (Geryak & Kanarsky, 2006), less than 10 km away from the Romanian border and the Satu Mare and Maramureș regions
P. damon	Comănești	Bacău					46.41	26.42	Caradja (1895), taken over by various authors such as Fleck (1900) and Salay (1910)	Only literature data	Doubtful record. Caradja (1895) used data from L. Cosmovici, but mentioned that the specimen is not present in coll. Kemmingers

Table 1 (continued).

Taxon	Locality	County	Original name on label or in publication	Date	Speci-mens / Sex	Alt (m)	Lat (DD)	Lon (DD)	Leg. / Coll. / Source	Material examined by the authors	Comments
P. damon					1 m, 2 f				Coll. E. Worell, MBSR	Specimens examined at the MBSR	The specimens lack labels
P. ripartii	C.A.Rosetti, Letea forest	Tulcea		30.vi.1980	1 m		45.32	29.53	Leg. M. Brătășeanu, coll. F. König, MMM	Specimen examined at the MMM	Record requires confirmation
P. admetus	Greci, Ditcova Valley (Măcin Mts)	Tulcea		4.vii.2006	1 m	100	45.177	28.270	Leg. M. Skolka	Original data	
P. admetus	3 km south-west of Hamcearca (Măcin Mts)	Tulcea		2.vii.2007, 6.vii.2007	1 m, 1 f	170	45.091	28.373	Leg. M. Skolka	Original data	
P. admetus	3 km west of Hamcearca (Măcin Mts)	Tulcea		5.vii.2006	1 m	275	45.112	28.347	Leg. M. Skolka	Original data	
P. admetus	Clearings at the borders of Creasta Cardonului, Măcin Mts (Hamcearca)	Tulcea		16.vii.2014; 25.vii.2014	8 m, 2 f	165	45.097	28.388	Leg. M. Skolka; V. Dincă, L. Székely	Original data	
P. admetus	Creasta Cardonului, Măcin Mts (Hamcearca)	Tulcea		29.vi.2007; 25.vii.2014; 27.vi.2015	>25 m & >10 f observed	280	45.106	28.377	Leg. M. Skolka; V. Dincă, L. Székely	Original data	
P. admetus	ca. 8 km south of Babadag town	Tulcea		26.vii.2014	1 m, 1 f	130	44.817	28.688	Leg. V. Dincă, L. Székely	Original data	
P. admetus	Babadag	Tulcea		16.vii.1917	1 m		44.81	28.68	Fiebig (1927)	Only literature data	
P. admetus	Ciucurova	Tulcea		vi.1865	2 m		44.92	28.45	Mann (1866), taken over by various authors	Only literature data	
P. admetus	Horia	Tulcea		16.viii.1980	2 m, 1 f		45.01	28.45	Leg & coll. F. König, MMM	Specimens examined at the MMM	
P. admetus	C.A.Rosetti, Letea forest	Tulcea		30.vi.1980	1 m		45.32	29.53	Leg. M. Brătășeanu, coll. F. König, MMM	Specimen examined at the MMM	Record requires confirmation
P. admetus	Muntenia or Oltenia		Oláhország						Abafi-Aigner (1910)	Only literature data	Imprecise data, potentially taken over from other authors such as Caradja (1895)
P. admetus	Retezat Mountains, Bucura Lake	Hunedoara	Retyezát, Bukura	Possibly year 1912, since Pongrácz collected in the Retezat Mts during that year	1 m	2050	45.36	22.87	Leg. S. Pongrácz, coll. HNHM	Specimen examined at the HNHM	Doubtful record. Comment by A. Schmidt attached to the specimen's pin: *P. admetus* doubtful, lacking from entire Transylvania (Original text: "*admetus*, Kétséges, Egész Erdélyből hiányzik")
Phengaris alcon	River Zlaști inf.	Hunedoara		21.vii.1935	1 f				Leg R. Racovitza, coll. A. Ostrogovich, MGAB	Specimen examined at the MGAB	Originally published as *P. admetus* (Popescu-Gorj, 1964), it actually represents a female of *P. alcon*
Phengaris alcon	Sovata	Mureș		5.vii.1937	1 f				Leg C. Grințescu, coll. A. Ostrogovich, MGAB	Specimen examined at the MGAB	Originally published as *P. admetus* (Popescu-Gorj, 1964), it actually represents a female of *P. alcon*

Phylogenetic relationships were inferred using Bayesian inference (BI) through the CIPRES Science Gateway (Miller et al., 2010). Both BI analyses and the estimation of node ages were run in BEAST 1.8.0 (Drummond & Rambaut, 2007) with the data set partitioned by gene. Substitution models for each partition were chosen according to the Akaike's information criterion (AIC) values obtained in JMODELTEST 2.1.3 (Darriba et al., 2012), and were GTR + I + G for *COI* and GTR + G for *COII*. Base frequencies were estimated, six gamma rate categories were selected and a randomly generated initial tree was used. As previous studies have shown that *P. damon* is the most basal species of *Agrodiaetus* (Kandul et al., 2004; Wiemers et al., 2009; Talavera et al.,

Table 2. Specimens used for the DNA analyses. The sample IDs of specimens sequenced in this study are in bold.The sequence of sample RVcoll14U819, which was not submitted to GenBank because it was shorter than 200 bp, has been assigned the BOLD process ID corresponding to its COI sequence.

