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Erebia serotina Descimon & de Lesse 1953 (Lepidoptera: Nymphalidae), a recurrent hybrid between two distantly related species

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Summary. *Erebia serotina* was described in 1953 as a scarce, low-elevation endemic Pyrenean species flying late in the season. At least 34 individuals are known from various locations. However, the absence of females suggests a hybrid origin, and *E. epiphron* and either *E. pronoe* or *E. manto* have been proposed as possible parents. Electrophoretic analysis of five allozyme loci and sequencing of three mitochondrial DNA segments and one nuclear gene now demonstrate that *E. serotina* results from the cross between *E. epiphron* females and *E. pronoe* males. We have used our and previously published sequence data to generate a molecular phylogenetic tree of the genus *Erebia* which shows that these two species are only distantly related. The question of why they happen to hybridize on a seemingly routine basis is thus raised.

Résumé. Erebia serotina Descimon & de Lesse 1953 (Lepidoptera: Nymphalidae), un hybride récurrent entre deux espèces phylogénétiquement éloignées. *Erebia serotina* a été décrit en 1953 et a été d'abord considéré comme une espèce endémique des Pyrénées, tardive et de basse altitude, dont au moins 34 individus ont été récoltés jusqu'ici dans diverses localités. Cependant, l'absence de femelles suggère une origine hybride, avec *E. epiphron* et soit *E. manto*, soit *E. pronoe* comme parents possibles. L'analyse électrophorétique de cinq loci enzymatiques et le séquençage de trois segments de l'ADN mitochondrial et d'un segment de gène nucléaire démontrent qu'*E. serotina* est le résultat du croisement entre femelles d'E. *epiphron* et mâles d'*E. pronoe*. Sur la base d'un arbre phylogénétique présenté ici, ces deux espèces ne sont que lointainement apparentées au sein du genre *Erebia*. La fréquence notable et la régularité de leur hybridation dans les Pyrénées n'en est que plus intrigante.

Keywords: interspecific hybridization; Haldane's rule; hybrid DNA; allozymes; *Erebia* phylogeny

It is not an exceptional event that a hybrid should be described as a species. Among butterflies, noteworthy instances include *Ornithoptera allotei* (Rothschild 1914), now believed to be a hybrid between *O. priamus urvillianus* Doubleday 1847 and *O. victoriae* Gray 1856 (Rousseau-Decelle 1939; Straatman 1976), *Papilio nandina* (Rothschild & Jordan 1901), subsequently proposed to be a hybrid between *P. dardanus* Yeats in Brown 1776 and *P. phorcas* Cramer 1775 (Clarke 1980), and *Lysandra cormion*, which was described by Vladimir Nabokov (1941) and is most probably a hybrid between *L. coridon* Poda 1761 and *Polyommatus daphnis* Denis & Schiffermüller 1775 (Schurian 1989).

In other cases, hybrid individuals were attributed to a species already described and of similar aspect. A striking instance is provided by *Lysandra syriaca italaglauca*, which was described by Vérity (1939) from Italy as a form of a species from the Near East. This butterfly was subsequently shown by a karyological study (de Lesse 1960) to result from a cross between *L. coridon* and *L. bellargus*. Paradoxically, a hybrid of the same parentage, which had been described as a true species under the name *polonus* by Zeller (1845), and is not uncommonly encountered in some parts of Europe, has long been regarded as a L. coridon × *L. bellargus* hybrid by European lepidopterists (e.g. Jacobs 1958), a fact which was confirmed through chromosome examination by de Lesse (1960).

Erebia serotina was described by Descimon and de Lesse (1953) on the basis of two male individuals captured in September 1953 by HD around Cauterets in Central Pyrénées (Table 1), at two sites located 2.5 km apart (so that these two individuals were probably not issued from the same brood). The following year, two additional males were collected and dissected to allow H. de Lesse to perform a karyological study (Descimon & de Lesse 1954). Actually, HD first considered it likely that these individuals were hybrids, but a thorough examination of the 'pro' and 'con' arguments with de Lesse eventually led both authors to favour the hypothesis of a new species. Indeed, the morphological characters did not suggest a hybrid origin and the features of spermatogenesis seemed normal, which is usually not the case in hybrids. This impression was further confirmed (pers.

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Table 1. A. Localities of capture of *Erebia serotina.*

Location		Township	Department	Coordinates	Elevation	Number of captures
A	Pont de la Raillère	Cauterets	Hautes-Pyrénées	42°52′ N 0°06′ W	975 m	16
B	Catarrabes	Cauterets	Hautes-Pyrénées	42°54′ N 0°06′ W	800 m	6
C	Val d'Arrens	Arrens	Hautes-Pyrénées	42°55′ N 0°14′ W	1200 m	2
D	Vallée du Lys	Bagnères-de-Luchon	Haute-Garonne	42°43′ N 0°33′ E	1150 m	5
E	Granges d'Astau	Castillon de Larboust	Haute-Garonne	42°45′ N 0°30′ E	1140 m	1
F	Mines d'Anglade	Salau	Ariège	42°44′ N 1°12′ E	1200 m	1
G	Foz de Minchate	Isaba	Navarra (Spain)	42°52′ N 0°56′ W	800–1200 m	3

B. Captures of *Erebia serotina.*

Number	Year	Locality	Date	Collector
1	1953	A	07.IX	H. Descimon
2	d°	B	14.IX	d°
3	1954	B	07.IX	d°
4	d°	B	27.IX	R. Descimon
5	1955	A	23.VIII	H. Descimon
6	d°	A	18.IX	d°
7	d°	A	27.IX	d°
8	1957	A	07.IX	d°
9	1958	A	06.IX	R. Descimon
10	d°	A	09.IX	d°
11	d°	A	12.IX	H. Descimon
12	d°	A	16.IX	d°
13	d°	A	17.IX	R. Descimon
14	d°	B	24.IX	H. Descimon
15	1959	A	10.IX	d°
16	1960	A	06.IX	R. Descimon
17	d°	B	11.IX	d°
18	d°	B	12.IX	d°
19	1968	A	26.IX	B. & C. Lalanne-Cassou
20	1970	A	06.IX	d°
21	d°	A	19.IX	d°
22	d°	A	21.IX	d°
23–25	1979	G	16.IX	A. Campoy & A. Ederra
26	1983	C	18.VIII	J. Louis-Augustin
27	d°	C	22.IX	P. Robert
28–30	2000	D	05.IX	H. Descimon
31	d°	D	06.IX	d°
32	d°	D	08.IX	A. Descimon
33	2002	E	14.IX	H. Descimon
34	2007	F	11.IX	H. Descimon

comm. to HD, 1984) by Z. Lorković, whose pioneer studies constitute (jointly with those of H. de Lesse) the foundation of modern work on chromosomal speciation in Lepidoptera. *E. serotina* was thus considered to be a scarce, late-flying species, perhaps a relative of *E. christi* Rätzer 1890. The latter species was the rarest among previously known European *Erebia*, and exists only as scanty colonies in a very restricted region of the Simplon Alps.

From 1953 to 1960, Henri and Robert Descimon captured 18 *E. serotina* male individuals, all of them around Cauterets and in September, except for one, collected on 23.VIII.1955. A morphological study carried out on these specimens (Descimon 1963) confirmed the previous conclusions. Four individuals were collected at Cauterets in 1968 and 1970 by Lalanne-Cassou and Lalanne-Cassou (1972), and two additional ones in another valley of the French Pyrénées, above Arrens, by Louis-Augustin (1985). Lantero and Jordana (1981) reported it also on the Spanish side. The circumstances of the catches were similar: single individual captures, at moderate elevation and late in the season.

However, Bourgogne (1963) had drawn attention to the absence of female individuals among the first 18 collected butterflies, recalling that such an imbalanced sex ratio is often observed in interspecific hybrid broods. He pointed out that the haploid chromosome number of *E. serotina* (n = 18) was intermediate between those of *E. epiphron* Knoch 1783 (n = 17) and *E. pronoe* Esper

1780 (n = 19) and that its morphological features could also be interpreted as intermediate between these species (Figure 1). He concluded that *E. serotina* was likely to be a hybrid between *E. epiphron* and *E. pronoe*. Later on, Warren (1981) proposed again that *E. serotina* was a hybrid, but between *E. epiphron* and *E. manto* Denis & Schiffermüller 1775, although the latter hypothesis was not supported by karyological data. Lalanne-Cassou and Lalanne-Cassou (1989) noted that *E. serotina* sometimes bears androconial scales, whereas *E. epiphron* and *E. manto* never do; by contrast, *E. pronoe* has well-developed androconiae. These facts supported Bourgogne's rather than Warren's hypothesis. More recently, Chovet (1998) obtained from artificial pairing between *E. epiphron* and *E. pronoe* three adult butterflies that were rather similar to the wild-caught individuals.

