ESM Annex 4 – Species pairs that are not reliably identified through DNA barcoding

Carcharodus flocciferus (Zeller, 1847) – *Carcharodus orientalis* Reverdin, 1913

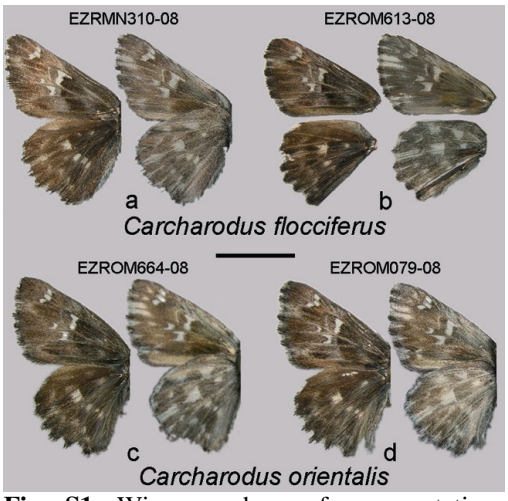

Fig. S1. Wing vouchers of representative barcoded *Carcharodus flocciferus* and *C. orientalis*. **a, b.** males of *C. flocciferus*. **c, d.** males of *C. orientalis*. Scale bar is 10 mm.

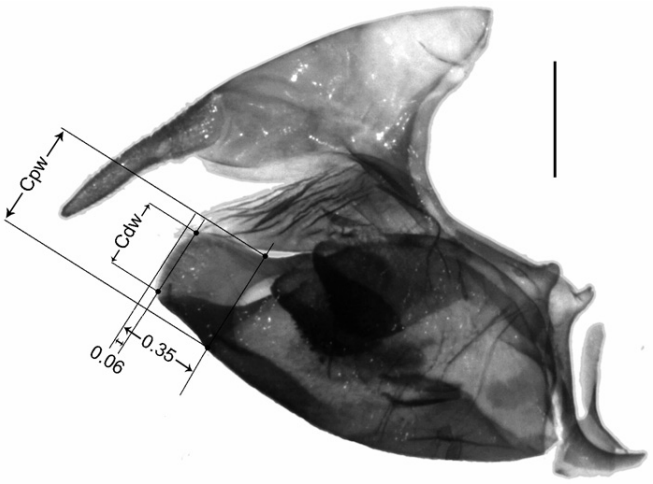

Fig. S2. Lateral view of representative male genitalia of *C. orientalis* indicating the elements measured for linear morphometry. Cpw – cuiller proximal width; Cdw – cuiller distal width. Scale bar is 0.5 mm.

Wing morphology: Does not allow for a reliable separation between the two species. No feature of the wing pattern is constant enough to be used as discriminative character (fig. S1a-d).

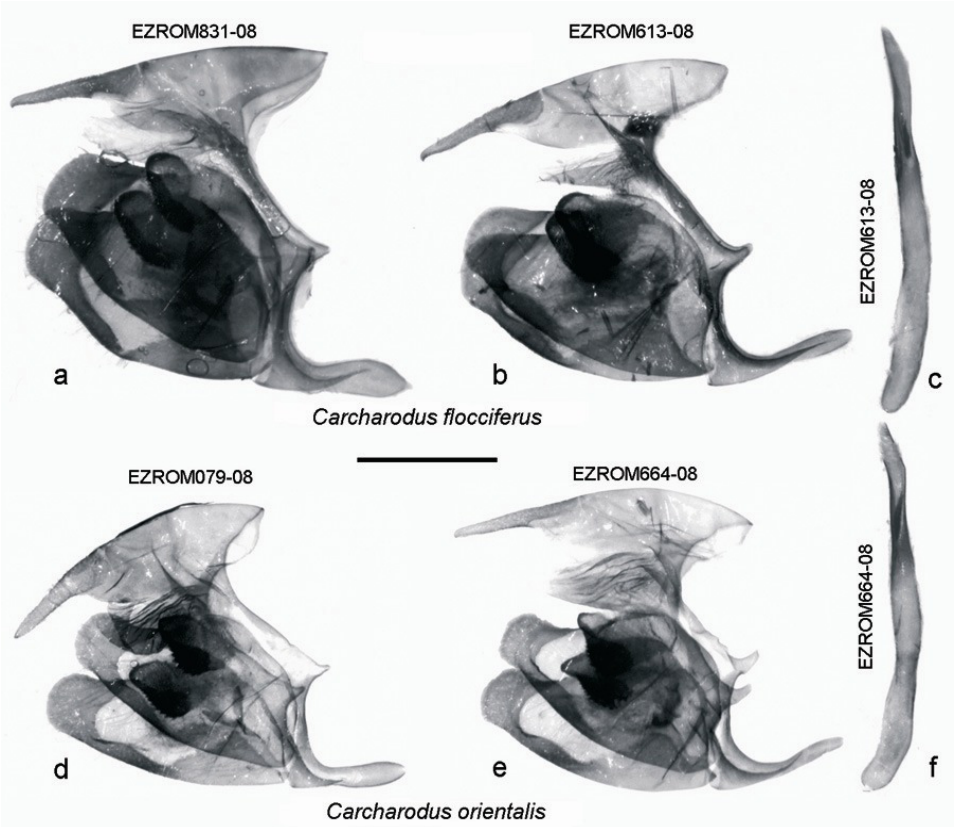

Fig. S3. Lateral view of representative male genitalia of *Carcharodus flocciferus* and *C. orientalis*. **a, b.** *C. flocciferus* (phallus removed). **c.** phallus of *C. flocciferus*. **d, e.** *C. orientalis* (phallus removed). **f.** phallus of *C. orientalis*. Scale bar is 1 mm.

Male genitalia: It can be used for the separation of the two species. The most useful character is the tip of the cuiller, which is broad in *C. flocciferus* and comparatively narrower in *C. orientalis* (Higgins 1975; Slamka 2004) (figs S2, S3a-f). The validity of this character was tested by employing both linear (fig. S2) and geometric morphometry (fig. 3 of the main text).

For linear morphometry, because the tip of the cuiller does not possess any constant landmarks, we measured the cuiller distal width (Cdw) at 0.06 mm from the tip of the cuiller and the cuiller proximal width (Cpw) at 0.35 mm (fig. S2). Based on our samples, these variables form two non-overlapping morphotypes that can be attributed to *C. flocciferus* and *C. orientalis* in a bivariate scatter plot (fig. S4).

For geometric morphometry, we defined two landmarks and 22 semi-landmarks. The two landmarks (marked as empty circles in fig. 3a of the main text) were defined as the intersection points with the margins of the cuiller generated by a transversal line crossing the cuiller at 0.35 mm distance from its tip (fig. 3a main text). The RWs explaining more than 1% of the variance were 15. A scatter plot of RW1 and RW2 (explaining respectively 37.01 and 19.63% of variance) revealed the presence of two discrete clusters of valva shape (fig. 3a main text) equivalent to those obtained with the linear morphometric analysis. By maximizing and minimizing inter-cluster and intra-cluster differences respectively, K-Means identified two clusters. RW1 revealed to be significantly different among the two clusters thus representing the variable responsible for the discrimination (table S1). When compared with the scatter plot of fig. 3a (main text), K-Means confirmed the validity of the two clusters.

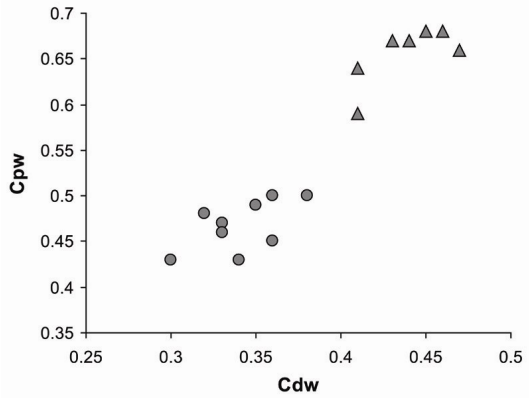

Fig. S4. Male specimen bivariate scatter plot using the cuiller proximal width (Cpw) and the cuiller distal width (Cdw) as discriminative characters. Circles – *Carcharodus orientalis*; triangles – *C. flocciferus*.

Table. S1. ANOVA table for K-Means clustering. In bold the variable showing significant differences between the two clusters.