Sample ID	COI haplotypes of *P. admetus*	Taxon	*P. ripartii* lineages	Accession number COI	Accession number COII	Locality data
RVcoll14B725	ha4	*P. admetus*		KY683542		Albania, Korcë, Boboshtiçë, 1187 m
RVcoll14B690	ha10	*P. admetus*		KY683538		Albania, Korcë, Marjan, 1250 m
RVcoll14B722	ha14	*P. admetus*		KY683536		Albania, Tepelenë, Gllavë, 912 m
RVcoll.09-V962	ha11	*P. admetus*		KC581753		Bulgaria, Pirin Mt., Paril - Nova Lovcha, 750 m
RVcoll.09-V963	ha3	*P. admetus*		KC581754		Bulgaria, Pirin Mt., Paril - Nova Lovcha, 750 m
RVcoll14C831	ha6	*P. admetus*		KY322826		Bulgaria, Satovcha, Godeshevo, 800 m
RVcoll14F364	ha7	*P. admetus*		KY322828		Bulgaria, Zemen, Zemen, above the gorges, 869 m
RVcoll15P063	ha21	*P. admetus*		KY683534		Croatia, Izvor Zrmanje
RVcoll15P066	ha8	*P. admetus*		KY322818		Croatia, Općina Trilj, Voštane, 900 m
RVcoll15P064	ha8	*P. admetus*		KY683533		Croatia, Paško polje
RVcoll14C330	ha15	*P. admetus*		KY683532		Greece, Drama, Granitis, 750 m
RVcoll14C663	ha3	*P. admetus*		KY683528		Greece, Drama, NW of Granitis, 750 m
RVcoll14C468	ha2	*P. admetus*		KY322823		Greece, Drama, Oros Orvilos, 960-1600 m
RVcoll14G365	ha4	*P. admetus*		KY683535		Greece, Dytiki Makedonia, Aposkepos, 924 m
RVcoll14G471	ha4	*P. admetus*		KY683537		Greece, Dytiki Makedonia, Kelli, 930 m
RVcoll14F601	ha2	*P. admetus*		KY683527		Greece, East Macedonia and Thrace, Oros Orvilos, 930 m
RVcoll14G676	ha23	*P. admetus*		KY683523		Greece, Epirus, Drosopigi, 1097 m
RVcoll14F939	ha13	*P. admetus*		KY683541		Greece, Peloponnese, Kato Lousi, 995 m
RVcoll14F936	ha16	*P. admetus*		KY683529		Greece, Peloponnese, Kato Lousi, 995 m
RVcoll14G212	ha19	*P. admetus*		KY683539		Greece, Peloponnese, Levidi, 756 m
RVcoll14G197	ha18	*P. admetus*		KY683531		Greece, Peloponnese, Manaris, 840 m
RVcoll14G186	ha4	*P. admetus*		KY683546		Greece, Peloponnese, Manaris, 841 m
JC01014	ha12	*P. admetus*		AY556867		Greece, Peloponnese, Mt. Taiyetos, 1200-1300 m
RVcoll14H493	ha4	*P. admetus*		KY683544		Greece, Peloponnese, Mt. Taygetos, below Anavriti, 400-780 m
RVcoll14G106	ha17	*P. admetus*		KY683547		Greece, Peloponnese, Oros Taygetos, Katafigio, 1645 m
RVcoll14O341	ha13	*P. admetus*		KY683545		Greece, Peloponnese, road to Rogi, 970 m
RVcoll14O340	ha20	*P. admetus*		KY683526		Greece, Sterea Ellada, Delphi to Arachova road, 665 m
RVcoll14O392	ha3	*P. admetus*		KY322829		Hungary, Kazincbarcika subregion, Trizs, 270 m
RVcoll15Q038	ha22	*P. admetus*		KY683540		Macedonia, Centar Zhupa, Mt. Stogovo, Brostica, 1250 m
RVcoll14O338	ha4	*P. admetus*		KY683525		Macedonia, Suva Planina, above Kozjak Lake, 1050 m
RVcoll14O336	ha10	*P. admetus*		KY683524		Macedonia, Veles, Topolka gorge, 185 m
RVcoll14O337	ha2	*P. admetus*		KY683543		Macedonia, Veles, Topolka gorge, 185 m
RVcoll15Q046	ha9	*P. admetus*		KY683530		Montenegro, Bar, Mt. Rumija, 837 m
RVcoll15Q045	ha9	*P. admetus*		KY322821		Montenegro, Bar, Mt. Rumija, 837 m
RVcoll14U819		*P. admetus*		OLEP007-15		Romania, Danube Delta, C.A. Rosetti
RVcoll14G780	ha1	*P. admetus*		KY322819		Romania, Tulcea, ca. 10 km south of Babadag, 130 m
RVcoll14L009	ha1	*P. admetus*		KY322831		Romania, Tulcea, ca. 10 km south of Babadag, 130 m
RVcoll14L001	ha1	*P. admetus*		KY322825		Romania, Tulcea, Hamcearca, Creasta Cardonului, 175 m
RVcoll14L006	ha1	*P. admetus*		KY322820		Romania, Tulcea, Hamcearca, Creasta Cardonului, 175 m
RVcoll14L007	ha1	*P. admetus*		KY322827		Romania, Tulcea, Hamcearca, Creasta Cardonului, 175 m
RVcoll14L002	ha5	*P. admetus*		KY322822		Romania, Tulcea, Hamcearca, Creasta Cardonului, 175 m
RVcoll14F242	ha2	*P. admetus*		KY322824		Serbia, Jalovik Izvor, Čeganica, 583 m
MW98084	ha2	*P. admetus*		AY556986		Turkey, Antalya, Çukurelma, 1300 m
VL03-F669		*P. alcestis karacetinae*		AY954018	AY954018	Iran, Markazi, Khiru
MAT99-Q841		*P. damon*		AY496732	AY496732	Spain, Girona, Urús
VL01-L342		*P. dantchenkoi*		AY496737	AY496737	Turkey, Van, Çatak
RE07-G106		*P. dolus virgilius*		HM210162	HM210162	Italy, L´Aquila, Rocca Pia, 1215 m
MAT99-Q923		*P. dolus vittatus*		AY496740	AY496740	France, Lozère, Mende
AD00-P303		*P. eriwanensis*		AY496742	AY496742	Armenia, Aiodzor Mts, Gnishyk
MAT99-Q984		*P. fabressei*		AY496744	AY496744	Spain, Albarracín, Puerto de la Losilla
RV03-H596		*P. fabressei*		EF104605	EF104605	Spain, Castelló, Coll d'Ares, 1150 m
MAT99-Q972		*P. fabressei*		HM210165	HM210165	Spain, Cuenca, Uña, 970 m
MAT99-Q894		*P. fulgens*		AY496712	AY496712	Spain, Lleida, Tremp, Rúbies
MAT99-Q910		*P. fulgens*		AY496746	AY496746	Spain, Tarragona, Santa Coloma de Queralt
RE07-G203		*P. humedasae*		HM210170	HM210170	Italy, Aosta, Val di Cogne, Ozien-Visyes, 1000 m
RE07-G191		*P. humedasae*		HM210169	HM210169	Italy, Aosta, Val di Cogne, Ozien-Visyes, 1000 m
VL03-F526		*P. khorasanensis*		AY954013	AY954013	Iran, Khorasan, Kopetdagh Mts.
VL01-L122		*P. menalcas*		AY496763	AY496763	Turkey, Gümüshane, Dilekyolu
RVcoll.09-V964		*P. nephohiptamenos*		KC581745		Bulgaria, Pirin Mt., Orelek, Popovi Livadi, 1550 m
JC00045		*P. nephohiptamenos*		AY556859		Greece, Macedonia, Mt. Orvilos, 1200-2100 m
JC00046		*P. nephohiptamenos*		AY556860		Greece, Macedonia, Mt. Orvilos, 1200-2100 m
NK00-P822		*P. ripartii colemani*		AY496781	AY496781	Kazakhstan, West Tian-Shan
AD00-P337		*P. ripartii paralcestis*		AY496782	AY496782	Armenia, Pambak Mts., Dzhur-Dzhur Pass
RVcoll.08-L387		*P. ripartii paralcestis*		KC581715		Armenia, Vayots Dzor, Yeghegnadzor suburbs
RVcoll.08-L389		*P. ripartii paralcestis*		KC581716		Armenia, Vayots Dzor, Yeghegnadzor suburbs
VL01-L103		*P. ripartii paralcestis*		AY496783	AY496783	Turkey, Gümüshane
VL01-L166		*P. ripartii paralcestis*		AY496784	AY496784	Turkey, Gümüshane, Şiran, Dilekyolu
RVcoll.12-M011		*P. ripartii pelopi*	Balkan	KC581748		Bulgaria, Chepelare, Hvoyna, 780 m
RVcoll.11-G195		*P. ripartii ripartii*	Eurasian	KC581740		Croatia, Dalmatia, Mosor Mt., 1000 m
RVcoll.11-G188		*P. ripartii ripartii*	Eurasian	KC581730		Croatia, Dalmatia, Mosor Mt., 1000 m
RVcoll.11-G190		*P. ripartii ripartii*	Eurasian	KC581734		Croatia, Dalmatia, Mosor Mt., 1000 m
RVcoll.11-G192		*P. ripartii ripartii*	Eurasian	KC581744		Croatia, Dalmatia, Mosor Mt., 1000 m
RE07-G266		*P. ripartii ripartii*	West European	HM210171	HM210171	France, Drôme, Col de la Chaudière, 1025 m
RVcoll.11-I851		*P. ripartii ripartii*	West European	KC581726		France, Drôme, Col des Roustans, 950 m
RVcoll.11-I888		*P. ripartii ripartii*	West European	KC581720		France, Drôme, Rossas, 930 m
RVcoll.08-L390		*P. ripartii ripartii*	West European	KC581717		France, Var, Lorgues
RE07-G436		*P. ripartii ripartii*	Eurasian	HM210167	HM210167	Italy, Calabria, Monte Pollino, 1650 m
RE07-G437		*P. ripartii ripartii*	Eurasian	HM210168	HM210168	Italy, Calabria, Monte Pollino, 1650 m
RE07-G229		*P. ripartii ripartii*	West European	HM210172	HM210172	Italy, Susa Valley, Urbiano, Mompantero, 720 m
RE07-G255		*P. ripartii ripartii*	West European	HM210164	HM210164	Italy, Torino, Novalesa-Moncenisio, 1155 m
RE07-G254		*P. ripartii ripartii*	West European	HM210163	HM210163	Italy, Torino, Novalesa-Moncenisio, 1155 m
NK00-P829		*P. ripartii ripartii*	Eurasian	AY496785	AY496785	Kazakhstan, Dzhungarian, Alatau Mts, Kolbai
2005-LOWA-767		*P. ripartii ripartii*	Eurasian	FJ663244		Kazakhstan, Semipalatinsk, Taskesken, 450 m
2005-LOWA-768		*P. ripartii ripartii*	Eurasian	FJ663243		Kazakhstan, Semipalatinsk, Taskesken, 450 m
NK00-P848		*P. ripartii ripartii*	Eurasian	AY496786	AY496786	Kazakhstan, Semipalatinsk, Taskesken, 450 m
ILL088		*P. ripartii ripartii*	Eurasian	JF343831		Mongolia, Arshantyn-Nuruu Mts., Hovd-aimak, 2100 m
RVcoll14U820		*P. ripartii ripartii*	Eurasian	KY322830		Romania, Danube Delta, C.A. Rosetti
AD00-P033		*P. ripartii ripartii*	Eurasian	AY496787	AY496787	Russia, Tula region, Tatinki
RV03-H463		*P. ripartii ripartii*	Eurasian	EF104603	EF104603	Spain, Barcelona, El Brull, 830 m
MW01014		*P. ripartii ripartii*	West European	AY556944		Spain, Burgos, Ubierna, 900 m
RVcoll.08-L946		*P. ripartii ripartii*	West European	KC617794		Spain, Cantabria, San Andrés, Valdeprado del Río, 977 m
RVcoll.08-L945		*P. ripartii ripartii*	West European	GU676213		Spain, Cantabria, San Andrés, Valdeprado del Río, 977 m
RVcoll.12-L138		*P. ripartii ripartii*	West European	KC581719		Spain, La Rioja, Arnedo, 850 m
RVcoll.12-L128		*P. ripartii ripartii*	West European	KC581721		Spain, La Rioja, Enciso, 815 m
RVcoll.12-Q453		*P. ripartii ripartii*	West European	KC581727		Spain, La Rioja, Hornillos de Cameros, 905 m
RVcoll.12-L140		*P. ripartii ripartii*	West European	KC567884		Spain, La Rioja, Larriba, Zarzosa
RVcoll.12-L137		*P. ripartii ripartii*	West European	KC581722		Spain, La Rioja, Peña Isasa, 1080 m
MAT99-Q878		*P. ripartii ripartii*	Eurasian	AY496780	AY496780	Spain, Lleida, Tremp, Rúbies
MW01105		*P. ripartii ripartii*	Eurasian	AY556962		Spain, Tarragona, Santa Coloma de Queralt, 700 m
RVcoll.08-P615		*P. ripartii ripartii*	Eurasian	GU675760		Spain, Teruel, Albarracín, 1250 m
NK00-P859		*P. ripartii ripartii*	Eurasian	AY496779	AY496779	Ukraine, Crimea , Karabi-Yaila
VL02-X474		*P. rjabovianus masul*		AY954006	AY954006	Iran, Gilan, Masuleh
VL03-F816		*P. rjabovianus rjabovianus*		AY954019	AY954019	Azerbaijan, Talysh, Zuvand
VL04-E365		*P. urmiaensis*		EF104631	EF104631	Iran, Azarbayjan-e-Gharbi
RV03-H558		*P. violetae subbaeticus*		EF104604	EF104604	Spain, Granada, Sierra de la Sagra, 1700 m
RV03-H555		*P. violetae subbaeticus*		HM210166	HM210166	Spain, Granada, Sierra de la Sagra, 1775 m
RVcoll.08-H299		*P. violetae violetae*		HM210175	HM210175	Spain, Andalucía
FGT05-J629		*P. violetae violetae*		HM210173	HM210173	Spain, Granada, Sierra de Almijara
FGT05-J630		*P. violetae violetae*		HM210174	HM210174	Spain, Granada, Sierra de Almijara
AD00-P016		*P. yeranyani*		AY496711	AY496711	Armenia, Aiodzor Mts, Gnishyk
F902		*P. yeranyani*		KJ906515		Azerbaijan, Talysh, Mistan, 1700 m
VL03-F903		*P. yeranyani*		EF104617	EF104617	Azerbaijan, Talysh, Zuvand
2002A01		*P. yeranyani*		KR265492		Turkey, Gümüshane Prov., Kelkit
VL01-L101		*P. yeranyani*		AY496710	AY496710	Turkey, Gümüshane, Torul