Actually, neither morphological evidence nor karyology, nor ecological data, nor even breeding provide indisputable criteria to distinguish hybrids from representatives of a 'good' species. The case of *Ornithoptera allotei* (Schmid 1970; Blandin 1973; Straatman 1976) illustrates the fact that the hybrid versus species debate can go on endlessly in the absence of solid genetic data. However, with the advent of molecular genetics and its formidable tools, such problems are no longer intractable. Protein electrophoresis and DNA technology offer insight into the genotypes of the organisms under scrutiny. Codominant nuclear markers, diagnostic for the putative parent species, should display heterozygote patterns in a

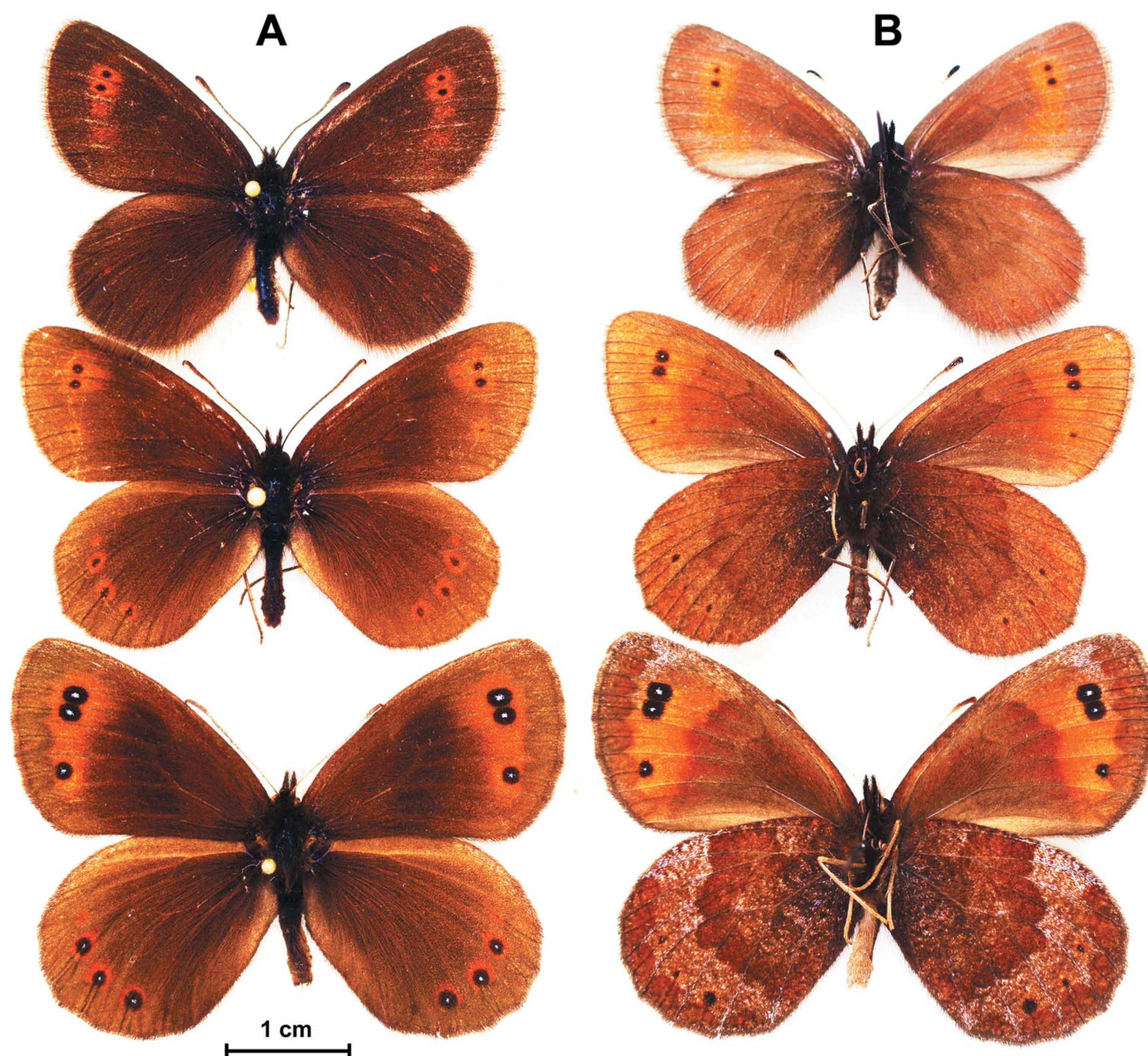

Figure 1. Habitus of *Erebias serotina* and its presumptive parent species. **A,** dorsal side: top, *E. epiphron*, vallée du Lys, 1800 m; middle, *E. serotina*, upstream of Salau, 11.IX.2007, 1050 m; bottom, *E. pronoe*, vallée du Lys, 1700 m. **B,** ventral side, same individuals and positions. The Pyrenean subspecies (*constans*, not illustrated) of *E. manto* is a uniform dark brown on both sides.

hybrid. Moreover, additional information can be expected from mitochondrial DNA (mtDNA), which is maternally inherited, so that its sequencing should make it possible to identify the maternal parent of hybrids (that is, as long as candidate parental species do possess different mtDNA sequences). Protein electrophoresis has already proved successful with butterflies, when allozymes were used as nuclear markers (Descimon & Geiger 1988; Aubert et al. 1997; Deschamps-Cottin et al. 2000). And a recent work based on DNA analysis of the nuclear gene '*engrailed*' and the mitochondrial gene cytochrome oxidase subunit 1 (CO1) demonstrated that *P. nandina* is actually a hybrid between a *P. dardanus* male and a *P. phorcas* female (Thompson et al. 2011).

We have now combined allozyme electrophoresis with the sequencing of both nuclear and mitochondrial DNA segments in order to demonstrate that *E. serotina* is actually a hybrid. Moreover, we have definitely identified its parents, which, remarkably, happen to be only distantly related within the genus *Erebia*.

Material and methods

Biological sampling

Fieldwork was conducted mainly by HD, whether in the times of the discovery of *Erebia serotina* (1953–1960), or in recent years (2000–2007). In the former period, searches were confined to the neighbourhood of Cauterets, whereas in recent years they were extended all along the Pyrenean range (Table 1).

During the early period, the research sites were visited on every sunny day between the middle of August and the end of September. In general, the morning was devoted to the bottom of the valley, between elevations of 900 and 1050 m, and the afternoon to higher altitudes, up to 1500–1800 m. From the year 2000 onwards, more extensive prospecting was carried out along the Pyrenean chain, after potential sites had been located on the maps, and a number of localities between 800 and 1500 m were visited on each day.

Captured individuals were kept alive at 0°C in an isotherm bottle until deep freezing at –80°C in the laboratory.

DNA extraction and mitochondrial DNA procedures

Individuals from which DNA was extracted are listed in Table 2. Total DNA was extracted as described in Aubert et al. (1999), following a standard phenol/chloroform procedure.

Segments of mitochondrial DNA coding for part of the large (16S) ribosomal RNA (LSU), NADH dehydrogenase subunit 1 (ND1) and CO1 were amplified by PCR following Aubert et al. (1999), Martin et al. (2002) and Michel et al. (2008). Automatic sequencing of purified PCR products was carried out either by Genome Express SA (Meylan, France) or GATC Biotech AG (Konstanz, Germany), using the same primers as in the PCR reactions.

Nuclear DNA amplification and sequencing

The ITS2 segment was PCR amplified with primers ITS3 (5′-GCATCGATGAAGAACGCAGC) and ITS4 (5′-TCCTCCGCTTATTGATATGC) (Wiemers et al. 2009). PCR was carried out with Taq polymerase (New England Biolabs, Ipswich, MA, USA) in its ThermoPol reaction buffer supplemented with 5% acetamide, 20 μM of each dNTP and 10 μM of each primer. Amplification was achieved with 40 cycles of 94°C/15 s, 50°C/45 s, 67°C/2.5 min and abundant material was obtained, except with the *E. manto* W298 extract, which repeatedly failed to yield any detectable product.