Rel. warp	Cluster Mean Square	df	Error Mean Square	df	F	Sig.
RW1	**0.0635453**	**1**	**0.0009129**	**14**	**69.607679**	**8.371E-07**
RW2	5.7327E-05	1	0.000751331	14	0.076300631	0.786410559
RW3	0.000157239	1	0.000597702	14	0.263072691	0.616014176
RW4	1.93113E-05	1	0.000172418	14	0.112002754	0.742836619
RW5	8.89821E-05	1	0.000114223	14	0.779017833	0.392344999
RW6	6.59647E-06	1	8.45746E-05	14	0.077995941	0.78411518
RW7	3.43123E-06	1	2.52897E-05	14	0.135677036	0.718126957
RW8	6.35191E-08	1	1.95141E-05	14	0.003255046	0.955309261
RW9	1.88692E-06	1	1.23221E-05	14	0.15313348	0.701449826
RW10	1.88742E-07	1	9.45028E-06	14	0.019972072	0.889628511
RW11	3.23497E-06	1	7.24223E-06	14	0.4466808	0.514784216
RW12	1.3563E-07	1	6.90462E-06	14	0.019643416	0.890534002
RW13	2.45177E-07	1	3.32574E-06	14	0.073721062	0.789956718
RW14	5.72565E-07	1	2.26356E-06	14	0.252949119	0.622825312
RW15	4.10348E-10	1	1.933E-06	14	0.000212286	0.988580812

DNA barcoding: *Carcharodus flocciferus* is paraphyletic with respect to *C. orientalis* (fig. 3b main text). The minimum interspecific distance between the two taxa is 0.46%. Since no barcodes are shared, species-level identifications seem possible given the current data, but more sampling is needed to test the robustness of these results.

Comments: Although described originally as a species, *C. orientalis* has for a long time been treated as a subspecies of *C. flocciferus* (e.g. Higgins 1975). However, karyological data (Lesse 1960) and further taxonomical studies (Jong 1974) suggested that *C. orientalis* is a distinct species so that this taxon is currently treated accordingly by most authors. Cohabitation between the two species has been reported from Greece (Lafranchis 2003) and it is strongly suspected in south-eastern Romania (Dincă *et al*. 2009).

In Romania, *C. flocciferus* is relatively widespread (except for Dobrogea where it appears to be very local) (Rákosy *et al*. 2003; Dincă *et al*. 2009). *Carcharodus orientalis* is

currently known only from the extreme east and south-east of the country (Dobrogea – relatively widespread, and a handful of sites in Moldavia) (Rákosy & Varga 2001; Székely 2008; Dincă *et al*. 2009).

***Colias crocea* (Fourcroy, 1785) – *Colias erate* (Esper, 1805)**

Wing morphology: The two species (but especially *C. erate*) display impressive intraspecific variability. Their separation is possible for more or less typical specimens (fig. S5a,e,f). However, given the large number of specimens bearing intermediate characters between the two species (fig. S5b-d), reliable identifications based on wing morphology alone can sometimes be unreliable. In fact, *C. erate* is known to display a large number of forms in Romania (Popescu-Gorj 1978), some of them closely resembling *C. crocea*. It is worth mentioning that the presence of an oval androconial patch at the base of the hindwing upperside is not necessarily a diagnostic sign for *C. crocea*, as some forms of *C. erate* may also have it (Popescu-Gorj 1978). Moreover, certain specimens with wing morphology closer to *C. crocea* than *C. erate* may lack this patch (e.g. fig. S5b).

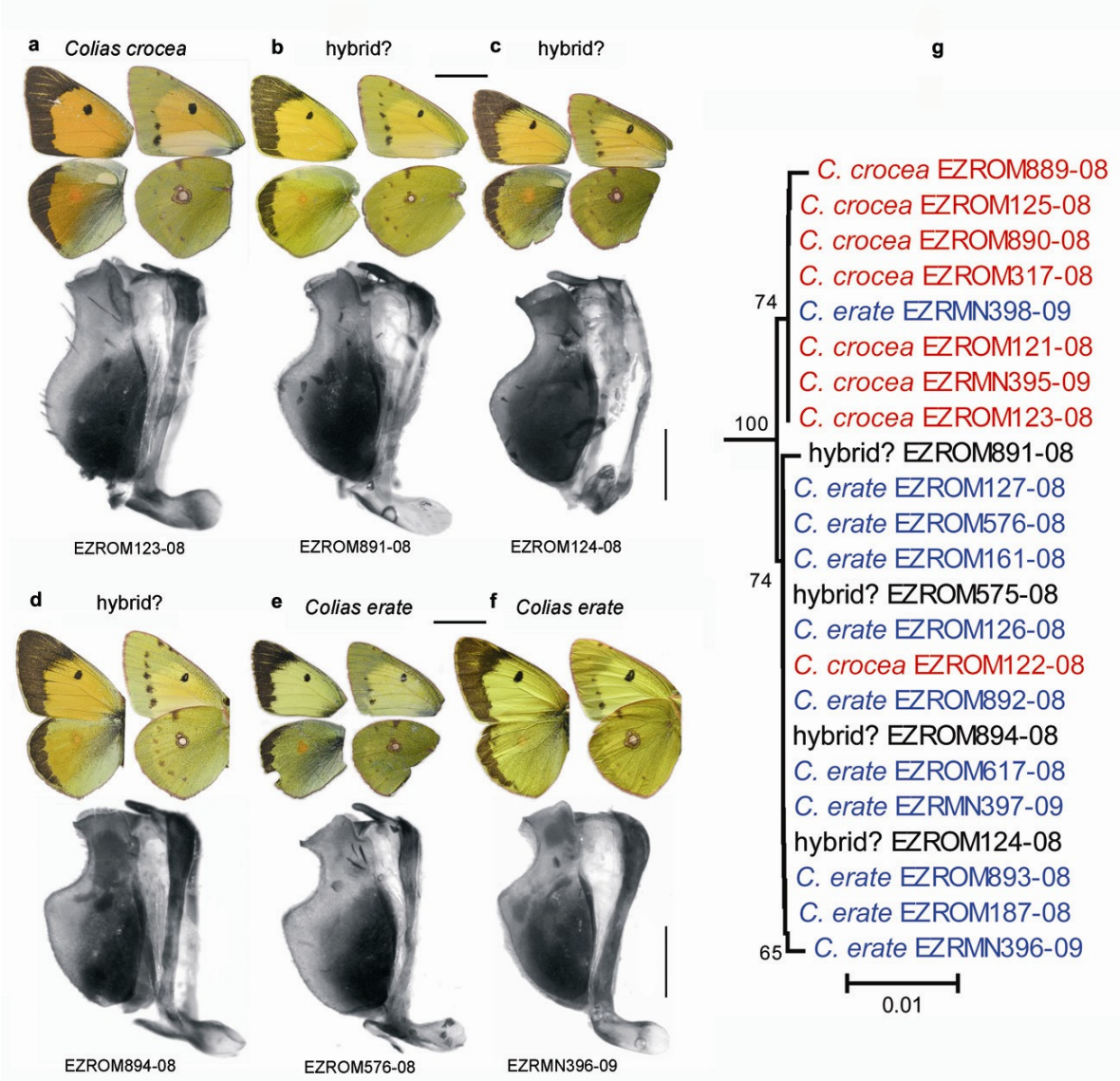

Fig. S5. Wing vouchers and male genitalia (lateral view) of representative barcoded *C. crocea* (**a**), *C. erate* (**e, f**) and three possible hybrids (**b-d**). **g.** Neighbour-joining tree of *COI* barcodes of Romanian *C. crocea* and *C. erate* with bootstrap values >50% indicated.

Male genitalia: Typical specimens of both species (fig. S5a,d-f) can be reliably differentiated due to differences in the posterior border of the valvae (evenly rounded in *C. crocea* and strongly angled in *C. erate*) (e.g. Niculescu 1963; Popescu-Gorj 1978; Higgins 1975; Slamka 2004). Nevertheless, based on our material, we found that this character is also subject to individual variability to the extent that it becomes difficult to assign certain specimens to any species (e.g. fig. S5e,f).
DNA barcoding: The two species share barcodes (fig. S5g). A similar situation was reported for Central Asia (Lukhtanov *et al.* 2009). This phenomenon is likely to reflect the presence of hybrids, or to be the consequence of historical introgression, as natural hybrids have been recorded (Alberti 1943; Tolman & Lewington 2008; Descimon & Mallet 2009).

***Pieris napi* (Linnaeus, 1758) – *Pieris bryoniae* (Hübner, 1806)**

Wing morphology: For the Romanian specimens, identification is reliable for females, which are much darker in *P. bryoniae* than in *P. napi* (fig. S6d,h,l), but can be difficult for males. This is due to possible confusions between *P. bryoniae* and certain specimens of the spring brood of *P. napi* that have the dark veins on the underside of the hindwings well developed (e.g. fig. S6c). The summer brood of *P. napi* is easier to distinguish from *P. bryoniae* (fig. S6e-h).
Male genitalia: It does not allow for the reliable separation of the two species (fig. S7a-d) (Higgins 1975, Gorbunov 2001).
DNA barcoding: 27 of the 29 specimens examined formed two clades with a minimum interspecific divergence of 1.85% (fig. S8). However, two specimens identified as *P. napi*, which could actually represent hybrids, (fig. S6a,h) clustered with *P. bryoniae*. Although *P. bryoniae* has not been recorded from the area where they were collected (the base of the Domogled Mountain, Pecinișca), it is known to occur between ca. 900-1700 m in the mountains situated about 50 km to the north (Retezat) (Rákosy 1997). However, it could occur much nearer as the whole area is connected by peaks of more than 1000 m. Even the area of the Domogled Mountain, which reaches 1000 m, hosts several mountainous species such as *Aricia artaxerxes* (Fabricius, 1793), *Erebia ligea* (Linnaeus, 1758), *Erebia euryale* (Esper, 1805), *Erebia melas* (Herbst, 1796).
Comments: *Pieris bryoniae* is a taxon with controversial status. Recently, it is most often treated as a distinct species, but also as a subspecies of *P. napi* (e.g. Higgins *et al*. 1991; Gorbunov 2001). Studies based on enzyme electrophoretic approaches pointed out the high similarity between the two taxa (e.g. Geiger & Scholl 1985; Geiger 1990). The two taxa are known to hybridize regularly where their ranges contact (Geiger & Shapiro 1992; Porter & Geiger 1995; Porter 1997; Descimon & Mallet 2009).