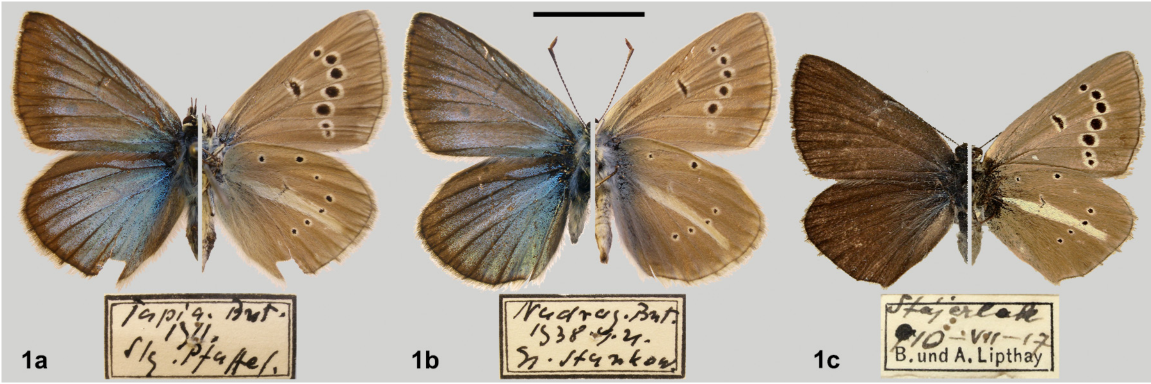

Fig. 1. Specimens of *Polyommatus* (*Agrodiaetus*) *damon* from Romania. Left sides represent dorsal views and right sides represent ventral views. The original labels of the specimens are illustrated (see Table 1 for further information). a – male of *P. damon* from Tapia (Banat) (coll. Sztankov, HNHM); b – male of *P. damon* from Nădrag (Banat) which is the most recent record from Romania; c – female of *P. damon* from Steierdorf (Banat) (coll. L. Diószeghy, MOSG) previously recorded as *P. admetus* by Căpușe & Kovács (1987). Scale bar: 10 mm. Figs 1a, b photos by G. Katona (HNHM), 1c photo by I. Kocs (MOSG).

2013), we enforced the monophyly of the *P. dolus* + *P. admetus* clade so that *P. damon* is recovered as sister to the rest.

Rough estimates of node ages were obtained by applying two molecular clocks with: 1.5% uncorrected pairwise distance per million years estimated for various invertebrates (Quek et al., 2004), and 2.3% estimated for the entire mitochondrial genome of several arthropods (Brower, 1994). A strict clock and a normal prior distribution was used, centred on the mean between the two substitution rates, and the standard deviation was tuned so that the 95% confidence interval of the posterior density coincided with the 1.5% and 2.3% rates, respectively.

Parameters were estimated using two independent runs of 20 million generations each, and convergence was checked using the program TRACER 1.6.

RESULTS

Polyommatus (*Agrodiaetus*) *damon*

Ten specimens (six males and four females), collected between 1896 and 1938, were found in museum collections (Fig. 1a–c, Table 1). The most recent Romanian record was a male collected at Nădrag (Timiș county, Banat region), on June 21, 1938 and stored in the collection of the HNHM (Fig. 1b, Table 1). A series of studies mentioned the occurrence of this species in several areas (mainly from western Romania), but without precise locality data and/or numbers of specimens (Table 1). These studies include records from as early as 1853 (Caradja, 1895) to 1917 (Czekelius, 1917), although the limit of the latest dates is hard to establish due to lack of exact data in the publications. A specimen of *P. damon* from Retezat Mountains, present in the L. Diószeghy collection at the HNHM, lacked a collection date (Table 1), but it was not likely collected later than 1940 (probably several years earlier) (Căpușe & Kovács, 1987).

A female specimen of *P. damon* examined by us in the coll. L. Diószeghy from the MOSG (Fig. 1c), was erroneously published as *P. admetus* (Căpușe & Kovács, 1987).

The distribution of *P. damon* in Romania based on the available data of acceptable precision, indicates that the species was relatively widespread in the western and south-western parts of the country, but probably very local (Table 1, Fig. 2).

Polyommatus (*Agrodiaetus*) *ripartii*

Our survey of museum collections identified a single male specimen of *P. ripartii* from Romania stored in the collection F. König at the MMM and also reported by Rákosy & Török (2013). This specimen was labelled as having been collected in the Danube Delta, on the 30th of June 1980 (Table 1, Figs 2, 3). In the same collection, there is also a specimen of *P. admetus* bearing identical collection data to that for *P. ripartii* (Table 1).

DNA results for *P. ripartii*

The Bayesian analysis did not recover *P. ripartii* as monophyletic (Fig. 4). This result is concordant with previous studies (e.g., Vila et al., 2010; Dincă et al., 2013; Vishnevskaya et al., 2016) and highlights the need for further study. However, there was good support for the three main European lineages of *P. ripartii* reported by previ-

Table 3. Primers used in this study.