After extended electrophoresis of PCR products on an agarose (Invitrogen) gel, apparently single bands at *c*.660 and *c*.600 nucleotides were observed in the *E. pronoe* W293 and *E. epiphron* W300 lanes, respectively (see Figure 3A). By contrast, both *E. serotina* extracts (W295 and W303) reproducibly generated two bands: a major product (band B) was found to comigrate with the *E. epiphron* band, while a minor one (band A) migrated approximately as the *E. pronoe* band (Figure 3A). Each of the two bands from a W295 PCR was cut out from the gel and 1 μl of melted agarose was used for reamplification. Material from band B generated a single PCR product that migrated as the initial one. On the other hand, reamplification of band A kept yielding multiple bands: a major one that migrated as the original band A and one or several minor bands that migrated as band B or in between bands A and B (not shown).

Products from amplifications and reamplification were purified (Sigma GenElute kit, St. Louis, MO, USA) and sequencing was attempted at GATC Biotech with primers ITS3 and ITS4. Complete sequences were readily obtained for *E. serotina* band B (Accession # KC184898) and *E. pronoe* (Accession # KC184896), despite the presence in the latter of a minor molecular population with four, rather than three GT repeats at ITS2 position 168. Reading of the *E. epiphron* sequence with primer ITS3 was hampered by the presence of two PCR products, with three and four TG repeats, respectively, at ITS2 position 14. However, a nearly complete sequence (Accession # KC184899) was obtained with primer ITS3epi (5′-TGTGTGTTGGACACGTGG), that partially overlaps onto the repeats. With *E. serotina* band A, primer ITS3 generated a sequence which could be deciphered only partially, but those segments that could be read proved identical or very similar to their *E. pronoe* counterparts. A clean, complete sequence (Accession # KC184897) could eventually be obtained by using a primer (ITS3pro, 5′-ATACCACACTGTCCAGCCC), the last two nucleotides of which, at ITS2 positions 31–32,

Table 2. DNA extraction vouchers and sequence accession numbers.

				Accession numbers			
Voucher	Taxon	Sex	Origin	LSU	ND1	CO1	ITS2
W293	*E. pronoe glottis*	Male	Site D	HE614703	HE614686	HE614681	KC184896
W298	*E. manto constans*	Male	Site D	HE614704	HE614687	HE614682	
W300	*E. epiphron pyrenaica*	Male	Site D	HE614705	HE614688	HE614683	KC184899
W295	*E. serotina*	Male #28	Site D 5.IX.2000	HE614706	HE614689	HE614684	KC184897KC184898
W303	*E. serotina*	Male #31	Site D 6.IX.2000	HE614707	HE614690	HE614685	
EM	*E. serotina*	Male #33	Site E 14.IX.2002	HE614708	HE614691		

matched the provisional *E. serotina* A sequence, but not the *E. epiphron* sequence.

Phylogenetic analyses

Sequences published prior to 1 January 2010 and claimed to pertain to *Erebia* and the mitochondrial genes encoding products ND1 and ND5 (NADH dehydrogenase subunits 1 and 5), CO1 and CO2 (cytochrome oxidase subunits 1 and 2) and LSU (large ribosomal RNA) were collected from databases (Appendix) and manually aligned. When several identical sequences were available for a single taxon, only one was retained. The following entries were discarded as well: (i) AY090212 ('*E. palarica* Chapman 1905 isolate 9-4', actually *E. ligea* L. 1758); (ii) FJ663463 ('*E. callias altajana* Staudinger 1901 voucher 2005-LOWA-581', actually a relative of *E. pharte* Hübner 1804 and *E. kindermanni* Staudinger 1881); (iii) AY346228 ('*E. gorge* Hübner 1804', in fact almost identical to entry AY346222, belonging to *E. cassioides* Hohenwarth 1793); (iv) EF545697 ('*E. alcmena* Grum-Grschimailo 1891 voucher H-026', actually not an *Erebia*, possibly a *Hyponephele*); (v) AF214625 ('*E. epipsodea* Butler 1868', actually *E. epiphron*); (vi) AF229963 ('*E. cassioides*', actually not an *Erebia*). *Boeberia parmenio* Boeber 1809 was used as outgroup.

Our final alignment (available from FM), which comprises sequences from 87 individuals and encompasses a total of 4101 sites with gaps interpreted as missing data, was fed to MrBayes-3.1.2 (Ronquist & Huelsenbeck 2003) as a single partition, with a GTR (General Time Reversible) model of nucleotide substitutions, gamma-distributed rate variation across sites and a proportion of invariable sites. The program was set to perform 3,000,000 iterations, of which the first 1,000,000 were discarded prior to checking convergence between the two parallel runs, calculating binary partitions and, from them, a consensus phylogram.

Allozyme electrophoresis

Nine to 12 imagines from each of the putative parental species (*E. epiphron*, *E. manto* and *E. pronoe*) were collected in Vallée du Lys (locality D), while the imago of *E. serotina* was from the neighbouring Granges d'Astau (locality E); they were stored at –80°C until electrophoresis. Five loci were examined for each sample: glutamate-oxalacetate dehydrogenase (GOT, EC 2.6.1.1), isocitrate dehydrogenase (IDH, EC 1.1.1.42), phosphoglucose isomerase (PGI, EC 5.3.1.9), Phosphoglucomutase (PGM, EC 2.7.5.1) and mannose-6-phosphate isomerase (MPI, EC 5.3.1.8). Electrophoreses were carried out on cellulose acetate gels according to Hebert and Beaton (1993), with slight modifications. Tris-glycine buffer (25 mM Tris, 192 mM glycine, pH 8.5) was used for MPI, PGM, PGI and GOT, and Tris-citrate buffer (50 mM Tris, 12.5 mM citric acid, pH 7.8) for IDH. Gels were run at 200 V during 25 min for MPI, 40 min for PGM, 50 min for GOT, 60 min for IDH and 75 min for PGI.

Genotypes of all the individuals were determined. Several individuals were run at least twice to check the repeatability and the relative position of all alleles in the different species.

Results and discussion

The pursuit of *E. serotina*: a narrative

Early observations (1953–1960)

Around Cauterets, most of the explored areas lie in the 'montane' zone, where beech and fir forest is the climax vegetation. All *E. serotina* individuals were collected at only two sites, one *c.*0.5 km upstream of Cauterets (Pont de la Raillère, locality A) and one *c.*1.5 km downstream (Catarrabes and Canceru hamlets, locality B). Both places consisted of flowery meadows; the former was lightly grazed and the second was mowed once or twice a year. At the time of the visits, sites at higher altitudes occasionally yielded old individuals of other *Erebia* species, but no *E. serotina*.

The two sites look rather different. The Pont de la Raillère is deeply set between two very steep mountain slopes, while the Catarrabes-Canceru meadows lie within a larger, sunnier valley floor. However, these places have in common that they look somewhat like the bottom of a funnel, the lower slopes of which are covered with forest and the upper ones with subalpine grassland.

Recent observations (1999–2007)

Pont de la Raillère and Catarrabes (Localities A and B) were visited in 1999 and 2002, with no success; the site itself underwent little change, but some of the neighbouring meadows had become afforested. The Catarrabes-Canceru hamlets had become more urbanized and the meadows were out of reach.

Site C had been discovered in the Arrens valley, about 20 km west of Cauterets, by Louis-Augustin (1985 and pers. comm. to HD, 1984), who collected there two *E. serotina* individuals in the 1970s. That site was visited by HD in September 1999; no *E. serotina* was captured, but the characteristics of the locality were found to be similar to those of the Cauterets neighbourhood: flowery meadow around 1000–1200 m elevation, funnel-bottom-like site.

Following these setbacks, more effort was devoted to exploring the eastern part of central Pyrénées, namely the Aure and Luchon valleys. A favourable locality (D) was spotted on the floor of the Lys valley, south of Luchon, and five *E. serotina* individuals were collected there between 5.IX.2000 and 8.IX.2000 (two of these individuals were further selected for molecular analysis under the references W295 and W303). The funnel-like overall topography of this site was obvious and extensive; moderately grazed and flowery meadows proved attractive to butterflies. The locality was visited again in early August 2001, in order to collect individuals of the putative parent species. On this occasion, it was observed that only *E. meolans* and *E. euryale* were to be found at the very place at which *E. serotina* had been captured in 2000. On the other hand, all three putative parental species were abundant above the continuous and extensive forest belt, in the lower subalpine grasslands.

In 2002, Vallée du Lys (site D) was explored again in the first half of September, with no success. However, an individual of *E. serotina* (further checked for allozymes under the reference EM) was found westwards of that site, in the neighbouring valley of Oo, once again in a typically funnel-shaped site, at the Granges d'Astau meadows

(site E). The same year, M. J. Lalanne Cassou (pers. comm., 2002) found an individual in the lower part of the Lys valley, close to Bagnères de Luchon, in early October.