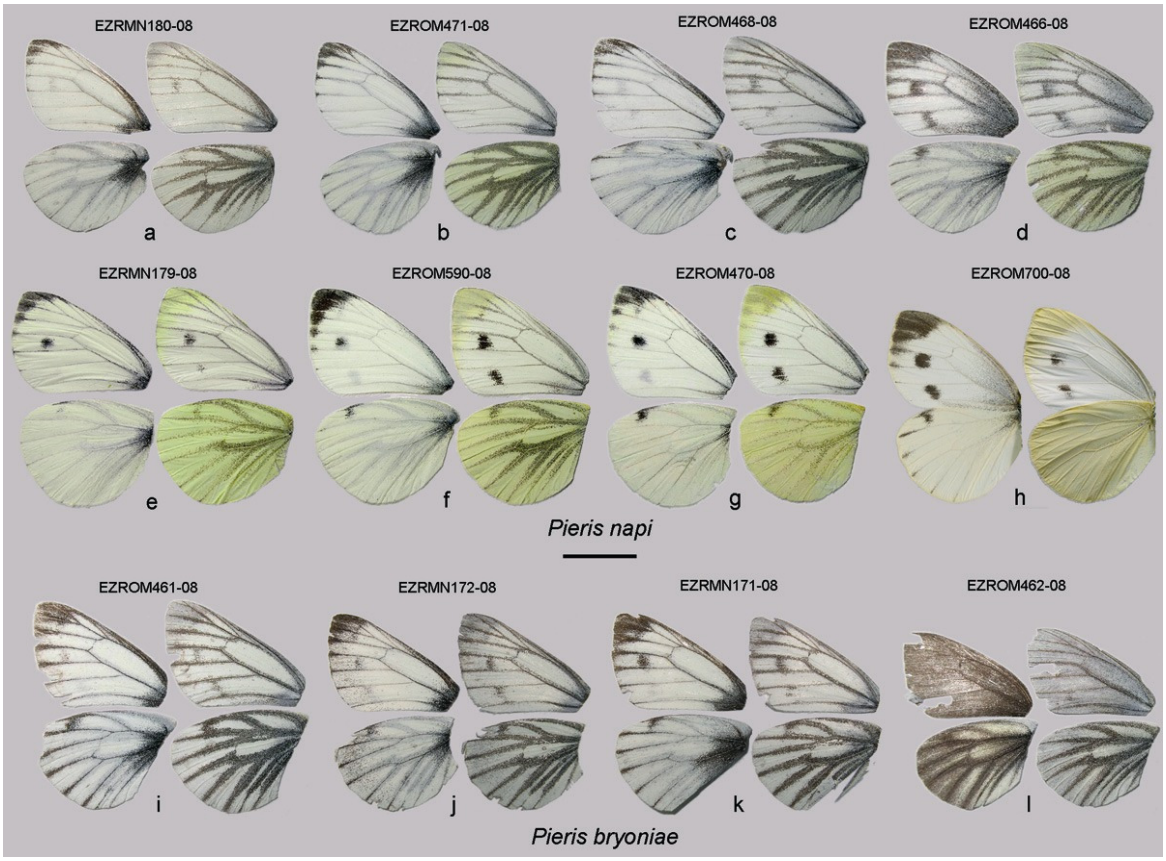

Fig. S6. Wing vouchers of representative barcoded *Pieris napi* and *P. bryoniae*. **a-c.** *P. napi* (males of the spring brood). **d.** *P. napi* (female of the spring brood). **e-g.** *P. napi* (males of the summer brood). **h.** *P. napi* (female of the summer brood). **i-k.** males of *P. bryoniae*. **l.** female of *P. bryoniae*. Scale bar is 10 mm.

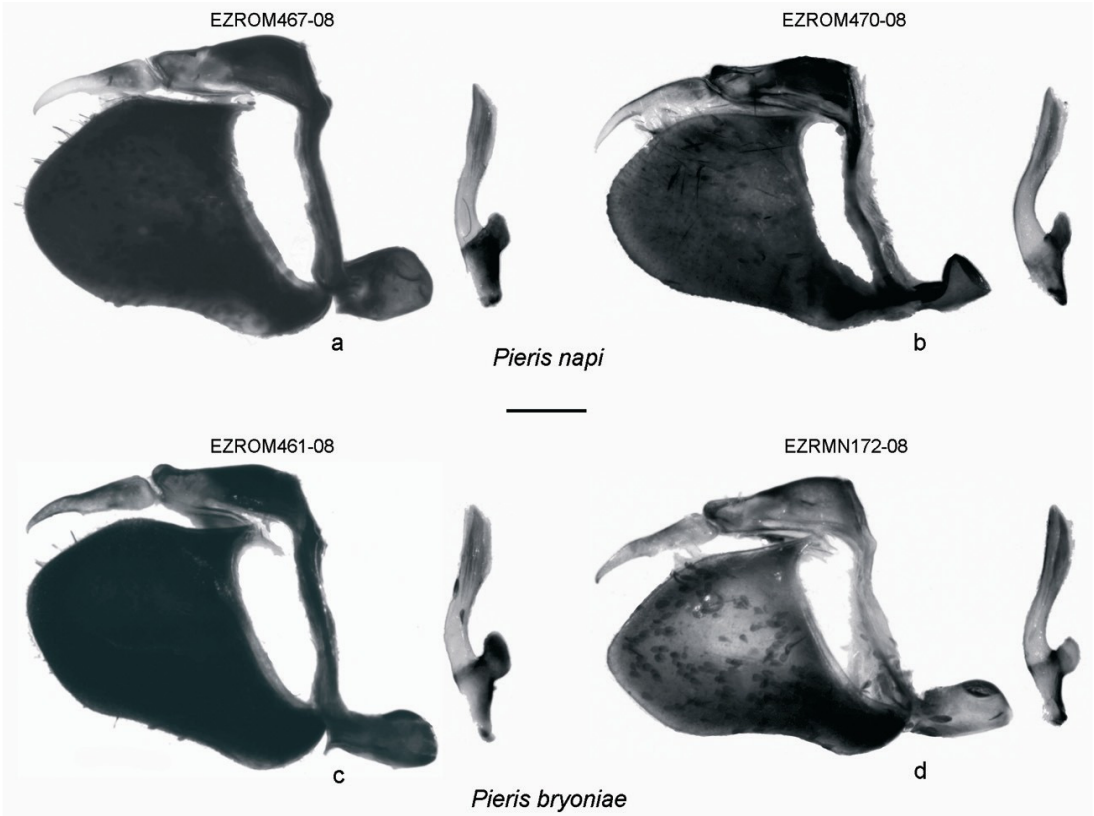

Fig. S7. Lateral view of representative male genitalia of *Pieris napi* (**a, b**) and *P. bryoniae* (**c, d**). Scale bar is 0.5 mm.

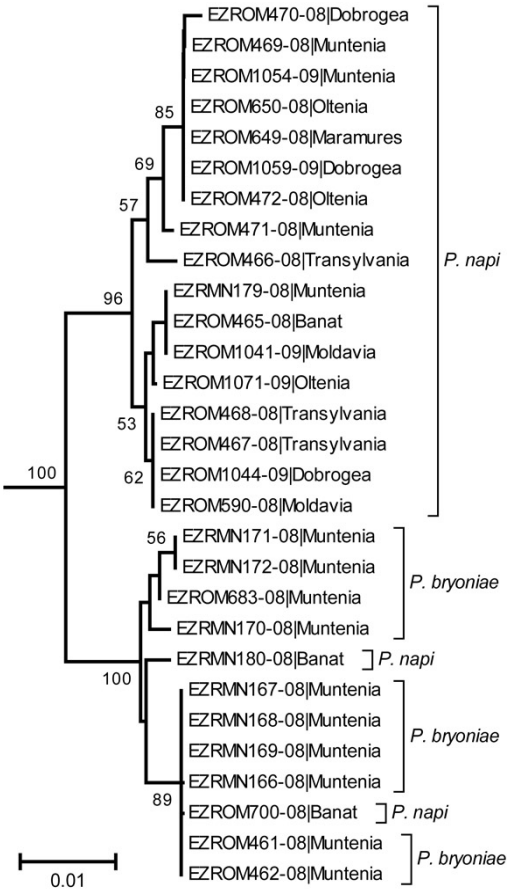

Fig. S8. Neighbour-joining tree of *COI* barcodes of Romanian *Pieris napi* and *P. bryoniae* with bootstrap values >50% indicated.