Primer cocktail	Primer name	Primer sequence (5´–3´)	Direction	Reference
N/A	LepF1	ATTCAACCAATCATAAAGATATTGG	Forward	Hebert et al., 2003
N/A	LepF1b	ATTCAACCAATCATAAAGATATTGGAAC	Forward	This study
N/A	LepR1	TAAACTTCTGGATGTCCAAAAAATCA	Reverse	Hebert et al., 2003
N/A	MLepF1	GCTTTCCCACGAATAAATAATA	Forward	Hajibabaei et al., 2006
N/A	MLepR2	GTTCAWCCWGTWCCWGCYCCATTTTC	Reverse	Hebert et al., 2013
C_microLepF1_t1	microLepF2_t1	TGTAAAACGACGGCCAGTCATGCWTTTATTATAATTTTYTTTATAG	Forward	Hebert et al., 2013
C_microLepF1_t1	microLepF3_t1	TGTAAAACGACGGCCAGTCATGCWTTTGTAATAATTTTYTTTATAG	Forward	Hebert et al., 2013
C_TypeR1	TypeR1	GGAGGRTAAACWGTTCAWCC	Reverse	Hebert et al., 2013
C_TypeR1	TypeR2	GGAGGGTAAACTGTTCAWCC	Reverse	Hebert et al., 2013
C_TypeR1	TypeR3	GGTGGATAAACAGTTCAWCC	Reverse	Hebert et al., 2013

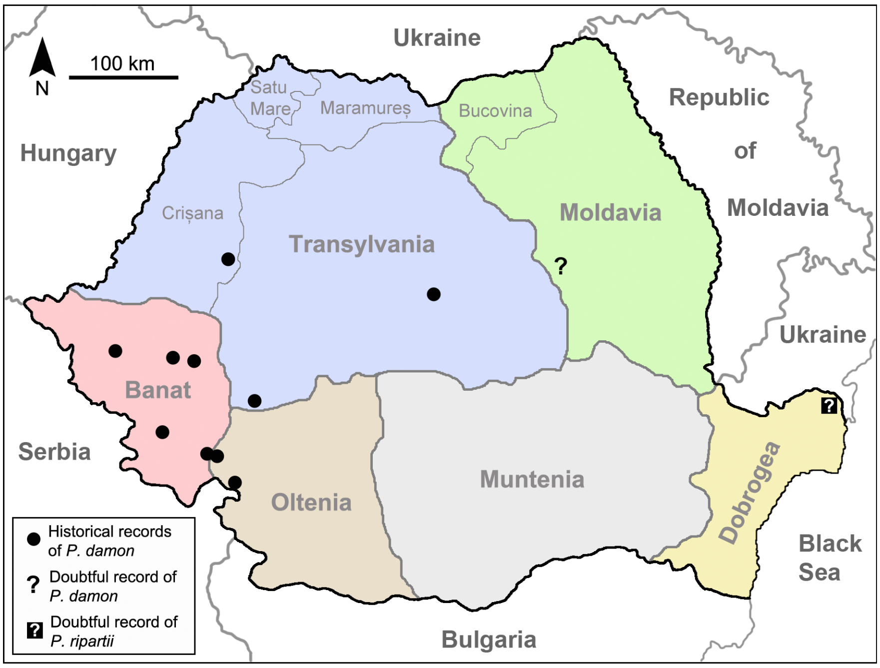

Fig. 2. Distribution of *P. damon* and *P. ripartii* in Romania. For *P. damon*, no record is available after 1938, while *P. ripartii* has only one record (requiring confirmation) from 1980 (Danube Delta). Further details are available in Table 1. Records of *P. damon* with vague locality data were not included on the map. The division of the Romanian territory illustrates the main historical regions of the country.

ous studies (Dincă et al., 2013; Vishnevskaya et al., 2016). The single specimen known from Romania (Danube Delta) (Fig. 3), which has been successfully DNA barcoded, was placed within the Eurasian lineage of *P. ripartii* (Fig. 4).

Polyommatus (*Agrodiaetus*) *admetus*

The first published records of this species in Romania date from 1865 and refer exclusively to northern Dobrogea (south-eastern Romania): two males from Ciucurova (Mann, 1866) and one male from Babadag (Fiebig, 1927) (Table 1, Fig. 5). In addition, our survey of material in museum collections located three more recently collected (1980) specimens from northern Dobrogea (Horia) (Fig. 6a), and one male specimen from the Danube Delta (also 1980), all stored in the coll. F. König at the MMM (Table 1, Fig. 5). These specimens have also been reported by Rákosy & Török (2013).

A male specimen of *P. admetus*, labelled as originating from the Bucura Lake in the Retezat Mountains was found in the collections of the HNHM. A comment by A. Schmidt (former curator of the Lepidoptera collections at the HNHM, see Bálint & Katona, 2014), attached to the pin of the specimen, expressed doubts regarding the origin of the specimen (Table 1). Two female specimens, stored in the coll. A. Ostrogovich at the MGAB, were published as *P. admetus* by Popescu-Gorj (1964) (Table 1), but our re-examination revealed that they actually represent females of *Phengaris alcon* (Denis & Schiffermüller, 1775) (Fig. 7). A specimen present in the coll. L. Diószeghy from the MOSG, was published as *P. admetus* by Căpușe & Kovács (1987), but we found that it actually represents a female of *P. damon* (Fig. 1c).

Our research in northern Dobrogea revealed the presence of *P. admetus* at several sites in the Măcin Mountains and also established its persistence in Babadag forest (Table 1, Figs 5, 6b–d). The species was local, but relatively abundant in the Măcin Mountains, while it was apparently much rarer in the southern part of Babadag forest (Table 1). At all sites, the adults were flying in forest clearings and along forest and shrub borders always in xeric areas with tall vegetation and little to no grazing pressure (Fig. 8a–c).

DNA results for *P. admetus*

Forty-two of the 43 DNA sequences from *P. admetus* analysed, representing well the range of the species (especially in Europe) (Kudrna et al., 2015) (Fig. 9a), were collapsed to 23 unique haplotypes. The six Romanian specimens originating from the recently discovered populations represented two haplotypes (h1 and h5) that were found exclusively in northern Dobrogea (Table 2, Fig. 9a, b). The single Romanian specimen of *P. admetus* collected in 1980 in the Danube Delta had only a short 164 bp COI sequence that was not used for haplotype inference. However, for the overlapping gene region, this sequence was identical to h3 from north-eastern Greece, southern Bulgaria and Hungary and to h7 from western Bulgaria, but differed from the other two Romanian haplotypes (h1 and h5) by three mutations.

The Bayesian analysis recovered *P. admetus* as monophyletic with good support (Fig. 4). It is worth noting that, in previous studies (e.g. Vila et al., 2010; Dincă et al., 2013), this species was not recovered as monophyletic due to the presence of a lineage distributed in eastern Turkey and southern Caucasus. However, Vishnevskaya et al. (2016) proposed that this lineage represents a distinct species (*P. yeranyani*), also recovered here as monophyletic with good support. Within *P. admetus*, haplotypes h1 and h5 from the Romanian specimens, sampled in this study in

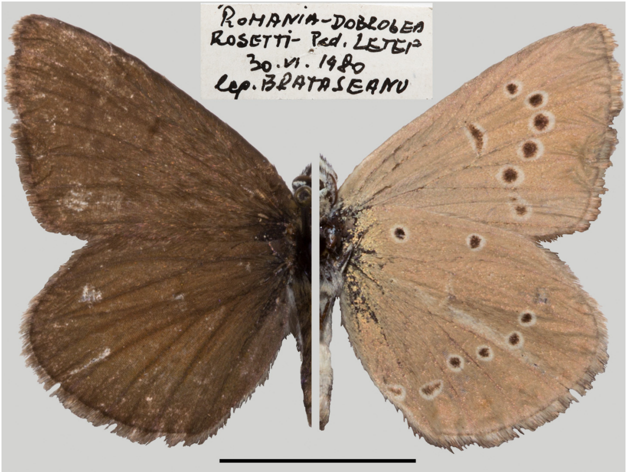

Fig. 3. The single specimen of *P. ripartii* (male; left, dorsal; right, ventral) known from the Romanian territory (DNA sample ID RVcoll14U820). The label on the specimen mentions C.A. Rosetti, Letea Forest, Danube Delta, 30.vi.1980, leg. M. Brătășeanu. Scale bar: 10 mm. Photo by S. Török.

northern Dobrogea (Măcin Mountains and Babadag forest) clustered together with good support and were part of a well-supported clade that included four more haplotypes (h2, h10, h11 and h15) from the central-eastern Balkans and western Turkey (Figs 4, 9a). The short COI sequence of the Romanian specimen from the Danube Delta (RVcoll14U819) clustered (albeit with relatively poor support) with haplotypes found in Albania (h14), north-eastern Greece, southern Bulgaria and Hungary (h3), as well as western Bulgaria (h7) (Figs 4, 9a).

DISCUSSION

Polyommatus (*Agrodiaetus*) *damon*

All specimens of this species are old with no reliable record from Romania after 1938. It is thus not surprising that its national extinction has been suspected (Székely, 2008; Rákosy, 2013).