In 2007, yet another exploration was carried out, first in sites D and E, with no result, and then eastwards, in the region of Salau (Ariège). There, 2.5 km SE of the village, in a balmy meadow within a typically funnel-like site, one *E. serotina* was captured on 11.IX. Some 400 m higher, *E. epiphron*, *E. pronoe* and *E. manto* were observed. Still further east, in the region of Vicdessos, mountains looked drier and neither *E. serotina* nor *E. pronoe* were noted.

To summarize, *E. serotina* has been collected at six Pyrenean sites in France and at a single one in Spain (Table 1), with its known range spanning 175 km from East to West. Practically all the other important valleys of the Pyrénées were explored at least once during the recent years, but in vain. This limited success might simply reflect inefficient prospecting, or else, the absence of funnel-like, *serotina*-attracting sites in some of these valleys. That is not true, however, for the upper Gave de Pau valley, which was thoroughly explored for tens of years by Rondou (1932), who never found *E. serotina*: this could be explained by the fact that *E. pronoe* is missing there.

Phenology

A total of 25 individuals were captured in 11 years of active research, to which can be added four and two individuals, from Lalanne-Cassou and Lalanne-Cassou (1972) and Louis-Augustin (1985), respectively. In Spain, Lantero and Jordana (1981) reported the capture by A. Campoy and A. Ederra of three individuals on 16. IX.1979. We never collected in October, but J. Lalanne-Cassou (pers. comm., 2002) found several individuals during this month. The median capture day is 11.5 September. The flight period is asymmetrically distributed, with the majority of individuals encountered between 5.IX and 15.IX and a tail which is likely to extend into October (Figure 2). At that time and elevation, apart from laggards of some earlier-flying species, most observed butterflies belong to second broods of multi-brooded species. Above 1500 m, thermal conditions do not allow a second developmental cycle to be completed and from late August onwards, only migrants are encountered in this zone. In fact, butterflies generally avoid it and seek shelter in the lower zones around 1000 m, where they find flowers for foraging and milder temperatures.

Potential parents among other Erebia *species*

Of the 13 species of *Erebia* that inhabit the central Pyrénées, three – *E. epiphron*, *E. manto* and *E. pronoe* – have been contemplated as potential parents of *E. serotina* based on morphological characters. These are medium elevation dwellers, which are more frequent in the lower subalpine zone, between 1500 and 2000 m. *E. epiphron* may occasionally form small colonies in wet, lightly grazed meadows around 1000 m, but this is exceptional. One such colony existed in the 1950s close to the Pont de la Raillère, but it disappeared when the meadows became invaded by trees. Actually, in the Catarrabes-Canceru meadows, no *Erebia* species is resident (not to mention *E. serotina*, of course).

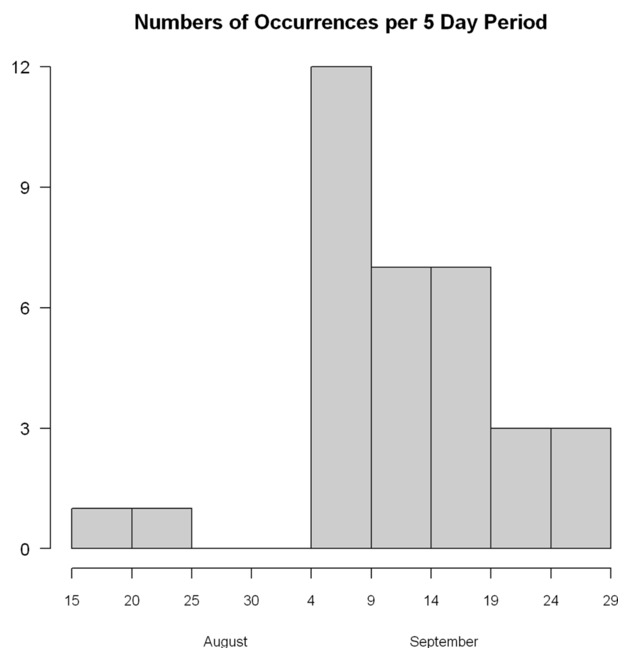

Figure 2. Histogram of the days of capture of *Erebia serotina*.

Molecular analyses

Mitochondrial DNA

Since mitochondrial DNA is maternally inherited in insects, it should be possible to identify the maternal parent of *E. serotina* – assuming it is a hybrid indeed – by comparing its mitochondrial genome to those of the candidate parental species. We chose to sequence three disjoint segments of mtDNA in order to preclude the possibility of undetected recombination (however unlikely the latter may be in the case of animal mtDNA; see in particular Maynard Smith & Smith 2002; Galtier et al. 2009). The LSU (513 nt) and ND1 (472 nt) segments are the ones analysed in Aubert et al. (1999) and subsequent publications from our and other groups, while the CO1 segment (652 nt) is the one used for 'barcoding' (Hebert et al. 2003). Sequences were obtained from two individuals of *E. serotina* (W295 and W303) from site D and one individual of each of the putative parental species from the same locality. A third *E. serotina* specimen (EM) from site E was also sequenced over the LSU and ND1 segments.

Table 3. Distances in nucleotides (and percentage) from *Erebia serotina* W303.
A. CO1 segment (419 nt, from positions 234 to 652 of the W303 sequence).

Taxon	Accession #	Origin	nt (%)
E. serotina W295	HE614684	Haute-Garonne	0 (0)
E. euryale	AY346221	Spain: Cantabrian Mt.	23 (5.5)
E. manto constans W298	HE614682	Haute-Garonne	23 (5.5)
E. epiphron pyrenaica W300	HE614683	Haute-Garonne	0 (0)
E. triaria	AY346219	Spain: Cantabrian Mt.	28 (6.7)
E. gorge	GU001961	Western Carpathians	21 (5.0)
E. cassioides	AY346222	Spain: Cantabrian Mt.	21 (5.0)
E. pronoe glottis W293	HE614681	Haute-Garonne	17 (4.1)
E. oeme	AY346227	France: Pyrénées	24 (5.7)
E. meolans	AY346223	Spain: Pyrénées	16 (3.8)
E. sthennyo	DQ338781	France: Pyrénées	21 (5.0)

B. LSU and ND1 segments (748 nt, from positions 51 to 388 and 31 to 439, respectively, of the W303 sequences)

Taxon	Accession #	Origin	nt (%)
E. serotina W295	HE614706 + HE614689	Haute-Garonne	0 (0)
E. serotina EM	HE614708 + HE614691	Haute-Garonne	1 (0.1)
E. euryale	AF214609 + AF229954	Hautes-Pyrénées	51 (6.8)
E. manto constans W298	HE614704 + HE614687	Haute Garonne	46 (6.1)
E. epiphron pyrenaica W300	HE614705 + HE614688	Haute-Garonne	1 (0)
E. cassioides pseudocarmenta	HE614716 + HE614699	Pyrénées Orientales	42 (5.6)
E. rondoui rondoui	HE614718 + HE614701	Pyrénées Centrales	61 (8.2)
E. pronoe glottis W293	HE614703 + HE614686	Haute Garonne	35 (4.7)
E. meolans	AF214599 + AF229944	Hautes-Pyrénées	43 (5.7)

The three *E. serotina* individuals share identical sequences over sequenced sections of their mitochondrial genome, except for a single nucleotide substitution at one end of the ND1 segment. Strikingly, the *serotina* CO1 and LSU sequences turned out to be identical as well to those of our *E. epiphron* specimen, which also has the same ND1 sequence as the EM individual. In contrast, the sequences of *E. manto* and *E. pronoe* are clearly divergent: 13 and 18 differences, respectively, from *E. serotina* over the LSU segment; 38 and 26 over the ND1 segment and 41 and 29 over CO1.

A majority of the remaining Pyrenean species of *Erebia* have been sequenced over at least part of the LSU, ND1 and CO1 segments. In Table 3, these sequences, which pertain mainly to individuals collected in the Pyrénées or Cantabrian mountains, are compared with ours over those segments they have in common. Distances to respective *E. serotina* sequences can be seen to be similar to, or higher than, those of *E. pronoe* and *E. manto*. Therefore, mitochondrial sequence data point unambiguously to *E. epiphron* being either a very close relative or, more likely, the maternal parent of *E. serotina*.

