

Genetics and extreme confinement of three overlooked butterfly species in Romania call for immediate conservation actions

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Abstract

A good knowledge of species distributions and their genetic structure is essential for numerous types of research such as population genetics, phylogeography, or conservation genetics. We document the presence of extremely local populations of three butterfly species (*Iolana iolas*, *Satyrus ferula* and *Melanargia larissa*) in the Romanian fauna. *Satyrus ferula* and *M. larissa* are reported for the first time in the country, while *I. iolas* is rediscovered following presumed extinction. Based on mitochondrial DNA (cytochrome c oxidase subunit 1 – COI sequences), we assessed the genetic structure of these populations and placed them into a broader context through comparisons with other populations from across the range of these species. Each of the three species had a single haplotype in Romania, suggesting low female effective population size possibly under genetic erosion. Two of the populations (*S. ferula* and *M. larissa*) are genetically unique, displaying endemic haplotypes in south-western Romania. The Romanian populations of the three species likely remained unnoticed due to

their extremely limited extent of occurrence. Their restricted range, close to the northern limits of distribution in the Balkans, their apparent low female effective population size, the presence of endemic haplotypes, and habitat vulnerability (especially for *I. iolas*) highlight the need for monitoring and conservation measures for the safeguarding of these populations.

Keywords: conservation, mitochondrial DNA, haplotypes, *Iolana iolas*, *Melanargia larissa*, *Satyrus ferula*

Declarations

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Conflicts of interest/Competing interests

The authors declare that they have no conflict of interest.

Ethics approval

Not applicable.

Consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and material

DNA sequences generated as a result of this study were submitted to GenBank and the entire dataset, including GenBank accession numbers and sampling sites, is publicly available in the dataset DS-ROLEP2 from the Barcode of Life Data Systems (<http://www.boldsystems.org/>).

Code availability

Not applicable.

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70 **Authors' contributions**

71 All authors contributed to the study's conception and design. Material preparation and data
72 collection were performed by all authors. Genetic analyses were performed by Vlad Dincă
73 and Raluca Vodă. The first draft of the manuscript was written by Bogdan Groza and Vlad
74 Dincă and all authors commented on previous versions of the manuscript. All authors read
75 and approved the final manuscript.

76

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84

85 **Introduction**

86 Wildlife populations occurring on the margin of a species' range can represent good systems
87 to study the dynamics and mechanisms shaping species distributions, local adaptation and the
88 effects of climate change (Jump and Peñuelas 2005; Antonovics et al. 2006; Gaston 2009;
89 Sexton et al. 2009). Such populations often face a high risk of extinction due to suboptimal
90 environmental conditions and may be genetically impoverished due to potentially complex
91 interactions between their biogeographic history and ecological factors (e.g. Kunin et al.
92 2009; Yang et al. 2016).

93 A good knowledge about these populations in relation to the overall genetic structure of the
94 species is particularly relevant to taxonomic groups that, given a long history of intensive
95 study, can be regarded as flagship for biodiversity research and conservation, and for which
96 new discoveries can have strong impacts on future research directions and conservation
97 actions. Such a group are the European butterflies, for which massive amounts of data
98 provide a good knowledge on species distributions (e.g. Kudrna 2019). Partly fuelled by the
99 advent of DNA barcoding (Hebert et al. 2003), the spatial distribution of European butterflies
100 is being increasingly linked to patterns of variation in mitochondrial DNA (e.g. Hausmann et
101 al. 2011; Dincă et al. 2011, 2015; Huemer and Wiesmair 2017; Dapporto et al. 2019), which
102 opens new possibilities for a wide range of studies integrating such data. In this context, it is

desirable that information on butterfly species distribution at a continental level is updated in parallel with the ongoing effort to document the genetic diversity of as many populations as possible.

Among the European countries, Romania has been the first to benefit from a comprehensive DNA barcode library for its butterflies (Dincă et al. 2011), but national data regarding butterfly species composition and distribution can be regarded as of medium quality only (Székely 2008; Rákosy 2013). Recent research has in fact revealed considerable gaps in knowledge reflected by discoveries of new species for the country (e.g. Dincă et al. 2008, 2010a), new populations of poorly known taxa and corrections of previous reports (e.g. Dincă and Vila 2008; Dincă et al. 2009, 2010b, 2011a, 2017; Rákosy et al. 2011).