This species formerly occurred in the western part of Romania (western Oltenia, Banat, Crișana, Transylvania and Maramureș) (Fig. 2). However, this general distribution is based on very few records (only ten specimens found in collections), many with vague data (e.g., Abafi-Aigner, 1911, reported the species from several large areas without precise information, such as Maramureș, Bihorului Mountains, or the valleys of the Criș rivers). For this reason, the distribution map in Fig. 2 includes only data that could be assigned to a locality with acceptable precision. The single record of *P. damon* from Moldavia (Comănești) was viewed as doubtful by Caradja (1895), who mentioned that the specimen was lacking from its original collection (Table 1). The labelling of the specimen from Saschiz (Mureș county, eastern Transylvania) (Table 1) may also be incorrect in our opinion; although not figured as doubtful in Fig. 2, this locality is quite isolated from reliable records and, while virtually impossible to verify, mislabelling cannot be discounted.

Although the species has been mentioned as frequent in Transylvania and Banat (e.g., Abafi-Aigner et al., 1896), the very low number of specimens in collections and the paucity of records with acceptable precision suggest that *P. damon* was probably rare in Romania even in the second half of the 19th and the beginning of the 20th century. Some recent publications also reported this species from south-eastern Romania (Dobrogea) (Székely, 2008; Rákosy, 2013), but our results do not support this. The core of its distribution in Romania seems to have been the Banat region (Fig. 2, Table 1). It is interesting that, although the most recent data known from Romania originate from Banat (June 1938, Nădrag, coll. Sztankov at the HNHM) (Table 1), records suddenly stopped after this date, although Banat has been relatively intensively studied. For example, F. König studied the Lepidoptera fauna of Banat for a very long time (1930–2000), but never reported this species.

Polyommatus damon also appears to be very localized in countries neighbouring Romania. In Hungary it has always been restricted to a few sites in the north of the country (Bálint et al., 2006) and in recent years only one highly endangered population survives near Budapest (Bálint, 2015). In Serbia, several records originate from the east and south of the country, but most are prior to 1988 (Jakšić, 1988; Miljević & Popović, 2014). One recent record (year 2012) originates from Suva Planina and requires confirmation (Jakšić, 2014; Popović & Đurić, 2014), while a population of the species has been found in 2016 in south-western Serbia, close to Sjenica city (Miljević & Popović, 2014). In Bulgaria, *P. damon* has only been reported from two localities in the south-west (Pirin and Rila Mountains), but the records are more than 50 years old. Moreover, since specimens are apparently lacking from collections, its occurrence requires confirmation in that country (Abadjiev, 2001). In Ukraine the species is declining and is currently known only from one restricted area in the west (Lviv district) (Nekrutenko & Tshikolovets, 2005; Geryak & Kanarsky, 2006). In this context, the historical distribution of *P. damon* in (mainly western) Romania (Fig. 2) represents an expected link between the populations in western Ukraine, northern Hungary and Serbia.

In Central Europe, *P. damon* is also very localized and declining. It was, for example, reported as extinct from Poland (where it occurred locally in the south-east) (Buszko & Masłowski, 2008), as near extinct (critically endangered) in the Czech Republic (Beneš et al., 2002; Šlancarová et al., 2012) and as also declining in Germany (Ebert & Rennwald, 1991; Nässig et al., 2004). In fact, according to Van Swaay et al. (2010), *P. damon* is near threatened in Europe.

The typical habitat for *P. damon* consists of xeric grasslands with an abundance of its larval food plant, the sainfoin (*Onobrychis* spp.). In Central Europe, grazing is suspected as the main cause for the decline of this species (Dolek, 1994; Dolek & Geyer, 2002; Kudrna, 1998). A study on *P. damon* and *P. thersites* (Cantener, 1835) performed in the Czech Republic (Šlancarová et al., 2012) found that *P. damon* is likely to be very sensitive to summer grazing and mowing since females lay their eggs high on the food plants (on sainfoin bracts). The same study also suggested

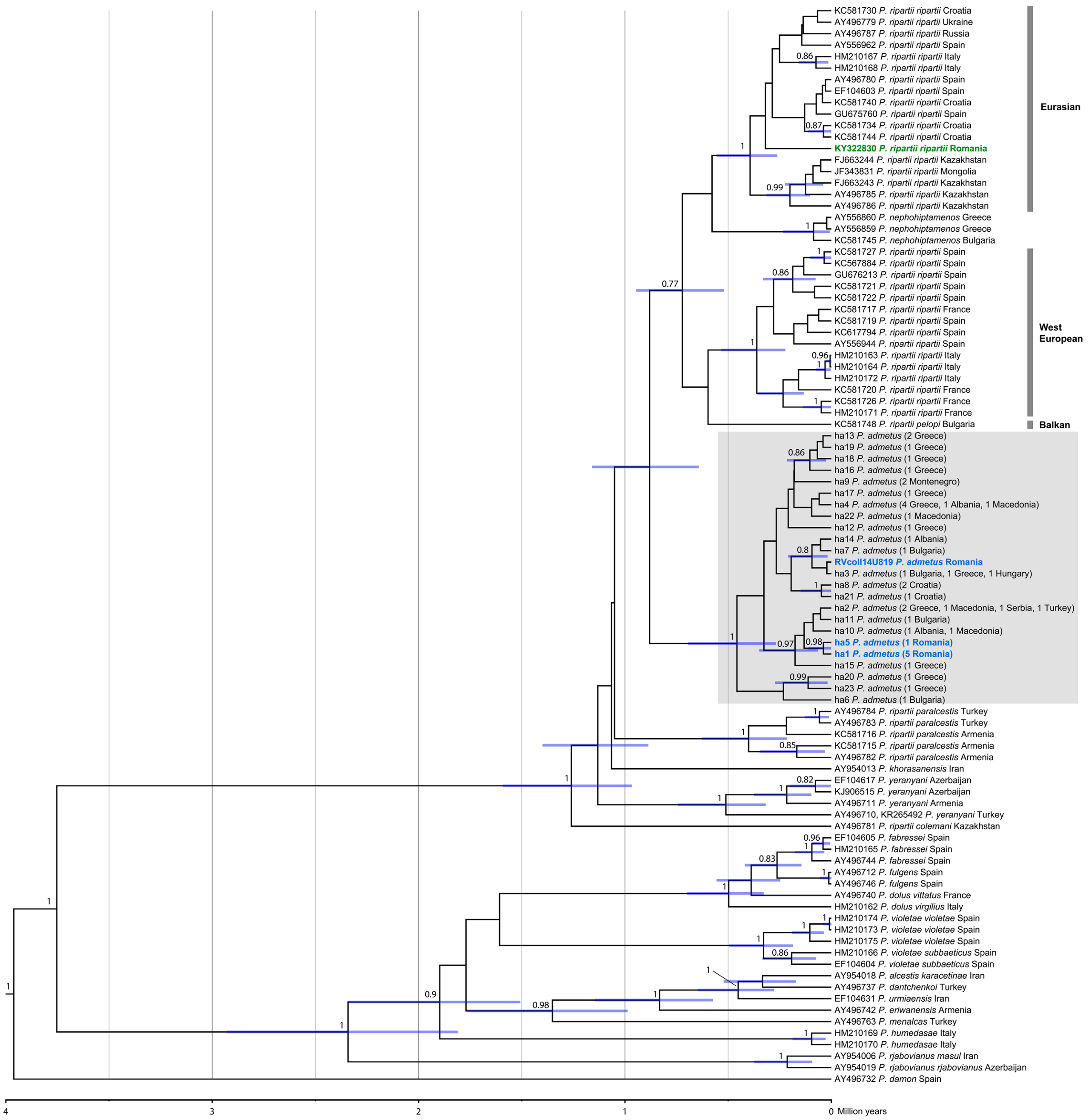

Fig. 4. Bayesian ultrametric tree based on cytochrome *c* oxidase subunit I (COI) and subunit II (COII) sequences. Bayesian posterior probabilities higher than 0.7 are displayed above recovered nodes. Node bars represent 95% highest posterior density for age estimations. The three main lineages of *P. ripartii* occurring in Europe as defined by Dincă et al. (2013) are indicated by grey vertical bars, and the clade of *P. admetus* highlighted in grey. Romanian specimens of *P. ripartii* and *P. admetus* are in bold green and blue, respectively. With the exception of sample RVcoll14U819, specimens are named using COI GenBank accession numbers. Sample codes, as well as COII GenBank accession numbers, are available in Table 2. For each unique haplotype of *P. admetus* the number of specimens and country of occurrence are indicated in parentheses. Haplotype codes correspond to those in Table 2.

that the occurrence of *P. damon* may be affected by metapopulation processes, so that only large areas of suitable habitat are sufficient to maintain the species.