Nuclear DNA

We chose to amplify and sequence the ITS2 (Internal Transcribed Spacer 2) segment of nuclear DNA because it offers multiple advantages. Being part of the ribosomal transcription unit, it is present in high copy numbers, which makes its amplification easier than that of other nuclear genes. It is flanked by extremely conserved sequences, so that universal primers are available. And it is itself highly variable, to the point that it has been suggested to offer a nuclear substitute to mitochondrial 'barcoding': as pointed out by Wiemers et al. (2009) in their extensive study of ITS2 variation in a rapidly evolving clade of Lycaenid butterflies, 'Even in the young radiation of *Agrodiaetus*, scarcely any two species have identical *ITS2* haplotypes'.

We were greatly helped in our analysis by the fact that the two ITS2 amplification products of *E. serotina* differ by almost 10% in length (Figure 3B), so that they could be separated by extensive migration on agarose gels (Figure 3A). The sequence of individual W295's band B product was found to be nearly identical to that of *E. epiphron*: the two sequences diverge only over a short segment (at position 368 of the *E. serotina* ITS2 sequence), which reads GGTACGCGA in *E. epiphron* and CGCGCGCC in *E. serotina*. As expected from the fact that during electrophoresis longer products are always contaminated by shorter ones, confirming the sequence of the W295 band A molecules required the synthesis of a specific primer (see Material and methods): the resulting sequence eventually proved indistinguishable from that of *E. pronoe* over its entire length.

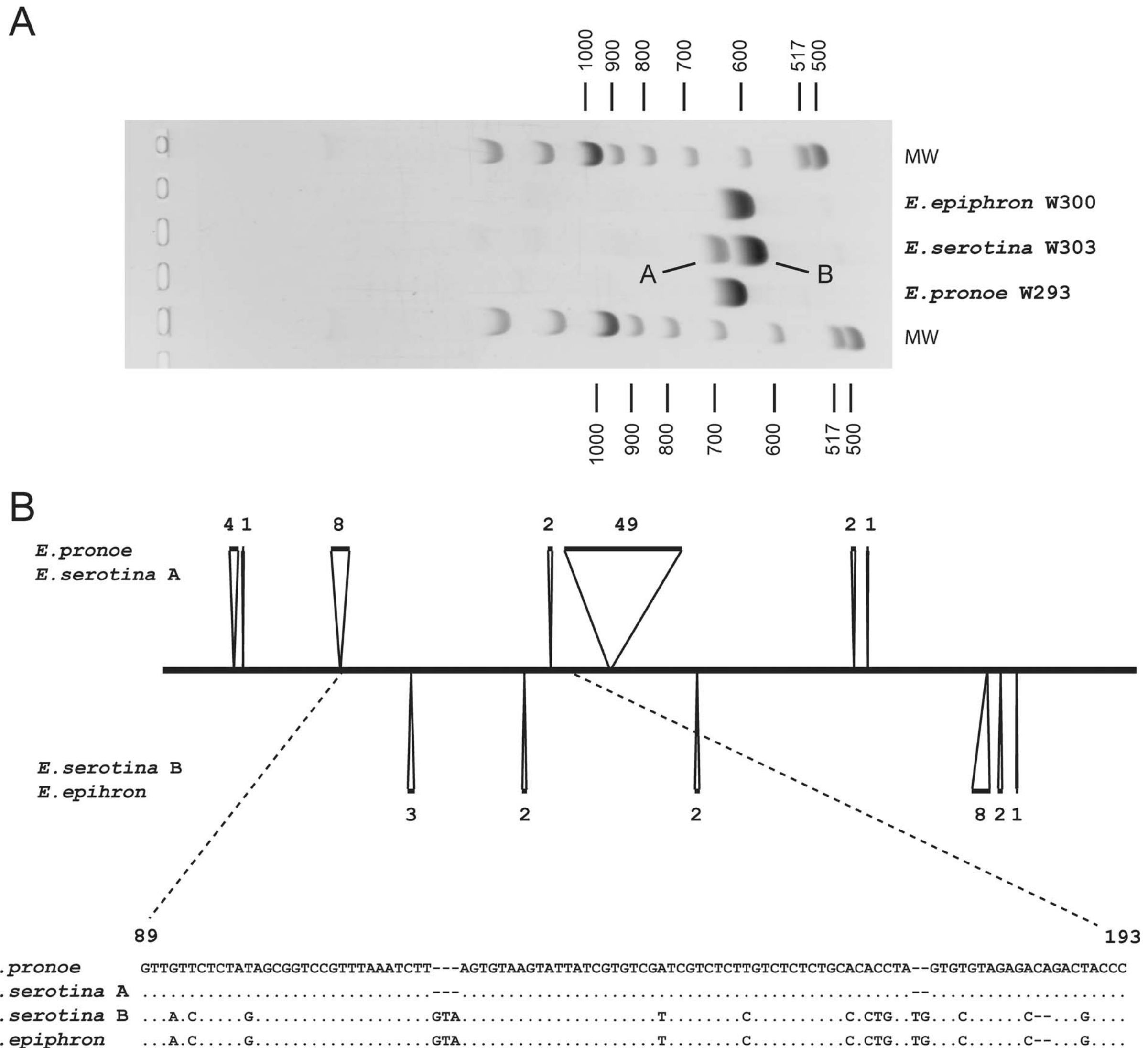

Figure 3. Comparison of the ITS2 amplification products of *Erebia pronoe*, *epiphron* and *serotina*. **A,** Electrophoresis of ITS2 PCR amplification products on a 1.2% agarose gel. Wells are at left, MW: 100 bp DNA ladder (New England Biolabs). The central lane (and the ones immediately flanking it) was slightly overloaded so as to make band A fully visible. Note that bands are slightly slanted, due to unusually extensive migration. **B,** Top part: location and length of indels in an alignment of the ITS2 sequences generated from the four bands in panel A (drawn to scale; 496 positions – including indels – were aligned; the rightmost, 1-nt indel is missing in *E. serotina* sequence B). Bottom part: detailed alignment of a representative section extending from ITS2 positions 89 to 193; identities to the sequence of *E. pronoe* are shown as dots, dashes stand for gaps.

The presence, in the *E. serotina* individuals examined, of two ITS2 amplification products with strikingly divergent lengths and sequences provides compelling support in favour of *E. serotina* actually resulting from interspecific hybridization. Moreover, even though *E. epiphron* and *E. pronoe* are the only *Erebia* species from which we determined ITS2 sequences, the rapid evolutionary divergence of the ITS2 segment makes it unlikely that any other Pyrenean taxon would generate sequences that would match so well those of *E. serotina*. Therefore, the identity of *E. serotina*'s parental species may be regarded as finally established. On the other hand, it must be emphasized that, since PCR amplification is expected to systematically favour shorter over longer molecules, it is impossible to infer the actual ratio of *E. pronoe* and *E. epiphron* genomes in the extracts that were analysed from the observed ratio of band A over band B material (about 1:3 on the average, but somewhat variable from one PCR experiment to the next).

Allozyme electrophoresis

Allozyme electrophoresis was expected to confirm the conclusions of our DNA analysis, and also to assess the

Table 4. Numbers of occurrences of each allele for each putative parental species and for individual #33 of *Erebia serotina*.

Loci	Allele	Number of occurrences of each allele			
		E. pronoe	*E. epiphron*	*E. manto*	*E. serotina*
PGM	A		1		
	B		2		
	C		13		1
	D		4		
	E			1	
	F			12	
	G	18		11	1
PGI	A			4	
	B		4	2	1
	C	1		16	
	D		13		
	E	2			
	F			2	
	G	6	2		
	H	1			1
	I		1		
	J	8			
GOT	A	18			1
	B		19		1
	C		1		
	D			2	
	E			22	
MPI	A	16			1
	B		1	1	
	C		17	15	1
	D		2	2	
	E			6	
IDH	A	18		24	1
	B		17		1
	C		3		

likelihood of the only *E. serotina* individual that could be examined actually being a F1 hybrid. Table 4 presents the numbers of alleles found at each locus in the three putative parental species and in the *E. serotina* EM specimen, which, strikingly enough, turned out to be heterozygote at all loci. The results are most straightforward for GOT, where none of the alleles were shared by the three different putative parental species and in all three species the frequency of the most common allele was higher than 0.9. Here, the EM individual proved heterozygote for the most common allele of *E. epiphron* and the unique allele of *E. pronoe*.