Coenonympha tullia (Müller, 1764) – *Coenonympha rhodopensis* Elwes, 1900

Wing morphology: Romanian specimens of *C. rhodopensis* lack (or have very little developed) ocelli on the hindwing underside (fig. S9c,d) and can be distinguished from *C. tullia* where these ocelli are more developed (fig. S9a,b) (Rákosy 1993).

Male genitalia: The material examined by us displayed differences in the shape of the valva of the male genitalia, which is more slender in the terminal part in *C. rhodopensis* compared to *C. tullia* (fig. S10a-c). Differences in the male genitalia were also reported by Rákosy (1993).

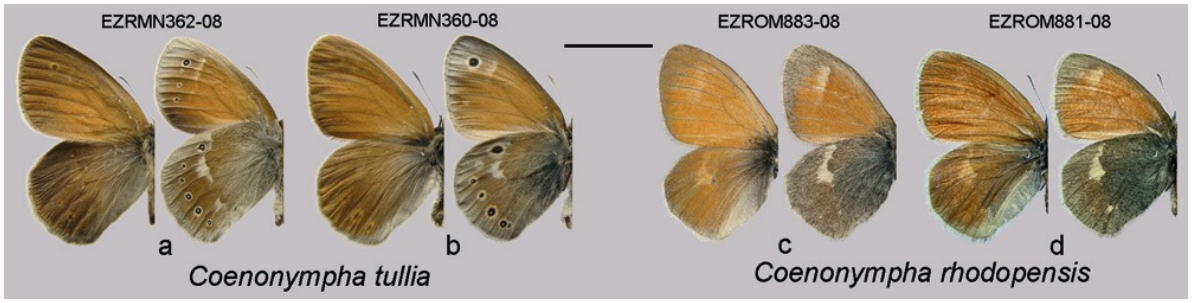

Fig. S9. Wing vouchers of representative barcoded specimens of *Coenonympha tullia* (**a, b**) and *C. rhodopensis* (**c, d**). Scale bar is 10 mm.

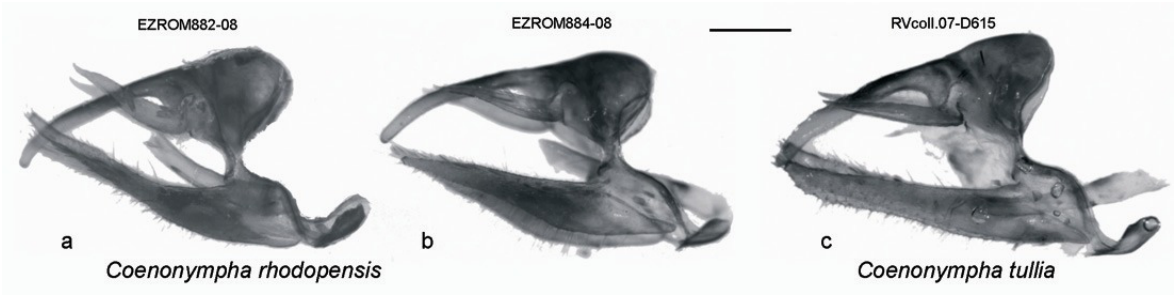

Fig. S10. Lateral view of representative male genitalia of *C. rhodopensis* (**a, b**) and *C. tullia* (**c**). Scale bar is 0.5 mm.

DNA barcoding: *Coenonympha rhodopensis* is recovered as paraphyletic with respect to *C. tullia* (fig. S11). The minimum interspecific distance is only 0.3%, but no barcodes are shared between the two taxa. Although species-level identifications seem possible based on unique haplotypes for each species, more sampling is needed to test the robustness of these results.

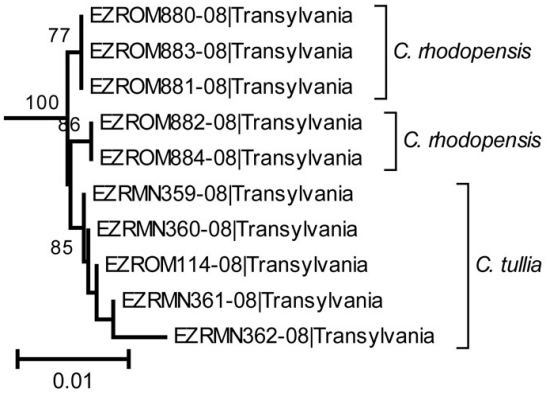

Fig. S11. Neighbour-joining tree of *COI* barcodes of Romanian *Coenonympha rhodopensis* and *C. tullia* with bootstrap values >50% indicated.

Erebia euryale (Esper, 1805) – *Erebia ligea* (Linnaeus, 1758)

Fig. S12. Wing vouchers of representative barcoded males of *Erebia euryale* and *E. ligea*. The detail of the forewing under strong transmitted light to the right of each specimen illustrates the absence/presence of androconial patches. **a, c.** *E. euryale*. **b, d.** *E. ligea*. Scale bars represent 10 mm.

Wing morphology: The two species can be separated through wing morphology. Males of *E. ligea* can be easily identified by the presence of androconial patches on the forewings (these are well visible in strong light) (fig. S12a-d). Such patches lack in *E. euryale* (Sonderegger 2005; Slamka 2004; Tolman & Lewington 2008). The females are especially distinct in the pattern on the underside of the hindwings.

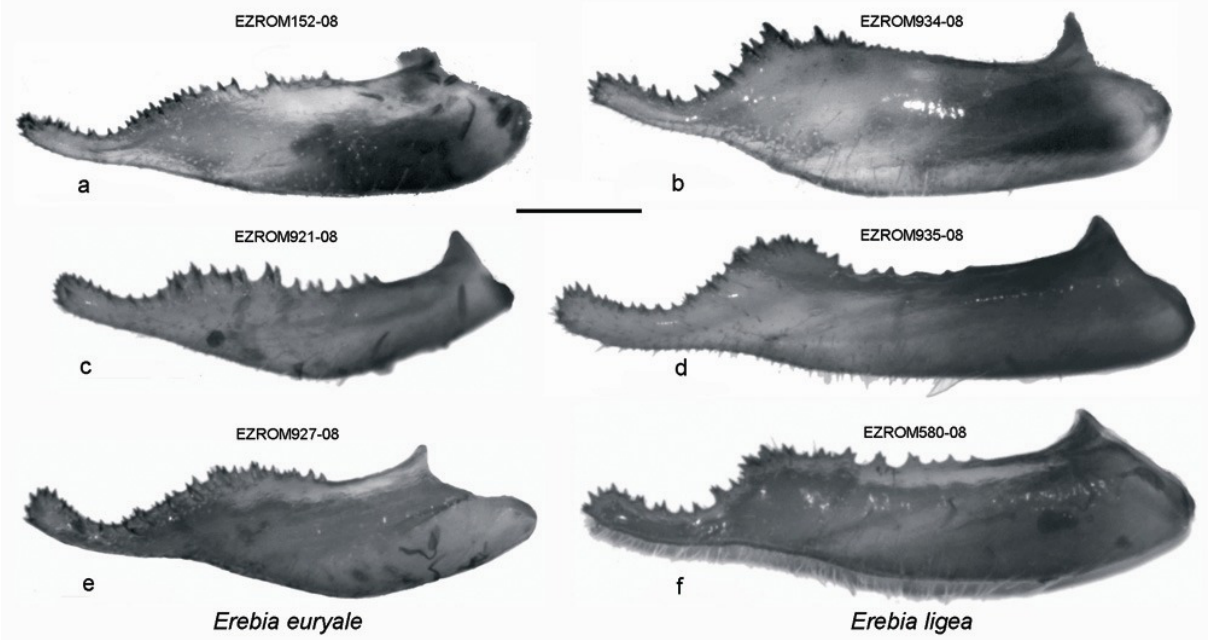

Fig. S13. Lateral view of representative valvae of male genitalia of *Erebia euryale* (**a, c, e**) and *E. ligea* (**b, d, f**). Scale bar is 1 mm.

Male genitalia: It is usually reliable for identification. Although the specimens examined by us were found to display considerable variability in the male genitalia, *Erebia ligea* has larger and more irregular teeth on the valva while in *E. euryale* these teeth are usually smaller and distributed in a partial double series (Higgins 1975). In *E. ligea*, the terminal part of the valva tapers in a more pronounced way than in *E. euryale* (Sonderegger 2005) (fig. S13a-f).

DNA barcoding: The NJ tree displays a complex pattern involving polyphyly (fig. S14). Although no barcodes are shared between the two species, the minimum interspecific distance is very low (0.15%). This situation suggests either incomplete lineage sorting or local hybridization events.