The causes for the apparent decline of *P. damon* in Romania are uncertain, its butterfly fauna still needs in depth studies. In fact, recent research has revealed new species for the country or confirmed older records, even in areas that were considered as well investigated (e.g., Dincă & Vila, 2008; Dincă et al., 2008, 2009, 2010, 2011; Rákosy & Craioveanu, 2016). On the other hand, it is also possible that the decline, or even extinction, of *P. damon* reflects its high sensitivity to habitat alteration (Šlancarová et al., 2012). For example, *P. damon* is suspected to have been introduced in the Benelux due to such agricultural practices and became extinct when sainfoin crops decreased (Bink, 2013). The availability of the larval food plant in western Romania may have also been higher in the 19th century, when the sainfoin was grown as fodder and as a bee plant, creating habitat for this species. At the end of the 19th and beginning of the 20th century, *Onobrychis* was also

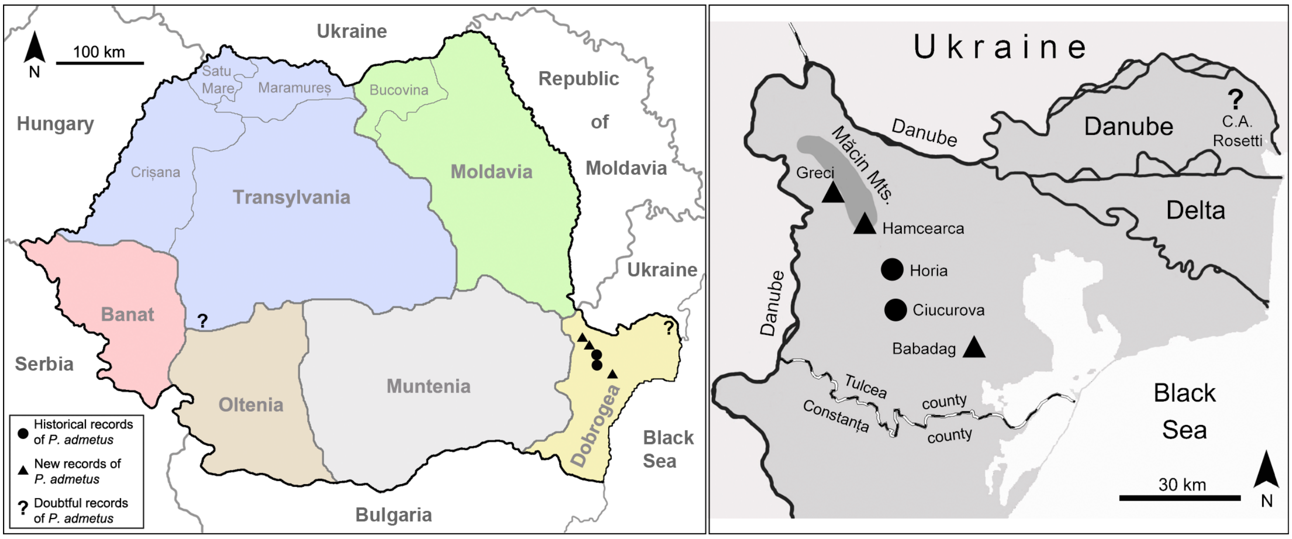

Fig. 5. Distribution of *P. admetus* in Romania. Further details are available in Table 1. The division of the Romanian territory illustrates the main historical regions of the country. The right side of the figure represents an enlarged portion of northern Dobrogea, from where all reliable records of *P. admetus* originate.

frequently used in the Carpathian Basin to cover barren hillsides and keep the ground after heavy works of railway construction. This practice promoted it as one of the most important elements dominating meadows for hay-making (Babai et al., 2014).

Until further research is done, *P. damon* should be treated in Romania as a data deficient, but potentially extinct species.

Polyommatus (*Agrodiaetus*) *ripartii*

The only specimen of this species known from Romania was collected in 1980 and, according to its label, originates from the Letea forest in the Danube Delta (Figs 2, 3, Table 1), a sylvo-steppe area with sand dunes. To place this record into a broader context, we consider the status of *P. ripartii* in the countries neighbouring Romania. From Hungary it is known from a single specimen collected near Budapest (Bálint, 1996). The label on this specimen does not mention the year, but it cannot be later than 1917, the year when the collection owner (E. Ulbrich) died. The species is also known from Serbia, some sites being less than 60 km from the south-western Romanian border, but almost 600 km from the Danube Delta (Miljević & Popović, 2014). In Bulgaria, *P. ripartii* occurs mainly in the west and south-west of the country, with the nearest records to the southern Romanian border being less than 80 km away, but almost 400 km from the Danube Delta (Abadjiev, 2001). In Ukraine, the species is known exclusively from Crimea (Kolev & De Prins, 1995; Nekrutenko & Tshikolovets, 2005), over 350 km from the Danube Delta. The easternmost records of *P. ripartii* in the Balkans originate from the European part of Turkey, over 400 km south from the Danube Delta (Hesselbarth et al., 1995; Kudrna et al., 2015). The distribution of *P. ripartii* in the neighbouring countries suggests that the species may be more likely to occur in south-western Romania than in the Danube Delta, but its presence in south-eastern Romania (Dobrogea) cannot be discounted, especially since the area also hosts populations of *P. admetus*. The habitat of *P. ripartii* ranges from xerothermic sylvo-steppe-like environments to shrubs and dry mountain slopes (the latter especially in the southern parts of its range), usually in limestone areas with its larval food plant, *Onobrychis* spp. *Polyommatus ripartii* is generally not known to occur near sea level in southern Europe, although towards its northern range limit it can fly at low elevations (e. g. Poland, southern Ural and Crimea) (Anikin, 1993; Kolev & De Prins 1995; Przybyłowicz, 2014). At least some of these habitat types occur in southern Romania. However, the Danube Delta is a surprising location for its occurrence due to both the very low elevation (0.52 m above mean Black Sea level) (Gâștescu, 2009) and the absence of *Onobrychis* species (Oprea, 2005; Ciocârlan, 2011; Doroftei et al., 2011).

It is interesting to note that F. König, who collected *P. admetus* in northern Dobrogea (Babadag forest area) without publishing the records, provided apparently contradictory information in private communications with colleagues, by mentioning both *P. admetus* and *P. ripartii* from the forests around Babadag (Rákosy & Török, 2013). In F. König's collection stored at the MMM, besides the *P. ripartii* from the Danube Delta, there is one specimen of *P. admetus* with the same labelling as *P. ripartii*. Besides these, there are three specimens of *P. admetus* from Babadag forest (Horia) captured by F. König. All five specimens were collected in 1980, although the labels for two specimens from the Danube Delta mention M. Brătășeanu as collector (Table 1). If the labelling is correct, it is unclear why F. König did not mention the presence of at least one *Agrodiaetus* species in the Danube Delta, not only in Babadag forest.

The COI sequence of *P. ripartii* from the Danube Delta belongs to the widespread Eurasian lineage of this species (Fig. 4). This eliminates the possible mislabelling involving specimens belonging to lineages that are unlikely to occur in the Danube Delta, such as the West European one or that of taxon *P. ripartii paralcestis* (Fig. 4). However, the fact that no species of *Onobrychis* is known from the