Because Romania lies at the northern limit of the Balkans, a number of butterfly species are known to reach their northern distribution limit in this country (Székely 2008; Rákosy 2013). However, large areas of high biodiversity remain insufficiently explored and it is possible that very local populations of other species, potentially threatened, have remained undetected. In this study we document extremely local butterfly populations from south-western Romania, close to their northern range limit in the Balkans, of *Iolana iolas* (Ochsenheimer, 1816), *Satyrus ferula* (Fabricius, 1793) and *Melanargia larissa* (Geyer, 1828). By combining field observations (distribution, habitat preferences, phenology, threats) with genetic data (mitochondrial DNA - mtDNA), we: (1) characterize the genetic structure of the newly discovered populations and place them into a broader phylogeographic context through comparisons with mtDNA of conspecifics from across each species' range; (2) document their habitat preferences and phenology; (3) estimate their extent of occurrence, potential threats to their habitats, and propose conservation actions.

Methods

Specimens and field observations

Directed fieldwork was conducted by the authors for four years (2013-2016), when several locations from south-western Romania in the Berzasca-Svinița area were visited (Tables S1, S2). For identification purposes, specimens were captured using insect nets and a small number of individuals were retained for molecular analyses. In this case, to ensure DNA preservation, samples were dried while in the field. Subsequently, wings were detached from

the body and stored as vouchers in glassine envelopes, while the bodies were stored at -20°C in 2 ml tubes with 99% ethanol.

Molecular analyses

Molecular analyses were based on 24 COI sequences for *I. iolas* (13 of them generated by this study), 65 for *S. ferula* (including four *S. virbius*) (34 of them generated by this study) and 76 for *M. larissa* (33 of them generated by this study) (Table S1).

DNA sequencing

We obtained DNA data from 80 specimens (13 specimens of *I. iolas*, 34 specimens of *S. ferula* and 33 specimens of *M. larissa*) from Romania and from other parts of the species' range (Table S1).

From 47 specimens (10 *I. iolas*, 25 *S. ferula* and 12 *M. larissa*) DNA was extracted using Chelex 100 resin, 100–200 mesh, sodium form (Biorad), employing 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 658-bp fragment near the 5' end of the mitochondrial gene cytochrome *c* oxidase subunit 1 (COI) was amplified by polymerase chain reaction using the primers LepF1 (5'-ATTCAACCAATCATAAAGATATTGG-3') and LepR1 (5'-TAAACTTCTGGATGTCCAAAAAATCA-3') (Hebert et al. 2004). For some samples, a 655-bp fragment was amplified using the primers LepF1b (a slightly modified version of LepF1, 5'-ATTCAACCAATCATAAAGATATTGGAAC-3') and LepR1. Double-stranded DNA was amplified in 25-µl volume reactions containing: 14.4 µl autoclaved Milli-Q water, 5 µl 5x buffer, 2 µl 25 mM MgCl₂, 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 Europe B.V. (Amsterdam, The Netherlands). Sequences were edited and aligned using GENEIOUS PRO 6.1.8 created by Biomatters (<http://www.geneious.com/>).

Thirty-three (three *I. iolas*, five *S. ferula*, four *Satyrus virbius*, and 21 *M. larissa*) of the novel sequences used by this study were generated at the Biodiversity Institute of Ontario, Canada.

In this case a glass fibre protocol (Ivanova et al. 2006) was employed to extract DNA and polymerase chain reactions (PCR) and DNA sequencing were carried out following standard DNA barcoding procedures for Lepidoptera (deWaard et al. 2008). Sequences generated as a result of this study were submitted to GenBank (see Table S1 for accession numbers) and the entire dataset is also publicly available in the dataset DS-ROLEP2 from the Barcode of Life Data Systems (<http://www.boldsystems.org/>).

Analyses of DNA sequences

We combined our COI data with 85 GenBank sequences (11 *I. iolas*, 31 *S. ferula*, 43 of *M. larissa*) that overlapped our alignment by at least 600 base pairs (Table S1). This resulted in final alignments of 24 COI sequences for *I. iolas*, 65 for *S. ferula* (including four *S. virbius*) and 76 for *M. larissa*. All sequences used were at least 632 bp-long and the vast majority were 655-658 bp (i.e. the full fragments amplified by the primers used, as described above). Sequences of each species were collapsed to haplotypes using TCS 1.21 (Clement et al. 2000). The same program was used to construct maximum parsimony haplotype networks, with a 95% connection limit.

Results

Iolana iolas in Romania

Presence data

Between 2014-2016, this species (Figs 1a,b) was observed yearly in the surroundings of Svinița (Mehedinți county) (Figs 1c, S1a; Table S2), close to the river Danube. Adults were on wing between mid-May and mid-June and were always found circling *Colutea arborescens* bushes (Fig. 1d), the larval food plant of the species. In the studied area, this plant often occurs along road margins. Additional information is available in the Electronic Supplementary Material.