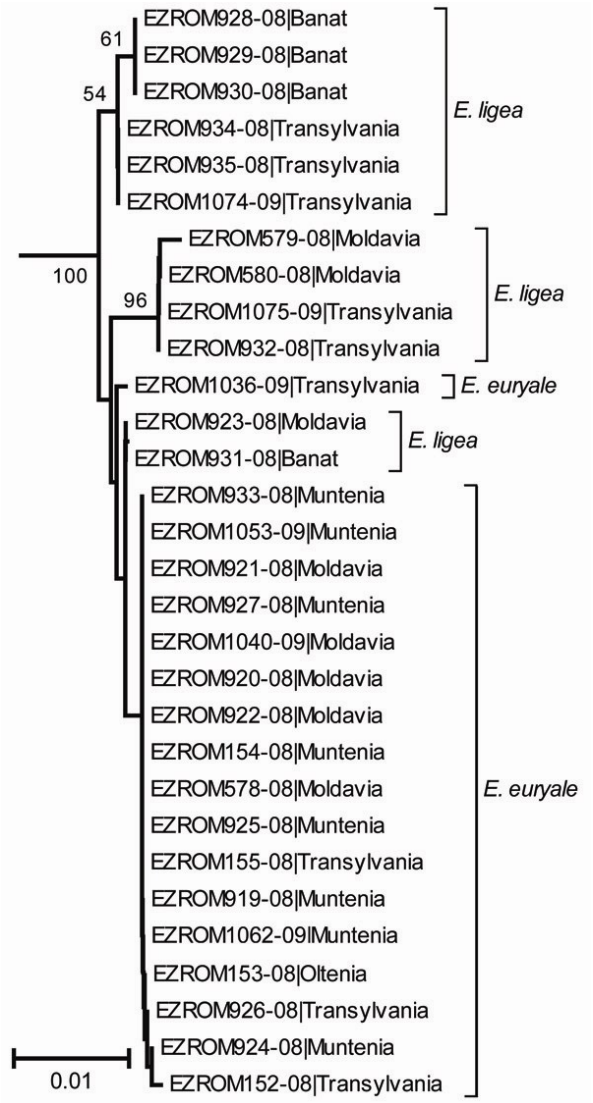

Fig. S14. Bootstrap neighbour-joining tree of *COI* barcodes of Romanian *Erebia ligea* and *E. euryale* with bootstrap values >50% indicated.

Hipparchia semele (Linnaeus, 1758) – *Hipparchia volgensis* (Mazochin-Porshnjakov, 1952)

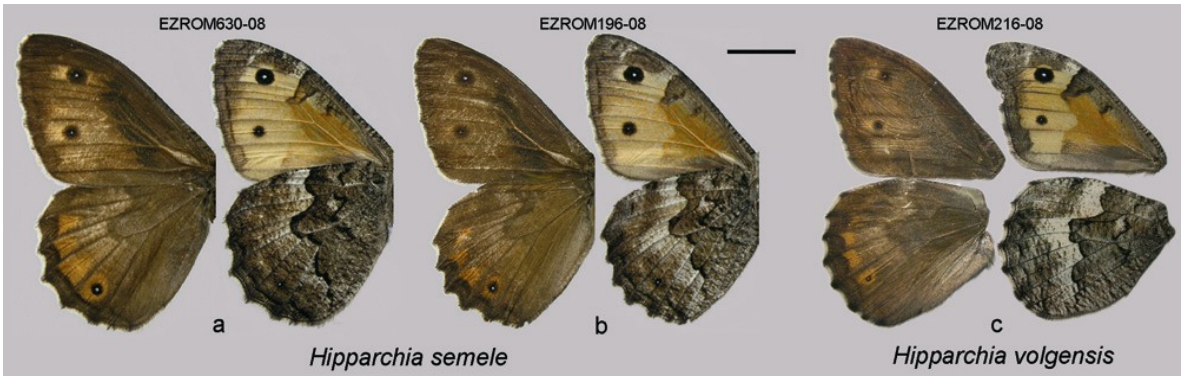

Fig. S15. Wing vouchers of representative barcoded *Hipparchia semele* and *H. volgensis*. **a, b.** males of *H. semele*. **c.** male of *H. volgensis*. Scale bar is 10 mm.

Wing morphology: These two species are indistinguishable based on wing morphology (fig. S15a-c) (Kudrna 1977; Lafranchis 2004; Tolman & Lewington 2008; Pamperis 2009).

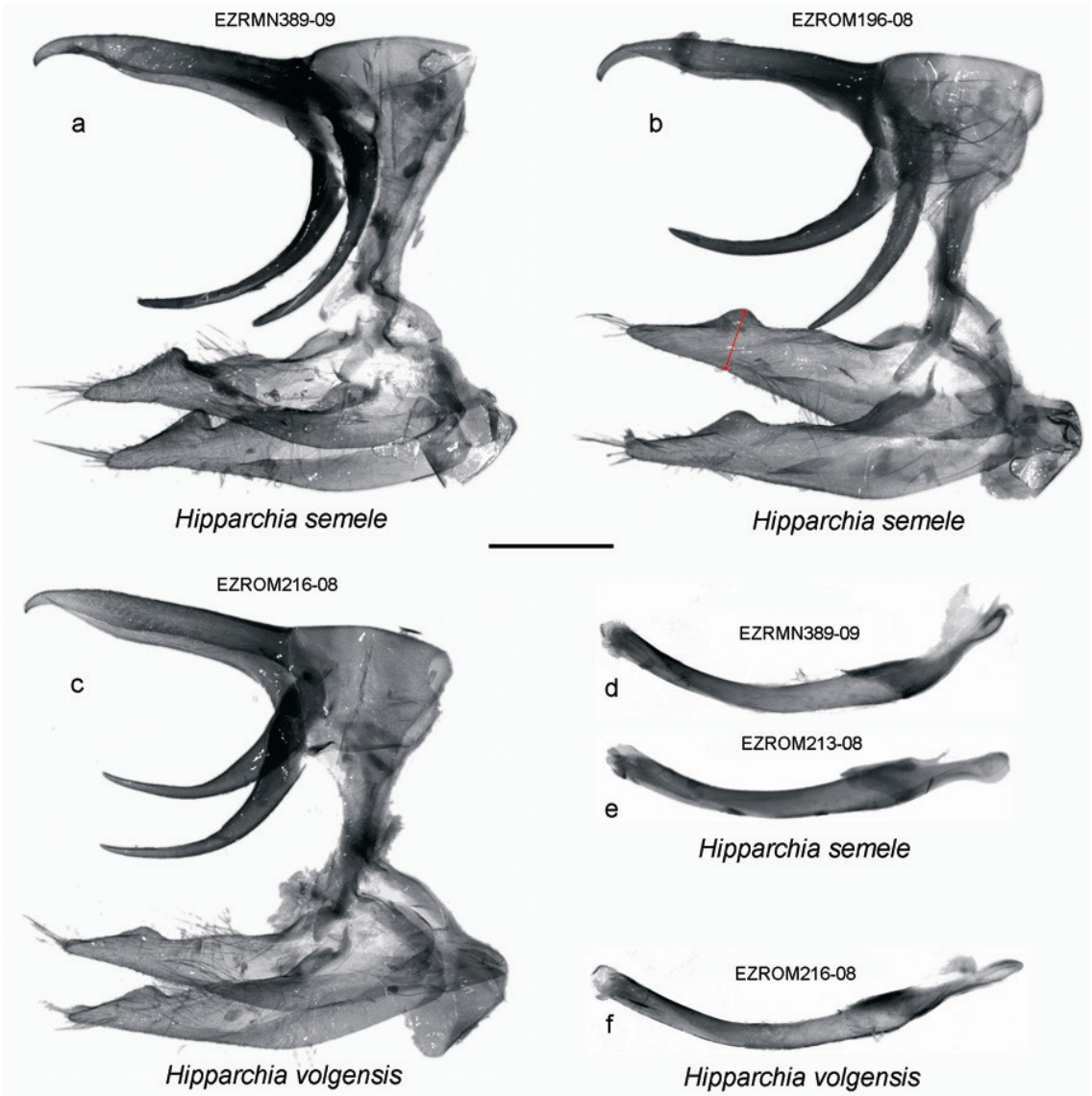

Fig. S16. Lateral view of male genitalia of representative *Hipparchia semele* and *H. volgensis*. **a, b.** male genitalia of *H. semele*. **c.** male genitalia of *H. volgensis*. **d, e.** phallus of *H. semele*. **f.** phallus of *H. volgensis*. Scale bar is 1 mm.

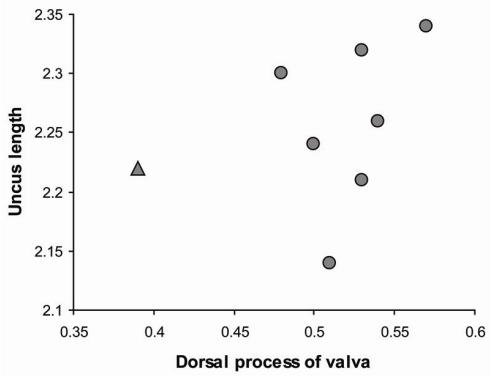

Fig. S17. Male specimen bivariate scatter plot using the dorsal process of valva and the uncus length as discriminative characters.