The genotype of the EM individual at IDH is clearly consistent as well with the results of DNA analyses. *E. manto* and *E. pronoe* are monomorphic for the same allele, while *E. epiphron* has two different alleles. *E. serotina* is heterozygote for the most common allele of *E. epiphron* and for the allele shared by *E. pronoe* and *E. manto*, indicating that *E. epiphron* is necessarily one of the parents. As for MPI, it clearly indicates that one of the parents is *E. pronoe*, while the other one could be either *E. manto* or *E. epiphron*.

Since the numbers of alleles at the PGM and PGI loci are high, data interpretation is more delicate. One PGM allele of *E. serotina* could come from *E. epiphron*, and the other one either from *E. pronoe* or from *E. manto*. One of the PGI alleles is probably from *E. pronoe*, while the other is either from *E. epiphron* or *E. manto*, so that, finally, these data are in good agreement as well with conclusions drawn from the other loci.

A provisional molecular phylogeny of the genus Erebia

E. epiphron and *E. pronoe* were placed in different species groups in the reference monograph of the genus *Erebia* (Warren 1936). These two taxa have distinct wing patterns (Figure 1), and their DNA sequences, especially over the ITS2 segment (Figure 3B), are markedly divergent. We sought to confirm that *E. serotina*'s presumptive parents

are only distantly related by building a framework – a molecular phylogeny – within which our sequence data could be put into perspective.

Although no comprehensive molecular phylogeny of the genus *Erebia* is available at present, the sequences of some 54 species (more than half of the generally accepted number of species in this large genus) have been determined over at least one of five mtDNA segments (LSU, ND1 CO1, CO2 and ND5; see Appendix for accession numbers). Analyses based on likelihood (as opposed to maximum parsimony) make it possible to combine heterogeneous sequence sets into a single, incomplete data matrix (e.g. Burleigh et al. 2009) and generate from it a consensus phylogenetic tree. A tree that incorporates all *Erebia* species and major subspecies for which mitochondrial sequences exist is shown in Figure 4. This provisional molecular phylogeny of the genus *Erebia* does provide support for most, although not all, species groups recognized by Warren (1936), based on male genitalia, and de Lesse (1949, 1960), based on female genitalia and chromosome numbers.

Of the groups proposed by Warren (1936), I, V, XI and XVI appear polyphyletic in Figure 4 (note that as taken into account in Figure 4, *E. epipsodea* was already transferred from Warren's group XI to group IX by Belik (2000); this author omitted to mention that much earlier, de Lesse (1960) had stressed that *E. epipsodea* and *E. medusa* share the same number of chromosomes and that Warren's group XI was heterogenous). The two members of group V (*E. triaria* and *E. rossii*), which stand quite far from one another according to molecular data, have markedly different female genitalia (de Lesse 1949, and in litt. to FM, 1968). The same author also pointed out that group XVI, which we found to be split amongst three clusters (Figure 4), was heterogeneous.

By contrast, the apparent association of *E. manto* (one of the prospective parents of *E. serotina*) with group XV rather than group I is poorly supported by molecular data (posterior probability 0.27). Most important, it is readily apparent from the tree in Figure 4 that the other two prospective parents of *E. serotina* – *E. epiphron* on the one hand, and *E. pronoe* on the other – are only distantly related; examination of the entire set of tree bipartitions indeed confirms the absence of any privileged relationship between the subgroups to which these species belong.

Erebia serotina: good species or hybrid?

Are all serotina *individuals F1 hybrids of* Erebia pronoe *and* E. epiphron*?*

Until now, the evidence for the hybrid nature of *E. serotina* had been indirect. As already stated in Descimon and de Lesse (1953), in the species-rich genus *Erebia*, almost any taxon may be considered to be 'intermediate' between at least two other ones. Thus, in addition to the previously mentioned *E. christi*, the species complex of *E. dabanensis* Erschoff 1871 from Siberia (Tuzov et al. 1997) displays morphological characters superficially close to those of *E. serotina*. The putative parental species (*E. epiphron* and *E. pronoe*) are of course not present and no doubt can be cast on the validity of these species, but *E. serotina* might have been deemed an European member of the *E. dabanensis* group – an error comparable to that made when describing *L.* '*syriaca italaglauca*' (Vérity 1939).

It is important to recall that the phenotypic characters of *E. serotina* (wing pattern, genitalia: Descimon 1963; Lantero & Jordana 1981) and also its phenology and habitat are sufficiently homogenous to attribute all collected individuals to a single 'entity'. This fact was formerly used to support the 'good species' hypothesis retained by Descimon and de Lesse (1953). But it could be used as well to support the alternative hypothesis of a F1 hybrid recurrently generated by crosses between two good species, namely *E. epiphron* and *E. pronoe* (Bourgogne 1963). Actually, the absence of females in the whole sample was the strongest argument in favour of the latter hypothesis (see further) but, by itself, it did not constitute much more than a clue. In that state of affairs, the '*E. serotina*' dilemma remained insoluble.

The amplification by PCR on *E. serotina* DNA extracts of two markedly distinct forms of the ITS2 nuclear gene – one of them nearly identical to that of *E. epiphron* and the other one undistinguishable from its *E. pronoe* counterpart – has now finally provided the long-sought proof that *E. serotina* individuals (or at least those specimens that we examined) are of hybrid origin. Confirmation of the hybrid status of *E. serotina* was provided by allozyme electrophoresis of a third individual, which turned out not to possess any specific allele at five allozyme loci; all of its alleles are also present in *E. epiphron* and *E. pronoe*. Moreover, this individual is heterozygote at all five loci for alleles characteristic of the two parental species, as should be an F1 hybrid. While we cannot formally conclude from these data alone that *all* serotina individuals will prove F1 hybrids of *E. pronoe* and *E. epiphron*, this constitutes by far the most reasonable hypothesis in view of the above-mentioned homogeneity of the *serotina* assemblage.

The three individuals (W295, W303 and EM) whose mtDNA genes have been amplified share their sequences with those of *E. epiphron*, and therefore must be issued from a female of *E. epiphron*. We acknowledge that in this particular case, morphological or phenological homogeneity cannot be invoked in order to generalize that statement to all *serotina* specimens: the reason is that in Lepidoptera, the F1 male progenies of reciprocal crosses inherit identical sets of parental chromosomes. Nevertheless, the possibility of a cross between an *E. epiphron* female and an