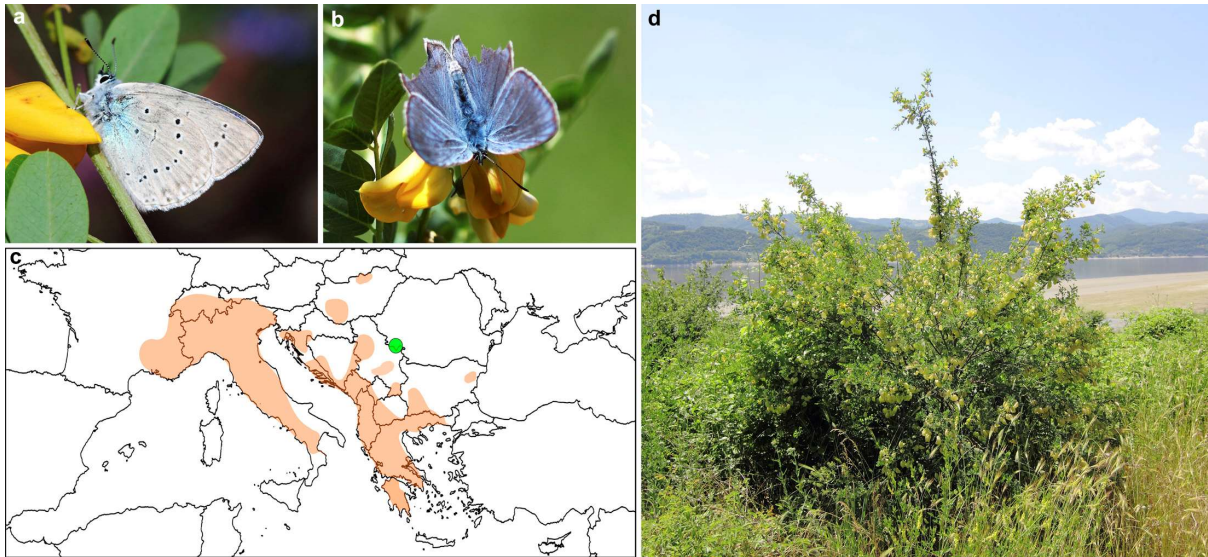


Fig. 1 *Iolana iolas* in south-western Romania. **a.** Male, 17.05.2015; **b.** Male carrying marks of a potential predator attack, 31.05.2015; **c.** Approximate distribution (orange) of *I. iolas* in the western Palearctic (the green dot indicates the Romanian population); **d.** Bush of *Colutea arborescens*, the larval food plant of *I. iolas*, on the Romanian bank of the river Danube. Photos: B. Groza.

Mitochondrial DNA

Five haplotypes were detected among the 24 specimens of *I. iolas* analysed (Figs 2a,b; Table S1). Levels of divergence were very low since no haplotype displayed more than two mutations with respect to its nearest haplotype. The three Romanian specimens analysed belonged to a single haplotype (h1), which was shared with other specimens originating from the Balkans (Bulgaria, Macedonia and Greece). Haplotypes h1 and h5 were detected only in the Balkans.