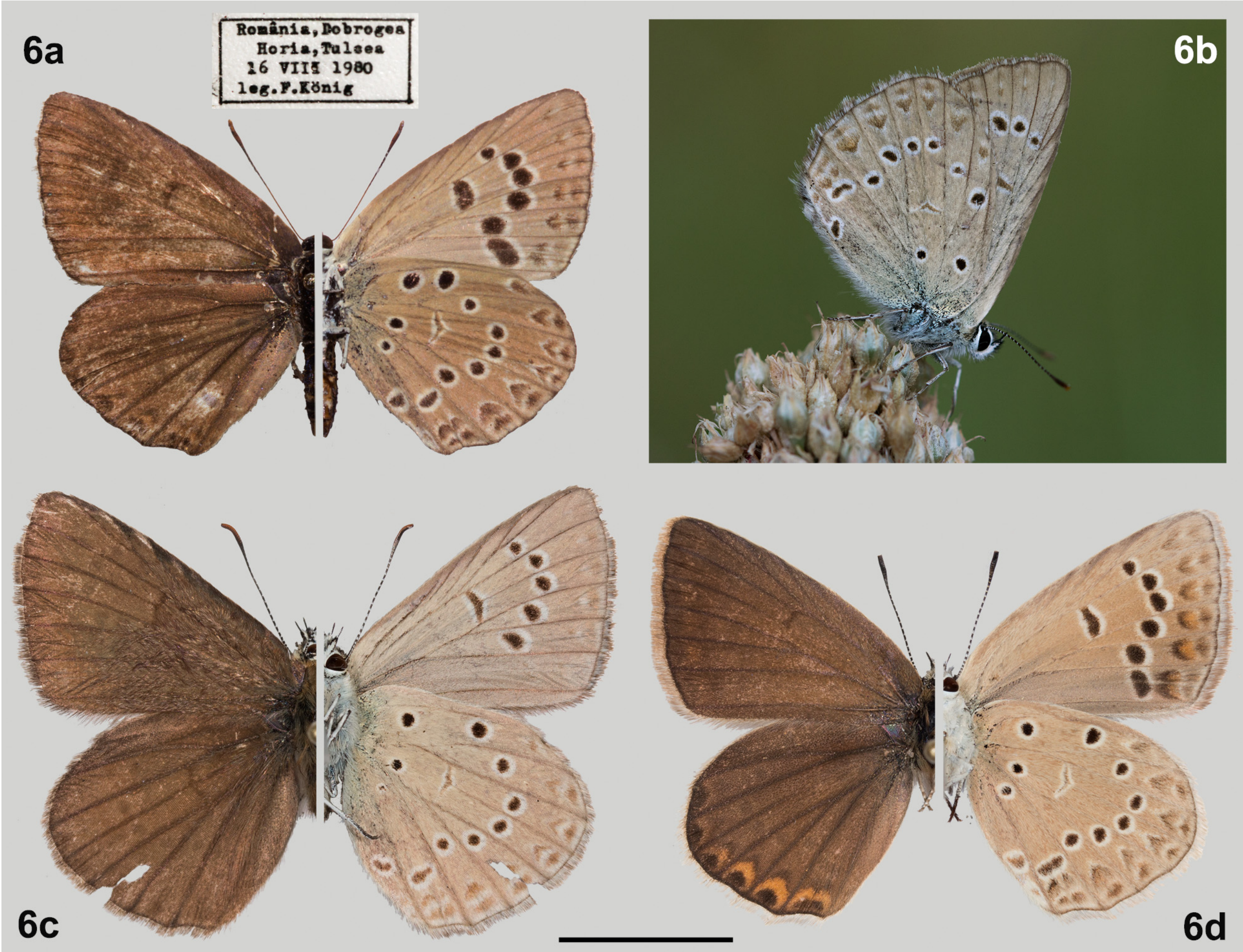

Fig. 6. Specimens of *Polyommatus* (*Agrodiaetus*) *admetus* from northern Dobrogea (Tulcea county), Romania. a – female from Horia, 16.viii.1980, coll. F. König at the MMM; b – male photographed near Hamcearca, 25.vii.2014; c – male, Hamcearca, 25.vii.2014 (DNA sample ID RVcoll14L007); d – female, ca. 8 km south of Babadag town, 26.vii.2014 (DNA sample ID RVcoll14L009). In both prepared specimens, the abdomens were removed and stored in tubes with ethanol for DNA preservation. Scale bar for the prepared specimens: 10 mm. Photo 6a by S. Török, photos 6b–d by V. Dincă.

Danube Delta (see above) raises doubts about the occurrence of *P. ripartii* (or any other species of *Agrodiaetus*) in this area. Therefore, a potential mislabelling cannot be fully excluded: the specimens from the Danube Delta could originate from northern Dobrogea, or even from outside the Romanian borders. *Polyommatus admetus* has already been confirmed from northern Dobrogea (see below), and that area offers more suitable habitats for *P. ripartii* compared to the Danube Delta, including the presence of *Onobrychis* spp. (Oprea, 2005).

Therefore, both the presence of *P. ripartii* in Romania and the occurrence of a population in the Danube Delta require confirmation. If proven, the occurrence of *P. ripartii* in the Danube Delta would represent the northermost locality for this species in the Balkans and one of the most northerly in Europe, reducing the distribution gap between the populations from the Balkans, Crimea (Nekrutenko & Tshikolovets, 2005) and the isolated ones from south-eastern Poland (Przybyłovicz, 2000). Such a population would also be of conservation concern since *P. ripartii* has been listed as near threatened in the European Union by Van Swaay et al. (2010).

Polyommatus (*Agrodiaetus*) *admetus*

The populations found in this study from the Măcin Mountains and Babadag forest, combined with historical records from Babadag (the most recent from 1980) (Mann, 1866; Fiebig, 1927, Rákosy & Török, 2013), confirm the presence and extend the known range of *P. admetus* in northern Dobrogea (Table 1, Figs 5, 6). The data also suggest that this species is local, but probably more widespread in the Babadag forest and especially in the Măcin Mountains, where suitable habitats are present (Fig. 8a, b) and where the species is relatively common. The single record from the Danube Delta (Table 1, Fig. 5) (Rákosy & Török, 2013), while plausible, requires confirmation (see below).

Our survey of museum collections revealed that none of the records for *P. admetus* from outside northern Dobrogea are reliable (Table 1). In fact, all supposed records from Transylvania and Banat represent misidentifications of *P. damon* (Căpușe & Kovács, 1987) (Fig. 1c) and *P. alcon* (Popescu-Gorj, 1964) (Fig. 7). These erroneous records resulted in overestimation of the real distribution of *P. ad-*

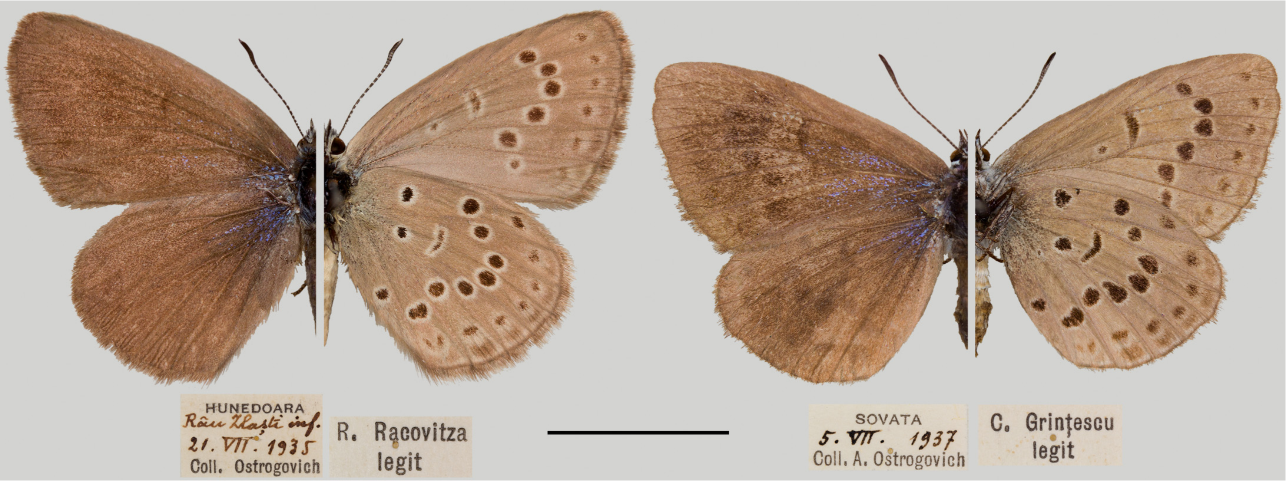

Fig. 7. Female specimens of *Phengaris alcon* (dorsal and ventral view for each) stored in the coll. A. Ostrogovich from the MGAB, erroneously attributed to *Polyommatus admetus* in previous studies (Popescu-Gorj, 1964). The specimen on the left was captured at River Zlaști, while the one on the right was collected at Sovata. For further information, see Table 1. Scale bar: 10 mm. Photos by V. Dincă.

metus in Romania (e.g., Tshikolovets, 2011; Kudrna et al., 2015; Eckweiler & Bozano, 2016). It is likely that published distribution maps indicating that *P. admetus* occurs in south-western and/or north-western Romania took over the erroneous literature data mentioned above.