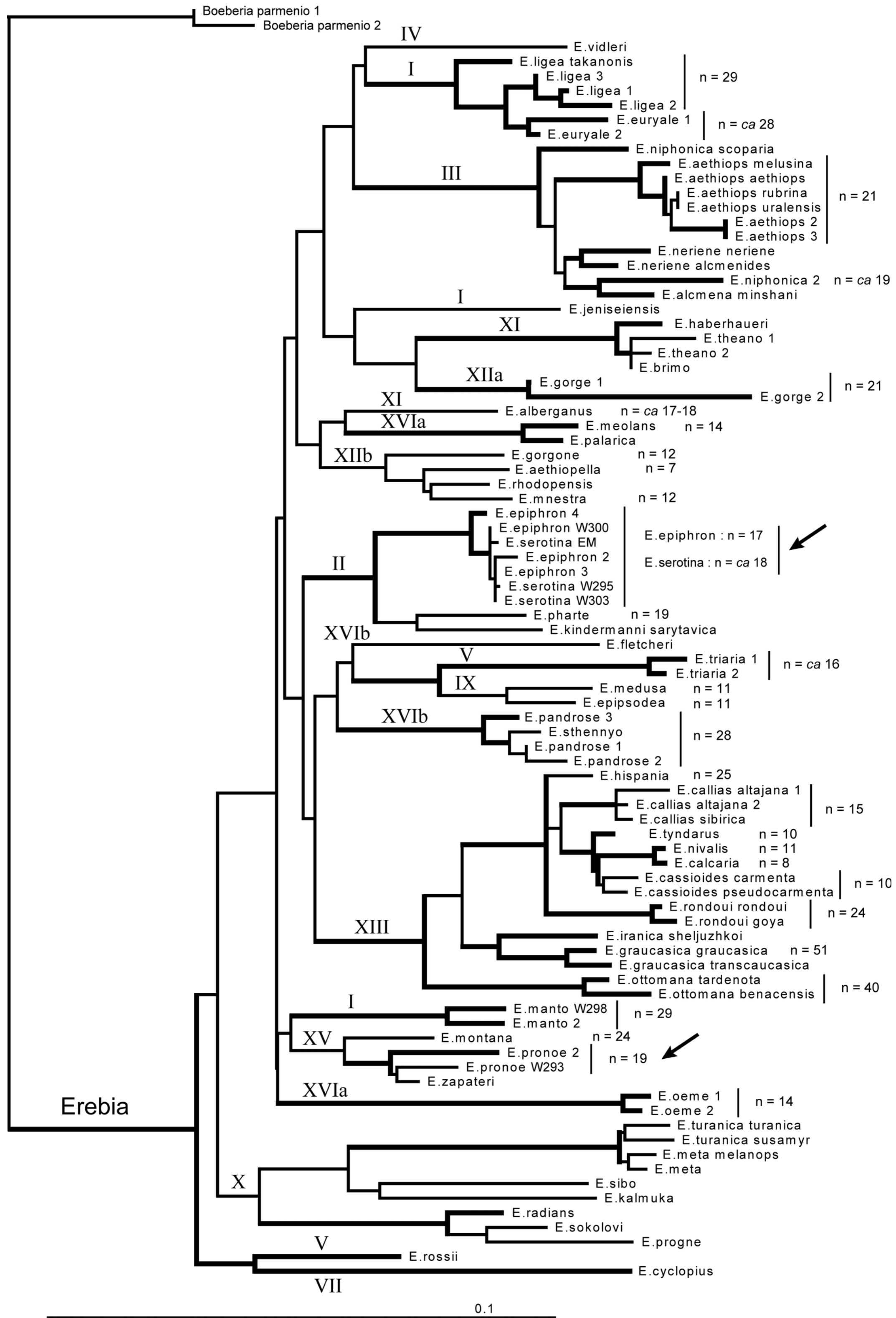

Figure 4. Provisional molecular phylogeny of the genus *Erebia*. Phylogram generated by MrBayes-3.1.2 from an alignment of the mitochondrial sequences listed in the Appendix. Thick branches have posterior probabilities greater than 0.9. Roman numbers refer to groups recognized by Warren (1936) and de Lesse (1949, 1960 and *in litt.* to FM, 1968), see Text. Arrows point to *E. serotina* and its parents, *E. epiphron* and *E. pronoe*. Chromosome numbers (de Lesse 1960; Maeki & Remington 1960) are indicated at far right.

E. pronoe male agrees best with the phenology of parental species (see below) and will constitute our working hypothesis in what follows.

Why the mistake?

As long as hybrids are scarce and observed at places where the parent species fly together, their origin should be obvious even to the least experienced observer. In some instances, however, hybrids can be so frequent that even a competent systematician may get confused. This has been especially the case in the Lycaenid genus *Lysandra*. In addition to the previously mentioned *L. polonus* and *L. italaglauca*, the hybrids formed between *L. coridon caelestissima* and *L. albicans* in their cohabitation zones in Montes Universales (central Spain) are generated at a frequency that can reach 5 to 10% of the total population (Descimon, unpublished observations). Consequently, they were regarded by Tutt (1909) as belonging to a distinct species, *L. caerulescens*. And, somewhat ironically, it was de Lesse (1969, 1970) who happened to play a major role in refuting this error.

Although *E. serotina* is rare, it is clear that, when sufficient sampling pressure is exercised, it can be found rather predictably. Moreover, the year-to-year regularity of its occurrence may give the impression of a discreet, overlooked species with a peculiar ecology. Actually, at the time of its description, both de Lesse and HD shared the idea that *E. serotina* had been overlooked because it was a late-flying species, localized in poorly accessible habitats (Descimon & de Lesse, 1953, 1954). Its flight period does not overlap with that of other *Erebia* species, a factor which suggests an original ecology, and hence a *bona fide* species. In fact, *E. serotina* is not much rarer than some 'true' Lepidoptera species, even in temperate regions.

Why is **Erebia serotina** *localized in habitats in which the parental species are not strictly resident?*

In general, hybrids are expected to occur at places where parental species fly together. However, *E. serotina* has been encountered in places where its parents are usually not resident: its elevation range is mostly between 850 and 1000 m, *below* the forest belt, while both parental species are mainly dwellers of subalpine grassland *above* the treeline. In the latter habitat, in spite of assiduous research, *E. serotina* has never been met with. Migration must therefore take place, either by the *pronoe*-mated *epiphron* females, or by the *serotina* adults. The former hypothesis is unlikely: the cause of female parent migration would have to be a perturbation of habitat choice by hybrid mating, since normally mated females do not migrate downwards from places which are perfectly hospitable at their flight period (mid-summer). It is far more likely that newly hatched imagines leave their inhospitable birthplace when they are confronted with the chilly temperatures of late summer and early fall in the subalpine zone and find shelter in the lower, balmier bottoms of the valleys, as do most butterflies at this time. Moreover, the funnel-like topography of the neighbourhood could account for the local concentrations observed in the field.

Why only males, and so late flying?

Univoltine butterflies are very generally protandric (Fagerström & Wiklund 1982; Nève & Singer 2007) and females have a less conspicuous behaviour, which often makes them appear scarcer. Therefore, it did not look too surprising at first that only males of *E. serotina* should be met with. However, as years passed by and catches accumulated, it became evident that the absence of females was abnormal. This situation finally led Bourgogne (1963), who had become acquainted with Haldane's rule by crosses in other Lepidopteran groups, to suggest the hypothesis that *E. serotina* was a hybrid. In Lepidoptera, it is the female sex that is heterogametic and, therefore, struck by genomic incompatibilities (Haldane 1922). In butterflies, this incompatibility often takes the form of diapause perturbations: the males benefit from hybrid vigour and undergo rapid development, whereas females prove unable either to enter or to leave diapause (i.e. become 'perpetual nymphs'; Clarke & Sheppard 1956; Aubert et al. 1997). In other taxa, where diapause takes place in the midst of the larval stage (as is mostly the case for *Erebia*), female hybrid caterpillars remain indefinitely at L3 stage, and perish (Descimon 1980). Anyway, the absence (or rarity, or impaired viability and fertility) of females is a major argument in favour of the hybrid nature of a set of individuals.

These facts account for the biological peculiarities of *E. serotina* and, combined with the known biology of the parental species, make it possible to draw a scenario of its life history. Adults of *E. epiphron* fly in July, while those of *E. pronoe* do so in August. Because of protandry, some *epiphron* females hatching in late July or early August could remain virgin for some time and get mated by some early hatched *pronoe* males. Due to hybrid vigour, egg to adult development should occur readily in males, which might skip the normal L3 diapausing stage and yield adults in September or even October. Conversely, females would stop development before the adult stage. Both the absence of females and the abnormal, late flight period of the males may thus be explained in a rather likely way.

A noticeably parallel example is provided by *E.* '*sinaica*', described by Popescu-Gorj (1974) as a rare hybrid between *E. pronoe* and *E. medusa*. In addition to some morphological similarities with *E. serotina*, the most striking fact is the late flight period (23 September) and, of course, the implication of *E. pronoe* as a parent.

Conclusion

Sixty years have elapsed since the discovery of *Erebia serotina* in 1953–the very year in which the double helix structure of DNA was elucidated. At that time, most French entomologists were not at all or barely aware of either the 'new synthesis' in evolutionary systematics (Huxley 1942) or the biological species concept (Mayr 1942). H. de Lesse, who was regarded by Brown (1972) as the keenest professional French lepidopterist of the 20th century, was one of the few naturalists who began to practice an evolutionary approach of systematics. However, even using those advanced insights, it was impossible to settle the '*E. serotina* dilemma': good species, or hybrid?

In fact, theory was not faulty, but powerless: the technical tools that could provide solid empirical support were in infancy or just remained to be discovered; no information on the genotypes of the individuals under scrutiny was available. Actually, the only genetic data available, the karyotypes, led de Lesse (and Lorkovic) to spurious conclusions.

Finally, molecular criteria have refuted (or, at the very least, strongly weakened) the hypothesis that the individual butterflies referred to *E. serotina* are members of a species (no matter 'good' or 'bad': Descimon & Mallet 2009). The only reasonable alternative to it is that they are hybrids and in the present state of our knowledge, we must accept it. The senior author of this paper, HD, is thus obliged to admit that he committed an error, jointly with H de Lesse, 60 years ago.

The riddle of 'Descimon's ringlet' is now solved, but only to be replaced by new questions. Both morphology (Warren 1936; Sonderegger 1980) and molecules (see Lörtscher et al. 1998, for allozymes, and this work, Figure 4, for mtDNA) make it clear that *E. epiphron* and *E. pronoe* belong to remotely related subdivisions of the genus *Erebia*. Hybrids are known to be rather scarce in *Erebia* (de Lesse 1960). Why should such relatively distant species as *E. epiphron* and *E. pronoe* regularly yield viable hybrids, whereas much more closely related taxa do not? An obvious research direction suggested by this work is the analysis of sex pheromones in *Erebia*, and in particular, the two parents of *serotina*. It is a safe bet that their pheromones should be, at the very least, closely similar. The conundrum of the medium-scale difference of habitat between *serotina* and its parents is likely to be more difficult to address, since it implies migration, which should be difficult to observe.