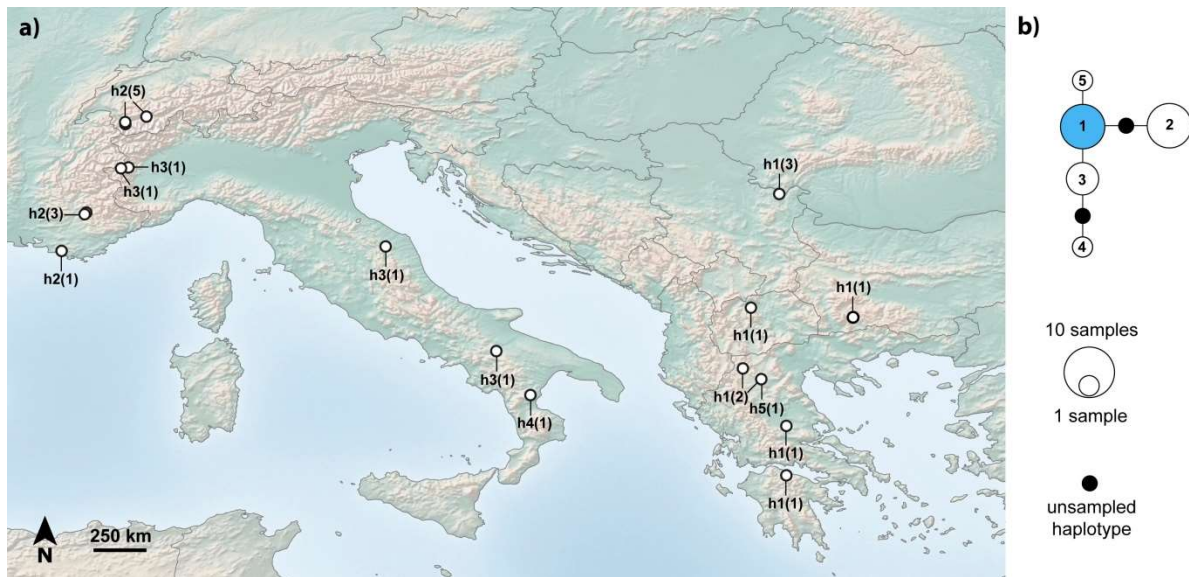


Fig. 2 a) Geographic distribution of COI haplotypes of *Iolana iolas*. Numbers in parentheses indicate the number of specimens for each haplotype; **b)** Maximum parsimony haplotype network based on COI sequences of *I. iolas*. The haplotype found in Romanian specimens (h1) is coloured in blue. The circles are scaled to the relative frequency of each haplotype in the data set; branches represent one-point mutational step

Satyrus ferula in Romania

Presence data

Between 2013-2016, this species (Figs 3a,b) was observed yearly in the surroundings of Svinița (Mehedinți county) and Cozla (Caraș-Severin county) (Figs 3c, S1b; Table S2).

Adults were present between the second half of June and mid-July and appeared to be mostly confined to a few rocky areas with sparse vegetation (Fig. 3d). Only occasionally, a few specimens were also found in flowery meadows or mud-puddling on forest tracks.

In the collections of the Hungarian Museum of Natural History (HMNH) Budapest, we also found one specimen of *S. ferula* collected in south-western Romania. This male specimen was labelled as originating from Piatra Lungă, Berzasca, 2.07.1911 (Fig. S2). The precise location of Piatra Lungă remains somewhat unclear to us, but it likely refers to a rocky area near the river Danube, between Berzasca and Cozla, approximately ten kilometres away from the location of our initial field observations.

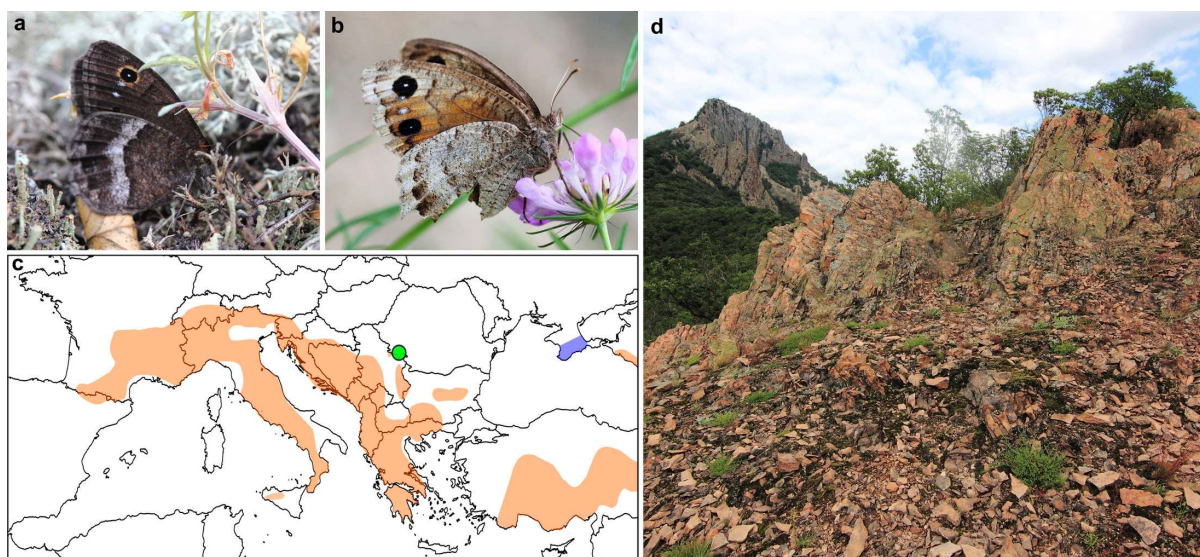


Fig. 3 *Satyrus ferula* in south-western Romania. **a.** Male, 21.06.2014; **b.** Female, 04.07.2015; **c.** Approximate distribution (orange) of *S. ferula* in the western Palearctic (the green dot indicates the Romanian population; the blue area represents the range of *Satyrus virbius*); **d.** Typical habitat, a rocky area near the Trescovăț peak, 21.06.2014. Photos: B. Groza

Mitochondrial DNA

Thirty haplotypes were detected among the specimens of *S. ferula* (61 specimens) and *S. virbius* (four specimens) analysed (Figs 4 a,b; Table S1). The most diverged were haplotypes attributable to *S. ferula altaica* from south-eastern Kazakhstan, Mongolia and south-eastern Russia (h27, 28, 29), displaying between five and seven mutations with respect to the closest haplotypes from other regions. Specimens from the southern Ural Mountains, sometimes also attributed to *S. f. altaica* (h11 and h13), did not belong to the same haplogroup as the above-mentioned specimens.