In countries neighbouring Romania, *P. admetus* is local in the northern part of Hungary (Bálint et al., 2006), and a little further to the north it was reported as local and endangered in the Pannonian part of Slovakia (Kulfan & Kulfan, 1992). In Serbia *P. admetus* occurs mostly in the southern and south-eastern regions (Miljević & Popović, 2014), while in Bulgaria it is relatively widespread in the central, eastern and southern parts (Abadjiev, 2001), and it is also present in nearby areas including the European part of Turkey (Hesselbarth et al., 1995; Kudrna et al., 2015). In Ukraine the presence of *P. admetus* requires confirmation (Nekrutenko & Tshikolovets, 2005) because there are only two records: one specimen collected in 1892 from the Lviv region (western Ukraine) (Garbowski, 1892) and another found in 1904 in the Odessa region (southern Ukraine) (Shugurov, 1906). In this context, *P. admetus* from Dobrogea represents the north-eastern range limit for this species in the Balkans, and the nearest confirmed populations occur in north-eastern Bulgaria, circa 200 km to the south-east (Nevsha village, N. Shtinkov personal comment to V. Dincă, 2015).

The six analysed specimens of *P. admetus* from northern Dobrogea represent two unique haplotypes (h1 and h5), that were differentiated by at least one (h1) and two mutations (h5) from the other haplotypes found in the Balkans (Fig. 9b). The Romanian haplotypes also differed by four (h1) and five (h5) mutations from the Hungarian specimen that was analysed. The genetic data, combined with the historical faunistical data available from Romania, suggests that the presence of *P. admetus* in Romania does not reflect a recent range expansion potentially related to climate or other environmental change. The uniqueness of these haplotypes also likely reflects the geographical isolation of the populations of *P. admetus* from northern Dobrogea and highlights their conservation value. Interestingly, the COI sequence of the single Romanian specimen labelled as from the Danube Delta, although only 164 bp-long, was placed into a different clade compared to the specimens

Fig. 8. Habitats of *P. admetus* in northern Dobrogea (south-eastern Romania). a – Hamcearca, Creasta Cardonului (Măcin Mountains), 25.vii.2014; b – Hamcearca, below Creasta Cardonului (Măcin Mountains), 26.vii.2014; c – Babadag forest (ca. 8 km south of Babadag town), 26.vii.2014. Photos by V. Dincă.

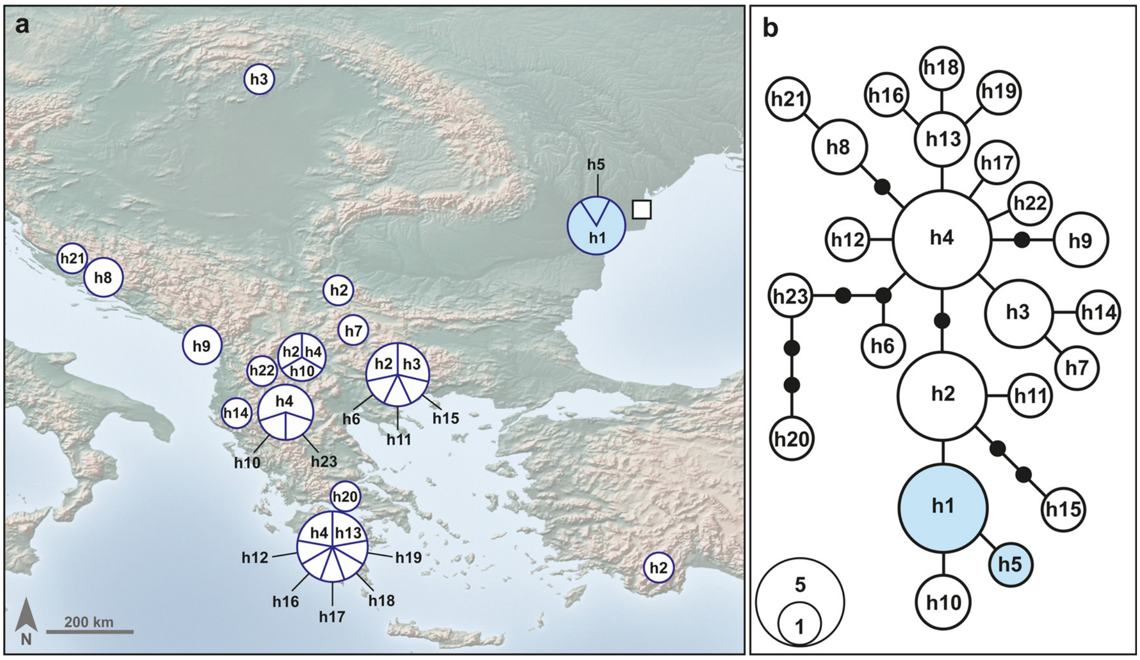

Fig. 9. a – geographic distribution of COI haplotypes of *P. admetus*. The white square corresponds to a 164 bp COI sequence of *P. admetus* from the Danube Delta (Romania) that, for the overlapping COI region, differed by three mutations from the Romanian haplotypes from northern Dobrogea (h1 and h5). b – maximum parsimony haplotype network based on 42 COI sequences of *P. admetus*. The circles are scaled to represent the relative frequency of each haplotype in the data set. Each branch represents one point mutational step, and the black dots represent unsampled haplotypes. The Romanian haplotypes are coloured in light blue.

from northern Dobrogea (Fig. 4) and, despite geographical proximity (Fig. 9a), differed by three mutations from the haplotypes from northern Dobrogea.

This result can have three explanations: (1) DNA analysis of additional specimens would reveal the presence of the Danube Delta haplotype in northern Dobrogea as well; (2) the presence of *P. admetus* in the Danube Delta reflects a colonization event independent from that involving northern Dobrogea; (3) the specimen from the Danube Delta is mislabelled and actually originates from outside Romanian territory, or at least from outside Dobrogea. Assuming that the first two hypotheses are not correct, a recent colonization of the Danube Delta by *P. admetus* from northern Dobrogea is unlikely due to the genetic differences found between the two regions. Moreover, as in the case of *P. ripartii*, the lack of *Onobrychis* spp. from the Danube Delta raises further doubts regarding the presence of *P. admetus* in the area so further studies are needed to clarify this situation.

Although historical records of *P. admetus* from outside northern Dobrogea have been shown to be unreliable by our study, it is possible that the species occurs in other parts of the country, especially in the south-west and west, where potentially suitable habitats are present. Indeed, the nearest populations from Hungary are circa 150 km away from the western Romanian border, while the ones from eastern Serbia and western Bulgaria are less than 70 km away from the south-western Romanian border. It is therefore possible that directed research would reveal populations of *P. admetus* outside northern Dobrogea.

The populations of *P. admetus* recently discovered in northern Dobrogea are within protected areas, namely the Măcin Mountains National Park (ROSCI0123) and the Babadag-Codru forest (ROSPA0091). Based on our observations (years 2014–2016), the impact of grazing is low in Măcin Mountains and moderate in Babadag forest so it probably does not severely affect the species and its food plant, *Onobrychis* spp. However, in Babadag forest, where *P. admetus* seems rarer than in Măcin Mountains (Table 1), the areas with forest clearings and meadows where the species has been found, appear to be decreasing because of overgrowth by shrubs and trees, including *Pinus* plantations (Fig. 8c). Without a suitable management plan aiming to maintain the sylvo-steppe character of the area, the species may severely decline and even disappear from its currently known location.

CONCLUSIONS

By critically examining literature and museum collection data and by adding new data, this study provides an overview of *Agrodiaetus* in the Romanian fauna. Three species belonging to this subgenus have been reported from Romania: *P. damon*, *P. ripartii* and *P. admetus*.

We showed that the most recent record of *P. damon* from Romania dates back to 1938 and that urgent research is needed to determine whether the species is now extinct.

The single specimen of *P. ripartii* known from Romania was collected in 1980 and originates from the Danube Delta. DNA sequencing has shown that this specimen belongs to the widespread Eurasian lineage of *P. ripartii*. However, the lack of *Onobrychis* spp. (the larval food plant of this butterfly) in the Danube Delta suggests that the presence of *P. ripartii* in this area is unlikely and hence the single available record requires confirmation.

Recent findings prove the presence of *P. admetus* in Romania (northern Dobrogea) by confirming historical records from Babadag forest and, for the first time, identifying populations in Măcin Mountains. All records of *P. admetus* from outside Dobrogea (Transylvania, Banat) were found to represent either unreliable labelling or confusion with *P. damon* and *Phengaris alcon*. The Romanian populations from northern Dobrogea represent haplotypes that are distinct from all other material analysed and likely do not represent recent colonizations due to climate or other environmental change. The single specimen from the Danube Delta is, unexpectedly, genetically differentiated from the relatively near populations in northern Dobrogea. This result and also the lack of *Onobrychis* spp. in the Danube Delta, indicate the need to confirm the presence of *P. admetus* in this region.

Given the current data, *P. admetus* certainly occurs in the Romanian fauna, while *P. damon* may be extinct, and *P. ripartii* requires confirmation.

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