Cases of recurrent, natural hybridization involving such distantly related taxa as *E. pronoe* and *E. epiphron* are few. As mentioned in the Introduction, another example in Europe is provided by the rare *cormion* hybrid between *L. coridon* and *P. daphnis*. In fact, the extent of molecular divergence between *cormion*'s parent species on the one hand, and *serotina*'s on the other, is rather similar: the ITS2 segments of *L. coridon* and *P. daphnis* were found to differ by 11 indels and 45 substitutions (Wiemers et al. 2009), as against 13 indels and 65 substitutions between *E. pronoe* and *E. epiphron* (this work). According to the latest time calibration for the Polyommatina section of Lycaenidae (Talavera et al. 2012, figure 1), L. *coridon* and *P. daphnis* diverged 5.0 ± 1.5 million years ago. This date, which happens to be just above the minimum age threshold that was chosen by these authors to define genera, may therefore be used as a rough estimate of the time of divergence of *serotina*'s parent species.

Mallet (2005) argued that interspecific hybridization is likely to be much more frequent than usually thought and that 'really good' species are perhaps the exception rather than the rule (Descimon & Mallet 2009). But what are the genetic consequences of such hybridizations? The 'classical' thesis is that they amount essentially to nothing: they result from a counter-natural process (*hubris* in Greek) or, in other words, from an imperfection of interspecific isolation. This is certainly not the opinion of Gilbert (2003), who believes that significant introgression can take place for a long time during the course of cladogenesis. Numerous cases of introgression between related butterfly species have in fact recently been uncovered by molecular approaches and one of them involves *L. bellargus* and *L. coridon*, the parent species of the *L. polonus* hybrid (see Introduction), which is sufficiently frequent in the southern part of Europe to be found almost certainly when sufficient research pressure is exercised in suitable sites (Diringer & Castelain 2011). As discussed in Dinca et al. (2011), *L. bellargus* individuals from Romania (and neighbouring countries – data not shown) fall into two distinct mitochondrial lineages, one of which is separate from *L. coridon*, whereas the other one is deeply embedded within the *coridon* subtree. It must nevertheless be noted that despite *L. bellargus* and *L. coridon* having widely different chromosome numbers (n = 45 and 88, respectively) (de Lesse 1970), they show only limited molecular divergence – 2.3% at most over the CO1 barcoding segment, two indels and three substitutions over the ITS2 segment (Wiemers et al. 2009). The question now is, may recurrent hybridization in a natural context be the source of a significant gene flow when the parent species are as divergent as *E. pronoe* and *E. epiphron*, or *L. coridon* and *P. daphnis*? After having solved the *serotina* riddle, molecular genetics could provide the tools needed to address this possibility.

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Appendix. Accession numbers of *Erebia* and *Boeberia* mitochondrial sequences used for phylogenetic analyses.

	Segments				
Taxa	LSU	ND1	CO1	CO2	ND5
Boeberia parmenio Boeber 1809 1			FJ663326		
B. parmenio 2			AB324847		AB324828
E. ligea L. 1758 1	AF214608	AF229953	AY346224		
E. ligea 2			DQ338779		
E. ligea takanonis			AB324844		AB324825
E. ligea 3			FJ628431		
E. euryale Esper 1805 1	AF214609	AF229954	AY346221		
E. euryale 2			FJ628423		
E. jeniseiensis Trybom 1877			FJ663504		
E. manto Denis & Schiffermüller 1775 2			FJ628427		
E. epiphron Knoch 1783 2			DQ338778		
E. epiphron 3			AY346225		
E epiphron 4		GU001957	FJ628433		
E. pharte Hübner 1804	AF214610	AF229955	FJ628447		
E. kindermanni sarytavica Lukhtanov 1990			FJ663507		
E. vidleri Elwes 1898			AB324843		AB324824
E. niphonica scoparia Butler 1881			AB324849		AB324830
E. neriene neriene Boeber 1809			AB324842		AB324823
E. neriene alcmenides Sheljuzhko 1919			AB324839		AB324820
E. alcmena minshani Bang-Haas 1933			AB324836		AB324817
E. aethiops rubria Frühstörfer 1909			AB324834		AB324815
E. aethiops melusina Herrich-Shäffer 1847			AB324833		AB324814
E. aethiops aethiops Esper 1777			AB324831		AB324812
E. aethiops 2			FJ628434		
E. aethiops uralensis Goltz 1930					AB324813
E. aethiops 3			FJ663461		
E. niphonica Janson 1877			AB306501		
E. triaria de Prunner 1798 1			DQ338782		
E. triaria 2			AY346219		
E. rossii Curtis 1835	AF214629	AF229972	FJ663521		
E. cyclopius Eversmann 1844			FJ663475		
E. medusa Fabricius 1787		GU001959	FJ628426		
E. epipsodea Butler 1868	AF214625	AF229968			
E. turanica turanica Erschoff 1877			FJ663539		
E. turanica susamyr Lukhtanov 1999			FJ663537		
E. sokolovi Lukhtanov 1990			FJ663530		
E. sibo Alpheraky 1881			FJ663523		
E. radians Staudinger 1886			FJ663519		
E. progne Grum-Grshimailo 1890			FJ663517		
E. meta melanops Christoph 1889			FJ663515		
E. meta meta Staudinger 1886			FJ663513		
E. kalmuka Alpheraky 1881			FJ663506		
E. alberganus de Prunner 1798	AF214593	AF229937			
E. theano Tauscher 1806 1			AB324846		AB324827
E. theano 2			FJ663535		
E. brimo Boeber 1809			FJ663511		
E. haberhaueri Staudinger 1881			FJ663493		
E. gorge Esper 1805 1			GU001961		
E. gorge 2			FJ628430		
E. gorgone Boisduval 1833				EU037888	EU037849
E. mnestra Hübner 1804	HE614710	HE614693		EU037889	EU037850
E. aethiopella Hoffmannsegg 1806	AF214619	AF229964			
E. rhodopensis Nicholl 1900	HE614709	HE614692			
E. callias altajana Staudinger 1901 1			AB324845		AB324826
E. callias altajana 2			FJ663468	EU037868	EU037814
E. callias sibirica Staudinger 1881			FJ663471	EU037871	EU037818
E. graucasica graucasica Jachontov 1909			FJ663478	EU037881	EU037838
E. graucasica transcaucasica Warren 1950			FJ663486	EU037883	EU037841

(*continued*)

Appendix. (*Continued*).

Taxa	Segments				
	LSU	ND1	CO1	CO2	ND5
E. iranica sheljuzhkoi Warren 1935			FJ663499		EU037836
E. ottomana tardenota Praviel 1941	HE614711	HE614694		EU037885	EU037846
E. ottomana benacensis Dannehl 1933	HE614712	HE614695		EU037884	EU037842
E. nivalis Lorković & de Lesse 1954	HE614713	HE614696		EU037866	EU037810
E. tyndarus Esper 1781	HE614714	HE614697		EU037863	EU037805
E. cassioides carmenta Früstörfer 1907	HE614715	HE614698		EU037856	EU037803
E. cassioides pseudocarmenta de Lesse 1952	HE614716	HE614699	AY346222	EU037854	EU037801
E. calcaria Lorković 1953	HE614717	HE614700		EU037865	EU037808
E. hispania Butler 1868					EU037833
E. rondoui goya Frühstörfer 1909	HE614719	HE614702		EU037874	EU037823
E. rondoui rondoui Oberthür 1908	HE614718	HE614701		EU037876	EU037828
E. pronoe Esper 1780 2		GU001958	FJ628439		
E. montana de Prunner 1798	AF214617	AF229962			
E. zapateri Oberthür 1875	HE614720				
E. oeme Hübner 1803 1			DQ338780		
E. oeme 2			AY346227		
E. meolans de Prunner 1798	AF214599	AF229944	AY346223		
E. palarica Chapman 1905			AY346220		
E. pandrose Borkhausen 1788 1		GU001960	FJ628436		
E. pandrose 2			AY346226		
E. pandrose 3			FJ663516		
E. sthennyo de Graslin 1850			DQ338781		
E. fletcheri Elwes 1899			FJ663477		