Apart from *S. f. altaica*, no other haplotype displayed a minimum divergence higher than three mutations. The four specimens attributable to *S. virbius* from Crimea (h12), had a single mutation with respect to h13, a haplotype detected in northern Greece and southern Urals. The haplotype of the five Romanian specimens analysed (h15) was not shared with other regions and was differentiated by a single mutation from h13.

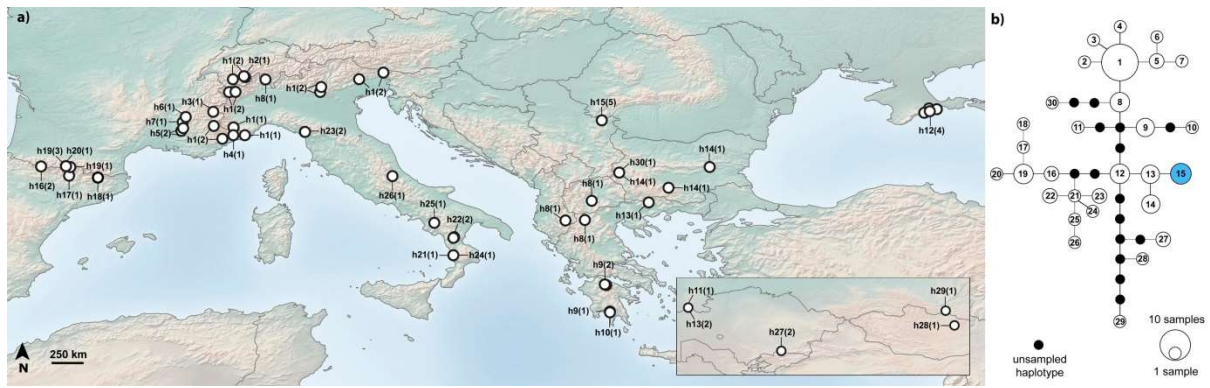


Fig. 4 a) Geographic distribution of COI haplotypes of *Satyrus ferula*. The lower right inset shows haplotype distribution in central Asia. Numbers in parentheses indicate the number of specimens for each haplotype; **b)** Maximum parsimony haplotype network based on COI sequences of *S. ferula*. The haplotype found in Romanian specimens (h15) is coloured in blue. The circles are scaled to the relative frequency of each haplotype in the data set; branches represent one-point mutational step

Melanargia larissa in Romania

Presence data

This species (Figs 5a,b) was observed in 2015 and 2016 south-east of Cozla (Figs 5c, S1b, Table S2). Adults were present between the end of June and the beginning of July and appeared to be restricted to a small area consisting of a rocky flowery slope (Fig. 5d). The congeneric butterfly *Melanargia galathea* (Linnaeus, 1758), a common and widespread species in Romania, was flying in sympatry with *M. larissa*.