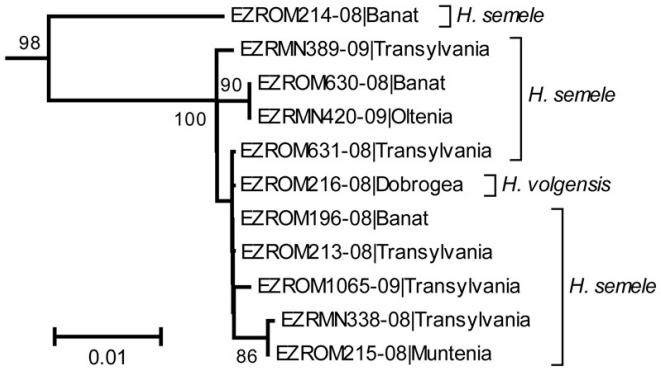

Fig. S18. Neighbour-joining tree of *COI* barcodes of Romanian *Hipparchia semele* and *H. volgensis* with bootstrap values >50% indicated.

Male genitalia: By using linear morphometry, we found that the dorsal process of the male valva (fig. S16b) is considerably less pronounced in our specimen of *H. volgensis* compared to the analyzed specimens of *H. semele* (fig. S16a-c, S17). This feature has been mentioned as one of the main diagnostic characters of *H. volgensis* (Kudrna 1977).
DNA barcoding: Our single specimen of *H. volgensis* appears to have identical barcode with several specimens of *H. semele* (fig. S18). Surprisingly, it is one specimen with genitalia of *H. semele* type that displays a rather high maximum intraspecific divergence of 3.5%. The correct interpretation of this pattern, especially taking into account the uncertainties on the taxonomic status of *H. volgensis*, is very difficult without additional material and further studies are needed.
Comments: *Hipparchia volgensis* is a taxon that still has a controversial status. According to Kudrna (1977), in the Balkans occurs *H. v. delattini* Kudrna, 1975. However, the taxon *delattini* was sometimes raised to species rank by some authors (e.g. Wakeham-Dawson *et al.* 2004; Pamperis 2009). Morphometrical analyses of the male genitalia involving *H. semele*, *H. volgensis* and *H. muelleri* showed rather inconclusive results (Wakeham-Dawson *et al.* 2003, 2004). In Romania, *H. v. delattini* has been recorded only recently (Rákosy 1998). The specimen barcoded here was collected in the same area from where it has been first recorded in Romania (Măcin Mountains). The locality where it was collected, plus the small dorsal process of the valva in the male genitalia, led us to assign it to *H. v. delattini*. The lack of genetic divergence in DNA barcodes is not in favour of the specific distinctness of the taxon *delattini* but it is also not sufficient to allow definitive conclusions. More material and additional studies are needed in order to correctly evaluate the relationship between *H. semele* and *H. v. delattini*.

***Hipparchia fagi* (Scopoli, 1763) – *Hipparchia syriaca* (Staudinger, 1871)**

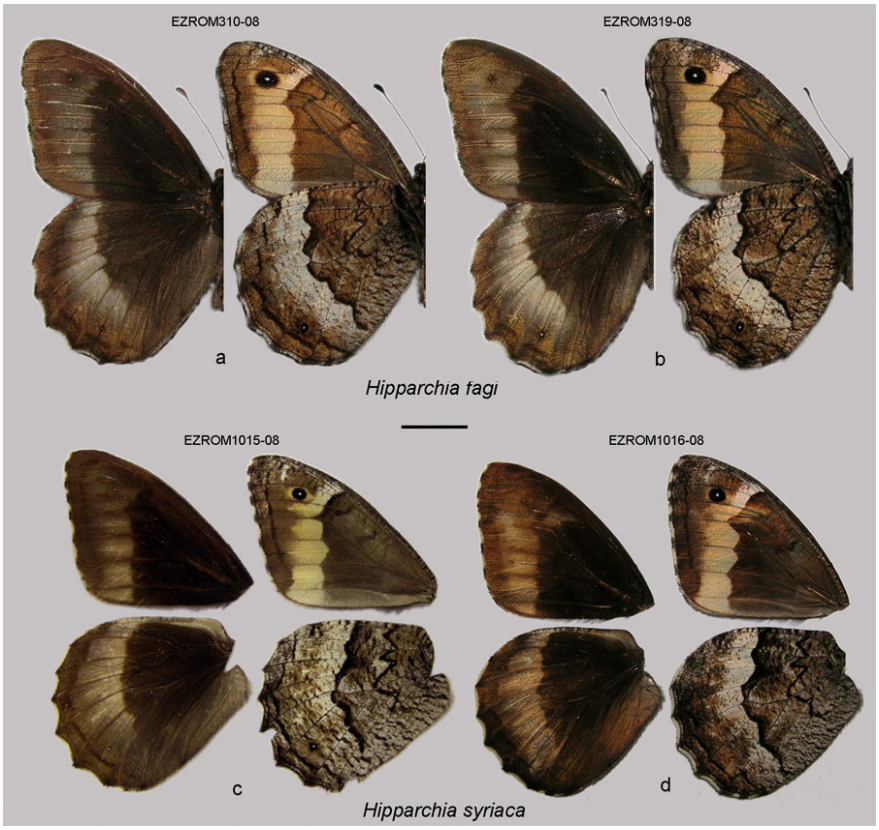

Fig. S19. Wing vouchers of representative barcoded *Hipparchia fagi* (**a, b**) and *H. syriaca* (**c, d**). Scale bar is 10 mm.

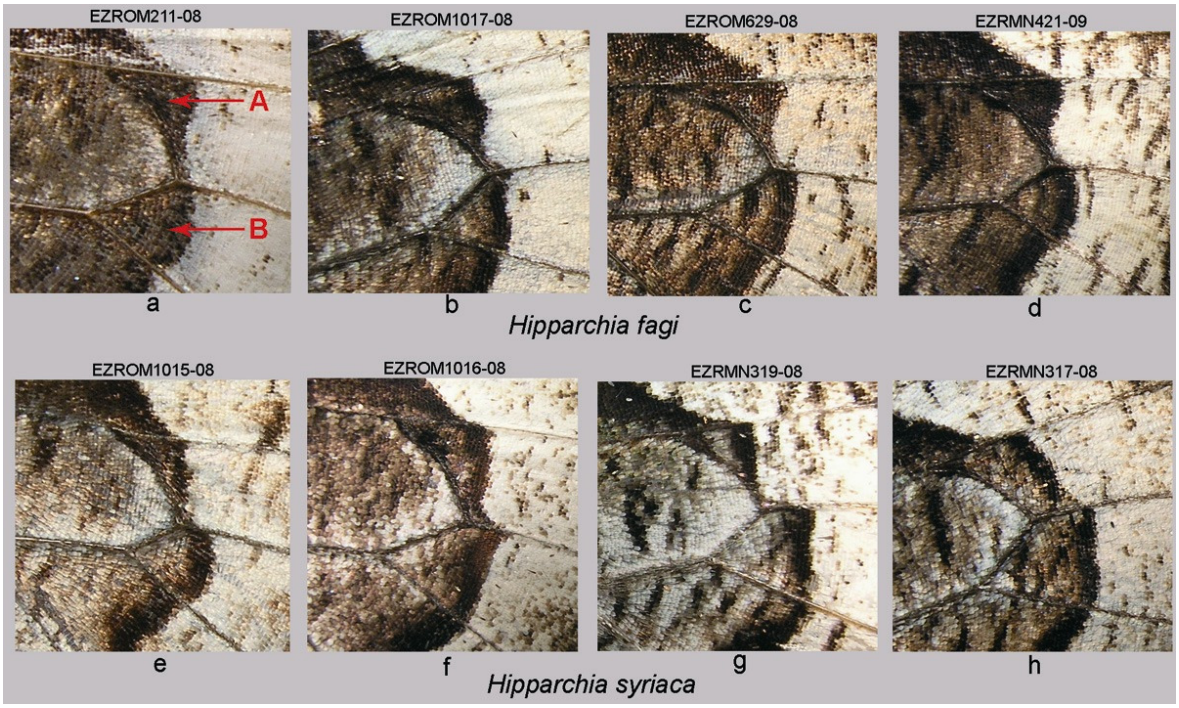

Fig. S20. Detail of the underside of the hindwings for representative barcoded specimens of *Hipparchia fagi* (**a-d**) and *H. syriaca* (**e-h**). A – indicates the dark base of s4; B – indicates the dark base of s3.