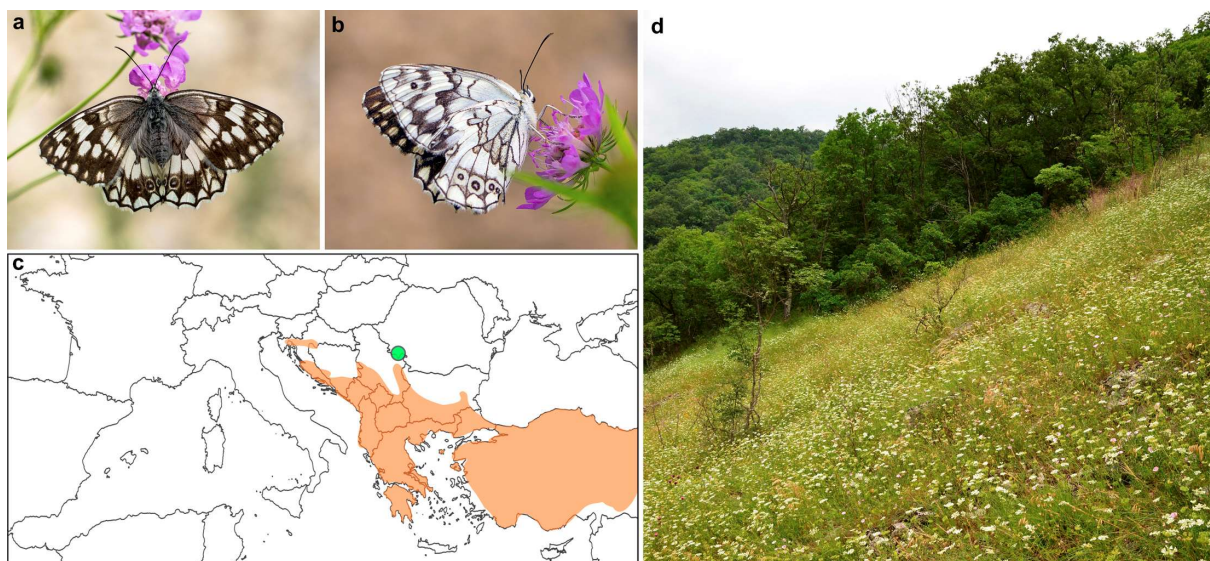


Fig. 5 *Melanargia larissa* in south-western Romania. **a.** Female, 19.06.2016; **b.** Female, 19.06.2016; **c.** Approximate distribution (orange) of *M. larissa* in the western Palearctic (the

green dot indicates the Romanian population); **d.** Typical habitat, a flowery slope near a rocky area with shrubs, 19.06.2016. Photos: B. Groza

Mitochondrial DNA

Thirty-seven haplotypes were detected among the 76 analysed specimens of *M. larissa* (Figs 6a,b; Table S1). The haplotype network displayed a complex structure but overall, haplogroups did not display strong geographic patterns, in agreement with previous research (Nazari et al. 2010). No haplotype displayed more than four mutations to its nearest haplotype. All six Romanian specimens analysed had the same haplotype (h16), which was not detected in other populations. This haplotype differed by one mutation from h17 from Serbia, representing the nearest sampled population to the Romanian site (approximately 50 km south).

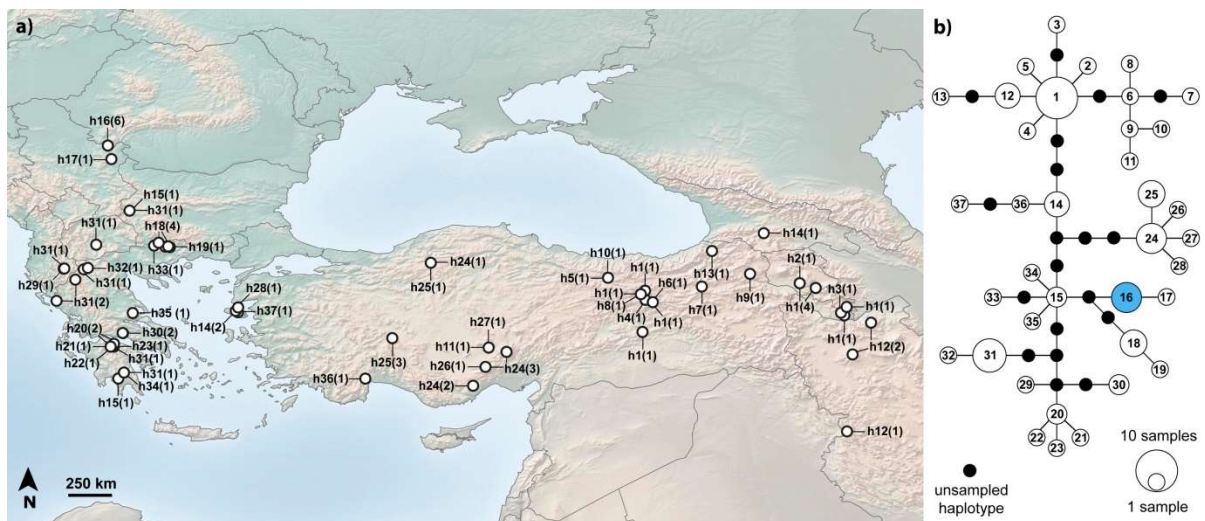


Fig. 6 a) Geographic distribution of COI haplotypes of *Melanargia larissa*. Numbers in parentheses indicate the number of specimens for each haplotype; **b)** Maximum parsimony haplotype network based on COI sequences of *M. larissa*. The haplotype found in Romanian specimens (h16) is coloured in blue. The circles are scaled to the relative frequency of each haplotype in the data set; branches represent one-point mutational step

Discussion

Iolana iolas, *S. ferula* and *M. larissa* in Romania: Populations close to the species' northern range limit

Iolana iolas was very rarely recorded in Romania (Székely 2008, Rákosy 2013) and was regarded as presumably extinct in the country (last published records in 1957, Stănoiu and Bobârnac 1965; and 1958, Popescu-Gorj 1959). From our study area, it has been recorded only once, based on a specimen found in 1911 (Rothschild 1912) (Fig. S1a).