Wing morphology: It does not allow for a reliable separation between the two species (fig. S19a-d). Pamperis (2009), based on data from Hesselbarth *et al*. (1995), mentioned that the dark base of s4 (area A) and s3 (area B) on the underside hindwings (fig. S20a) are useful for identification: if A > B - *H. fagi* and if A < B - *H. syriaca*. We tested this feature on our barcoded specimens and found that it is accurate only in some cases (fig. S20a-h). For example, the differences between samples EZROM629-08 (*H. fagi*) and EZROM1016-08 (*H. syriaca*) (fig. S20c,f) are small and, without examination of the genitalia, it would be very difficult to tell to which species these specimens belong to. Since this overlap already appeared in a relatively small sample, it is likely that such cases are not rare so that identifications based on this character should be regarded with reserves.

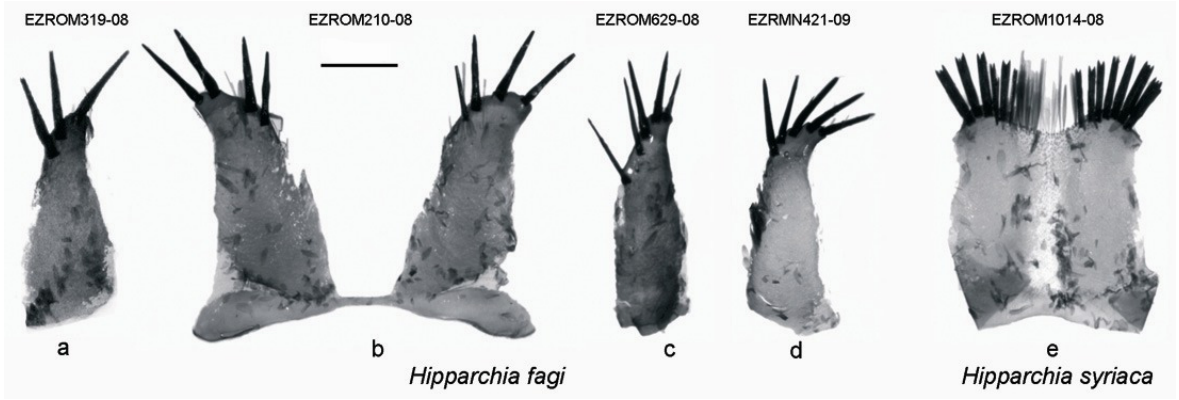

Fig. S21. Jullien organ of the male genitalia of representative *Hipparchia fagi* and *H. syriaca*. **a.** right lamella of the Jullien organ of *H. fagi* bearing three batons. **b.** Jullien organ of *H. fagi* bearing four batons. **c, d.** right lamella of the Jullien organ of *H. fagi* bearing five batons. **e.** Jullien organ of *H. syriaca*. Scale bar is 1 mm.

Male genitalia: It provides reliable characters for identification. On of the most obvious is related to the Jullien organ (Higgins 1975): lateral lamellae are narrow, each bearing three

to five long slender batons, in *H. fagi* (fig. S21a-d), while lamellae are wider and close together, each bearing eight thick batons, in *H. syriaca* (fig. S21e).

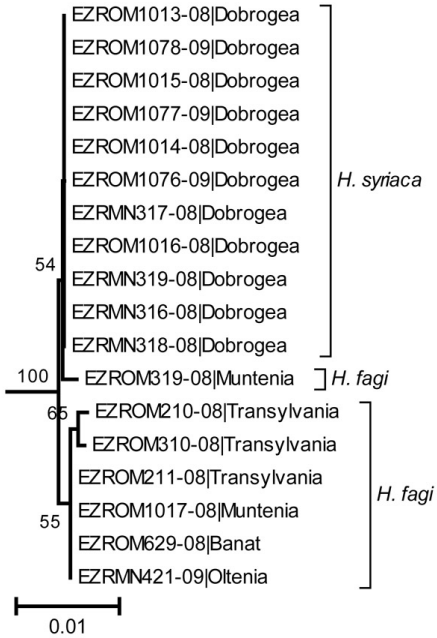

Fig. S22. Neighbour-joining tree of *COI* barcodes of Romanian *Hipparchia fagi* and *H. syriaca* with bootstrap values >50% indicated.

DNA barcoding: *Hipparchia fagi* is recovered as paraphyletic with respect to *H. syriaca* (fig. S22). Although no barcodes are shared, the minimum interspecific distance is very low (0.15%). Although species-level identifications seem possible given the current data, more sampling is needed to test the robustness of these results.

Apatura ilia ([Denis & Schiffermüller], 1775) – *Apatura metis* Freyer, 1829

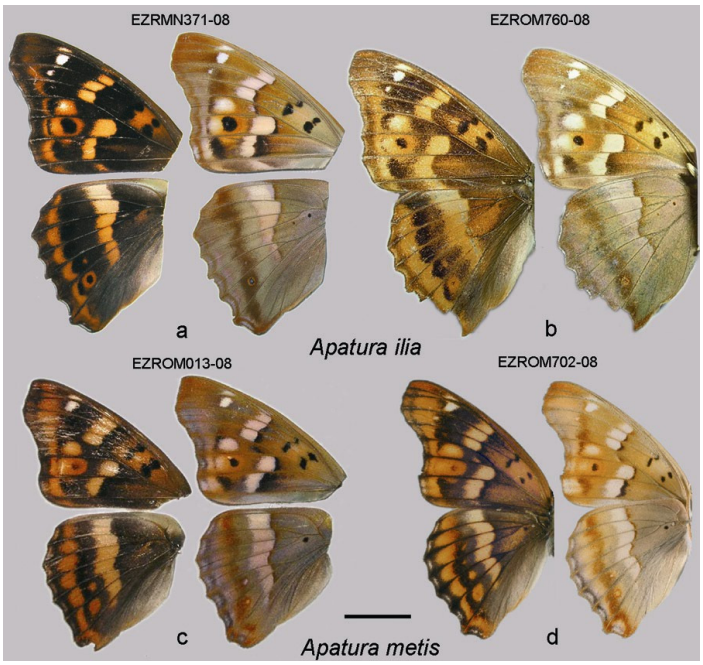

Fig. S23. Wing vouchers of representative barcoded *Apatura ilia* and *A. metis*. **a, b.** males of *A. ilia* f. *clytie*. **c.** spring brood of *A. metis* (male). **d.** summer brood of *A. metis* (male). Scale bar is 10 mm.

Wing morphology: The form *clytie* of *A. ilia* is very similar in wing morphology to *A. metis* and discrimination is possible based on subtle characters only (fig. S23a-d). The most often mentioned discriminative features are (Higgins *et al*. 1991; Slamka 2004, Lafranchis 2004; Tolman & Lewington 2008; Pamperis 2009):

- The size of the black ocellus in s2 of the forewings upperside, larger in *A. ilia* than in *A. metis*.
- The discal orange band on the hindwing upperside, which in *A. metis* has a marked discontinuity at v4.
- The spot in space 2 of the hindwing underside is small or often absent in *A. metis*.

For the Romanian specimens, these characters are usually enough to allow the separation between the two taxa. However, some specimens of *A. ilia* f. *clytie* may closely resemble *A. metis* as it can be seen in fig. S22b, where the black ocellus in s2 is quite small (forewings and hindwings) and there is a rather pronounced discontinuity at v4 (hindwings). The adults of *A. metis* tend to be smaller than those of *A. ilia*.

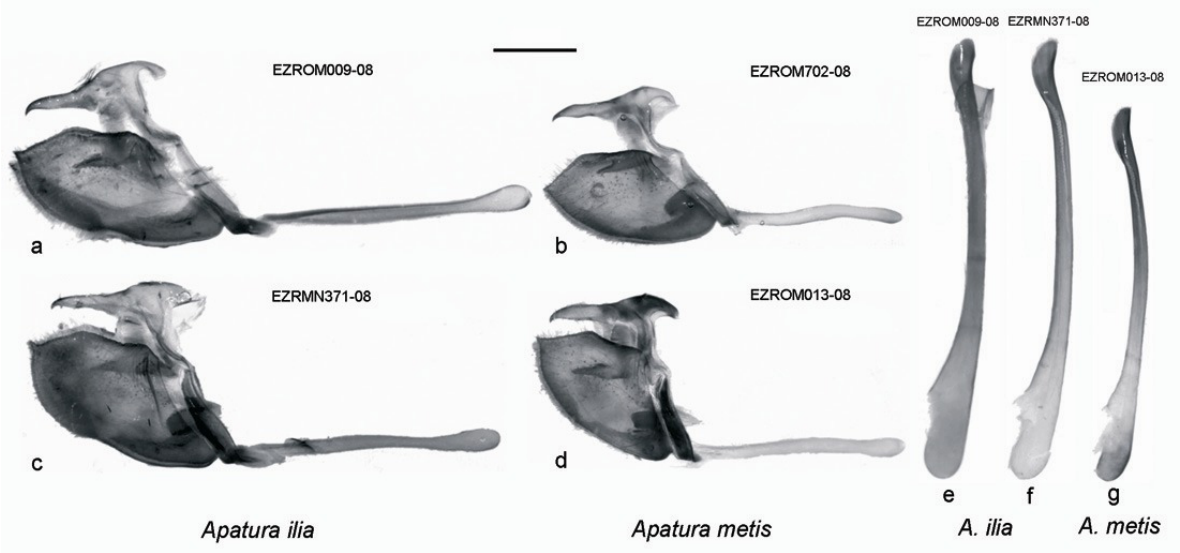

Fig. S24. Lateral view of the male genitalia of representative *Apatura ilia* and *A. metis*. **a, c.** *A. ilia* (phallus removed). **b, d.** *A. metis* (phallus removed). **e-f.** phallus of *A. ilia*. **g.** phallus of *A. metis*. Scale bar is 1 mm.

Male genitalia: Based on the material examined, we could find no clear discriminative character in the male genitalia (fig. S24a-g). We noticed however that the genitalia of *A. metis* are smaller compared to *A. ilia*, but this may be related to the smaller size of the adults of *A. metis*. Higgins (1975) also reported that the genitalia of *A. ilia* are slightly larger than those of *A. metis*. Studies based on more comparative material are needed in order to test if the differences in genitalia size can be considered as species-specific or they only reflect individual variability.

DNA barcoding: *Apatura ilia* is paraphyletic with respect to *A. metis* (fig. S25). The minimum interspecific distance is very low (0.3%), but no barcodes are shared between the two taxa and species-level identification is possible given the current data. However, because of the relatively small sample size, the ability of DNA barcoding of discriminating between the two species should be tested by adding more material.

Comments: The taxonomic status of *A. metis* has been subject to much debate (e.g. Niculescu 1977, 1980; Varga 1978) as it has for a long time been considered as a subspecies of *A. ilia*. However, currently most authors treat *A. metis* as a distinct species.

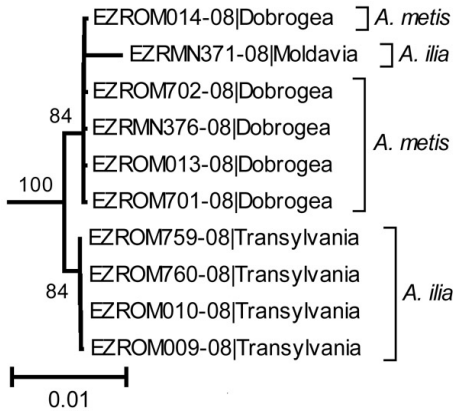

Fig. S25. Neighbour-joining tree of *COI* barcodes of Romanian *Apatura ilia* and *A. metis* with bootstrap values >50% indicated.

Polyommatus bellargus (Rottemburg, 1775) – *Polyommatus coridon* (Poda, 1761)

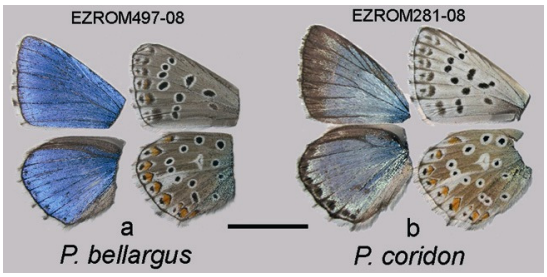

Fig. S26. Wing vouchers of representative barcoded *Polyommatus bellargus* and *P. coridon*. **a.** male of *P. bellargus*. **b.** male of *P. coridon*. Scale bar is 10 mm.

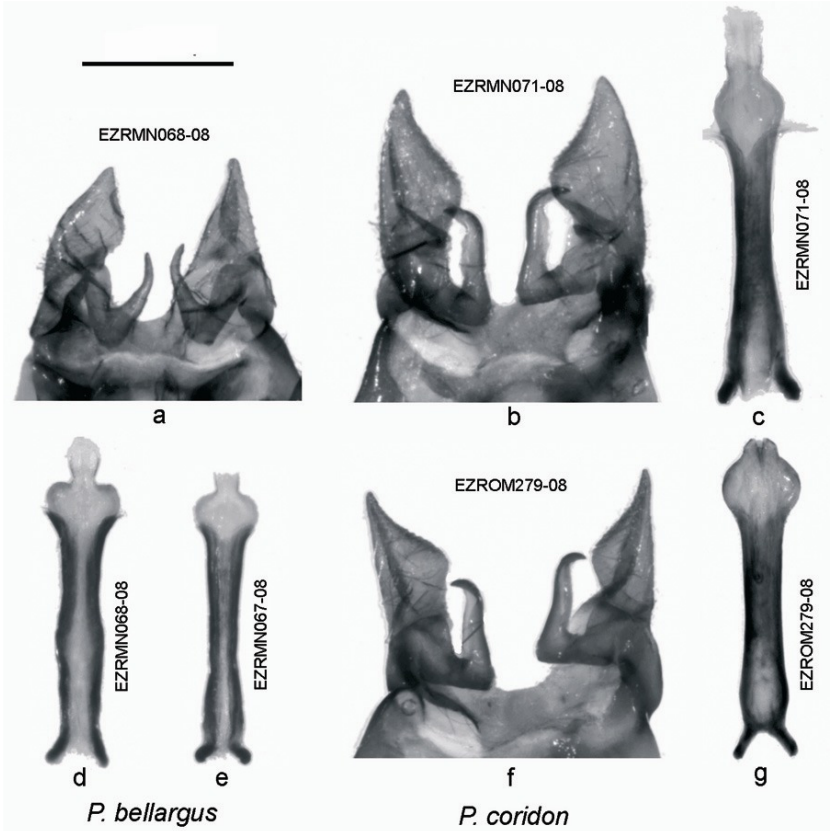

Fig. S27. Elements of the male genitalia of representative *Polyommatus bellargus* and *P. coridon*. **a.** labides and falces of *P. bellargus*. **b.** labides and falces of *P. coridon*. **c.** phallus of *P. coridon*. **d, e.** phallus of *P. bellargus*. f. labides and falces of *P. coridon*. g. phallus of *P. coridon*. Scale bar is 0.5 mm.

Wing morphology: These two species can be reliably separated based on wing characters (fig. S26a,b). The different blue colour on the upperside of the wings and the differences in the dark marginal border on the upperside of the wings (very narrow in *P. bellargus* and much thicker in *P. coridon*) are some of the most obvious characters that allow for their separation in the case of males. For females (not shown), differences are less evident, but they are generally enough for safe determination.
Male genitalia: Although rather subtle, certain elements of the male genitalia are useful to discriminate between the two species: the falces have a more robust appearance and are more strongly hooked in *L. coridon* by comparison to *P. bellargus* (fig. S27, a,b,f). The terminal part of the phallus is also slightly different (fig. S27c-e,g).

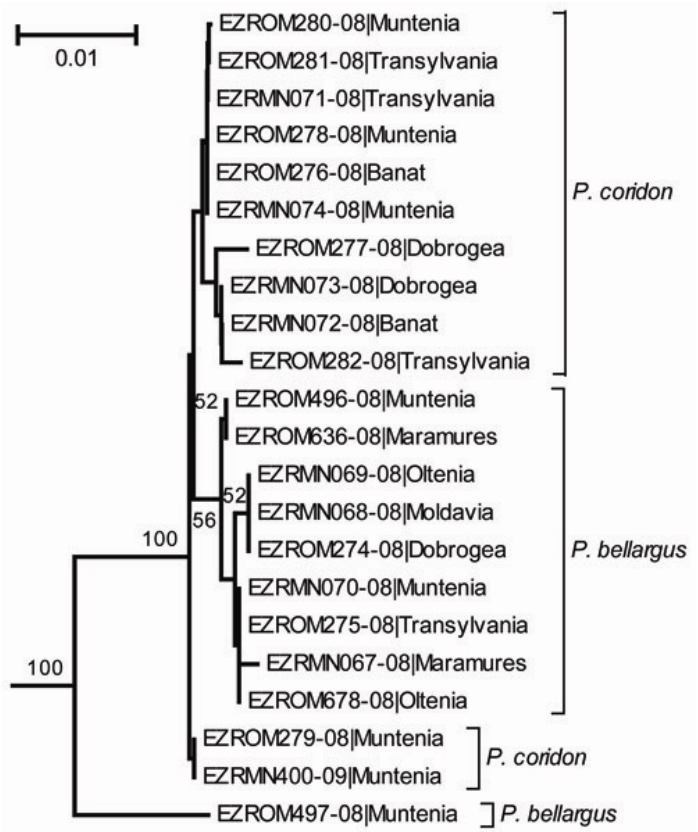

Fig. S28. Neighbour-joining tree of *COI* barcodes of Romanian *Polyommatus coridon* and *P. bellargus* with bootstrap values >50% indicated.

DNA barcoding: The NJ tree displays a complex pattern involving polyphyly (fig. S28). Although no barcodes are shared between the two species, the minimum interspecific distance is very low (0.3%). The relatively high maximum intraspecific distance within *L. bellargus* (2.3%) is most likely not an indicator of cryptic taxa. Instead, it may indicate a case of introgression from *L. coridon*.
Comments: These two species are known to produce natural hybrids such as *Polyommatus* x *polonus* (Zeller, 1845) (Lafranchis 2004; Tolman & Lewington 2008; Descimon & Mallet 2009).

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