Satyrus ferula and *M. larissa* were not reported from Romania prior to our findings (Rákosy et al. 2003; Rákosy 2003, 2013; Székely 2008), but we identified a museum specimen of *S. ferula* (collected in 1911 from the same area as our records) (Fig. S2) that has not been mentioned in previous publications.

Certain parts of south-western Romania can be regarded as relatively well-studied from a lepidopterological point of view. This is particularly the case of the Băile Herculane - Orșova area (Cerna river valley), which is situated between ca 30 and 45 km north-east of the areas where we report our findings (e.g. Rebel 1911; König 1975, 1979, 1998; Rákosy 1997).

However, publications targeting the area investigated by us are more sporadic and less comprehensive from a faunistic point of view (e.g. Rothschild 1912; Popescu-Gorj et al. 1972) (see the Electronic Supplementary Material for a more detailed overview of previous research in the area).

Both *S. ferula* and *M. larissa* from south-western Romania are close to their northern limit in the Balkans, while *I. iolas* is mainly distributed in Mediterranean Europe and populations of all three species are known from nearby sites in Serbia (Kudrna 2019, Miljević and Đurić 2020) (Figs 1c, 3c, 5c).

It is difficult to determine whether the presence of these species (or some of them) in south-western Romania is the result of range expansions and contractions (possibly northward expansions due to climate change), or if they have been surviving in this country for a longer time.

However, the mitochondrial DNA data suggest persistent populations of the three species in south-western Romania. The Romanian specimens of *S. ferula* and *M. larissa* represented haplotypes that were not shared with any other specimen in our dataset (Figs 4, 6). In the case of *I. iolas*, the Romanian specimens did share the single haplotype with other Balkan specimens, but the overall genetic variability of this species was very low (Fig. 2). Thus, the

genetic data available, combined with historical records from the study area and our field observations (i.e. extremely localized populations), suggest that these three species were not recently established in south-western Romania. Instead, they have probably been overlooked owing to insufficient research and the inaccessibility or very small area of their habitats. The discovery of these species close to their northern range limit in the Balkans also contributes to a better knowledge of their climatic niches across Europe, as it has been done with CLIMBER for European butterflies (Schweiger et al. 2014). Nevertheless, additional studies including more fine-scaled genetic analyses using other markers and additional sampling are desirable in order to further test the hypothesis of long-term populations in south-western Romania.

Apart from the data on Romanian populations, a notable genetic result is the minimal differentiation of the taxon *S. virbius*, occurring in Crimea and the middle part of Don and Volga rivers (Tshikolovets 2011). Although listed as a species in the latest checklist of European butterflies (Wiemers et al. 2018), this taxon has a debated status and it is unclear if it represents a distinct species or if it is conspecific with *S. ferula* (e.g. Tshikolovets 2011; Kudrna 2019). We found that this taxon, for which mitochondrial DNA data are provided here for the first time, represented a COI haplotype (h12) that displayed a single mutation with respect to a *S. ferula* haplotype documented in northern Greece and the southern Urals (Fig. 4). This minimal differentiation does not support a species status for this taxon, but further research on the topic using multiple sources of data in addition to mitochondrial DNA is needed. Finally, specimens of *S. ferula* from the southern Urals (h11 and h13), attributed to the subspecies *S. f. altaica* by some authors (e.g. Tshikolovets 2011), were not genetically similar to other *S. f. altaica* from Asia (h27-29), suggesting that this subspecies does not penetrate so much westwards.

Habitat and conservation status of *I. iolas*, *S. ferula* and *M. larissa* in south-western Romania

Iolana iolas is clearly associated with the presence of *Colutea* plants, which represent its unique larval host plant (Fig. 1d). We are unable to find any reasonable explanation to why the butterfly is apparently absent from all areas north-east of Dubova, where we did find *C. arborescens* plants in similar numbers as to the west of the same locality. Most of the flight period in Romania seems to be concentrated between mid-May and the end of June.

Satyrus ferula appears to be confined to certain rocky areas with sparse vegetation (Fig. 3d) in the extreme south of Banat region. It is somewhat surprising that no specimen was found in other nearby areas that appear to offer similar habitats. These areas include: the rocky formations near Topleț, Ciucaru Mare and Ciucaru Mic in the Danube Gorges, Domogled Mountain, Cerna Valley as well as Arjana Mountain.

Although usually associated to rocky areas, adults sometimes venture outside this habitat, likely in search for nectar or minerals (mud-puddling). While most specimens were generally found between 300-500 m of altitude, we were able to observe the species as low as 100 m. In terms of phenology, adults of *S. ferula* are on wing from mid-June to mid-July, probably with a peak towards the end of June, depending on the season. Although flight times partially overlap, *S. ferula* emerges earlier than *M. dryas*, the latter occurring in sympatry with *S. ferula* in all discovered locations.

Melanargia larissa occurs only on hot rocky areas with xeric herbaceous vegetation and shrubs (Fig. 5d). In contrast to *S. ferula*, no specimens were found descending to 100 m altitude near the Danube, or in open rocky areas where *S. ferula* was present. The distribution of *M. larissa* appears to be the most restricted of the three species reported here. Adults fly from June to mid-July. Given that specimens found on 04. and 05.07.2015 were very worn, we suspect that the butterfly may emerge as early as the first week of June. *Melanargia larissa* occurs in sympatry with *M. galathea*, and their phenologies partially overlap (the latter tends to start emerging slightly later than *M. larissa*).

Melanargia larissa is the most localised of the three species and we estimate that its extent of occurrence (EC) in Romania is 5-10 km². *Satyrus ferula* seems to be somewhat less demanding in terms of habitat and we estimate its EC to 10-20 km². *Iolana iolas* occurs along the shores of the Danube, with a possible EC of 10-20 km², but it requires the presence of *C. arborescens* plants, which are quite scarce in the area and their precise distribution is not known. Outside Romania, the nearest known populations from Serbia are approximately 90 km south for *I. iolas*, and 40 km south for *M. larissa*. Regarding *S. ferula*, one site in Serbia was reported just across the Danube, about 20 km west of our records, while other known populations are at least 45 km south (Miljević and Đurić 2020). Therefore, it appears that the Romanian populations are fairly isolated and immigration from neighbouring areas is unlikely, except perhaps for *S. ferula*, provided that the species can easily cross the river Danube.

399

400 Given the potentially suitable habitats that can be found in close vicinity, it is also possible
401 that these butterflies will expand their range on the Romanian territory. But it is also worth
402 noting that, due to the significant economic development in the area, these populations can be
403 affected by human activities. Both *S. ferula* and *M. larissa* may be impacted by shepherding
404 with goats, which can represent a serious threat. For example, about 40 km to the north-east,
405 near Topleț, we found potentially suitable habitats but most of them were overgrazed by
406 sheep and goats. Therefore, herding should be controlled in areas where *S. ferula* and *M.*
407 *larissa* reside. *Iolana iolas* is even more vulnerable especially due to landslides and human
408 activities (e.g. road margin burning, road works) that lead to the disappearance of *C.*
409 *arborescens*. We do recommend quick action to be taken by protecting the *Colutea* plants. As
410 a matter of fact, likely due to the instability of habitats with *Colutea* plants, *I. iolas* is
411 regarded as near threatened at European level, while *S. ferula* and *M. larissa* are listed as
412 least concern (van Swaay et al. 2010). *Colutea arborescens* is also considered as rare in the
413 Romanian flora and regarded as near threatened (Oprea 2005).

414

415 Finally, the COI haplotypes of Romanian *S. ferula* and *M. larissa* appear to be endemic.
416 While their level of differentiation is very low (one mutation) and further sampling might
417 reveal their presence outside Romania, they can be currently regarded as a unique fraction of
418 mitochondrial DNA diversity for these species. The fact that no genetic variability was
419 detected for any of the three species –a single COI haplotype was detected for each– also
420 suggests that the population sizes in Romania are very small and are possibly under severe
421 genetic erosion, highlighting the need for conservation actions.

422

423 **Conclusions**

424 Based on field observations, this study reported for the first time the presence of *M. larissa* in
425 the Romanian fauna. *Satyrus ferula* is also reported for the first time, based on field
426 observations and on a specimen (collected in 1911) found in the collections of HMNH,
427 Budapest. Both species are in south-western Romania close to their northerly distribution in
428 the Balkans (Kudrna 2019). *Iolana iolas* is rediscovered after having been regarded as extinct
429 in Romania (Rákossy et al. 2003; Rákossy 2003, 2013; Székely 2008) (last records in 1957 and
430 1958). Available mitochondrial DNA data suggest that *S. ferula* and *M. larissa* have endemic
431 COI haplotypes in south-western Romania, while *I. iolas* shares the single haplotype detected
432 with other specimens from the Balkans. No genetic variability was observed in any of the

Romanian populations based on the marker studied, suggesting low female effective populations sizes.

All three species seem to have very restricted extents of occurrence in south-western Romania. The discovered populations of *S. ferula* and *M. larissa* are threatened by overgrazing and *I. iolas* is threatened because areas with its larval host plant (*C. arborescens*) are susceptible to destruction by natural processes (e.g. landslides) and human activities (road margin burning, road works). We suggest that these species should be monitored and, possibly, become protected at national level.

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