Eur. J. Entomol. **110**(2): 379–382, 2013
http://www.eje.cz/pdfs/110/2/379
ISSN 1210-5759 (print), 1802-8829 (online)

Phylogenetic relatedness of *Erebia medusa* and *E. epipsodea* (Lepidoptera: Nymphalidae) confirmed

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Key words. Lepidoptera, Nymphalidae, *Erebia medusa*, *E. epipsodea*, mtDNA, COI, ND1

Abstract. The extensive genus *Erebia* is divided into several groups of species according to phylogenetic relatedness. The species *Erebia medusa* was assigned to the medusa group and *E. epipsodea* to the alberganus group. A detailed study of the morphology of their copulatory organs indicated that these species are closely related and based on this *E. epipsodea* was transferred to the medusa group. Phylogenetic analyses of the gene sequences of mitochondrial cytochrome C oxidase subunit I (COI) and mitochondrial NADH dehydrogenase subunit 1 (ND1) confirm that *E. medusa* and *E. epipsodea* are closely related. A possible scenario is that the North American species, *E. episodea,* evolved after exclusion/isolation from *E. medusa*, whose current centre of distribution is in Europe.

INTRODUCTION

In most cases the species of genus *Erebia* Dalm. are morphologically very similar. The correct identification of many species is very often difficult due to their high variability and occurrence of subspecies and geographical forms. This variability also occurs in the morphology of the ectodermal copulatory organs. Molecular-biological methods, such as the study of allozymes or sequences of mtDNA, provide phylogenies for the genus *Erebia*. Surprisingly, in many cases they indicate that species originally thought to be related are genetically different and vice versa. *Erebia* (Dalman, 1816) is a species-rich genus with a Holarctic distribution. Numerous species occur in alpine and/or arctic habitats. *Erebia medusa* (Denis & Schiffermüller, 1775) (Woodland Ringlet) is currently distributed from central France over large parts of Central Europe and southern Siberia to the Pacific, while nearctic *Erebia epipsodea* Butler, 1868 (Common Alpine) occurs in an extensive area from Alaska through the Rocky Mountains to northern New Mexico and from the west coast across the prairie provinces to southwest Manitoba (Brussard & Ehrlich, 1970).

Warren (1936) placed *E. medusa* and *E. epipsodea* into two different groups: *E. medusa* together with *E. hewitsoni* and *E. polaris* into the so-called medusa group and *E. epipsodea* into the alberganus group, which includes *E. alberganus*, *E. maurisius*, *E. pawloskii*, *E. theano*, *E. youngi*, *E. kozantshikovi* and *E. dabanensis*. Recently, based on the detailed analysis of morphological traits, Belik (2000) suggested that *E. medusa* and *E. epipsodea* are closely related and proposed placing *E. epipsodea* into the medusa group. However, the results based on classical methods such as morphology, anatomy and ecology should be revised carefully using the molecular data that is currently becoming available (Martin et al., 2000; Peña et al., 2006).

In this paper the relatedness of *E. medusa* and *E. epipsodea* was determined based on phylogenetic analyses of sequences of the mitochondrial cytochrome C oxidase subunit I (COI) and part of the subunit 1 of the mitochondrial gene for NADH dehydrogenase (ND1) of *E. medusa* and *E. epipsodea*.

MATERIAL AND METHODS

Specimens of *Erebia medusa* were collected in the Western Carpathians (Belianske Tatry Mountains, Ondavská vrchovina Mountains, Muranska planina Mountains, Slovak Karst Mountains and Branisko Mountains). In the laboratory DNA was extracted from tissue from the thorax of adults and used for amplification. COI gene sequences were amplified using the primer pair 5'-ATAATTTTTTTTATAGTTAT-3' (forward) and COIR: 5'-GTTTCTTTTTTTCCTCTTTC-3' (reverse) and PCR cycling parameters 94°C for 5 min, 35 cycles of 94°C for 45 s, 45°C for 45 s, 72°C for 45 s and 72°C for 10 min as specified by Ban et al. (2007). NADH subunit 1 sequences were amplified using the primer pair 5'-CGTAAAGTCCTAGGTTATATTCA GATTCG-3' (forward) and 5'-ATCAAAAGGAGCTCGATTA GTTTC-3' (reverse, Martin et al., 2000) and cycling parameters 92°C for 5 min, 35 cycles of 94°C for 15 s, 52°C for 45 s, 72°C for 2 min followed by final extension at 72°C for 10 min.

PCR amplicons were purified and sequenced from both sides using the same primers as for PCR amplification at Macrogen sequencing facility (Macrogen, Seoul, Korea). The sequences were submitted to the GenBank database under Accession Num-

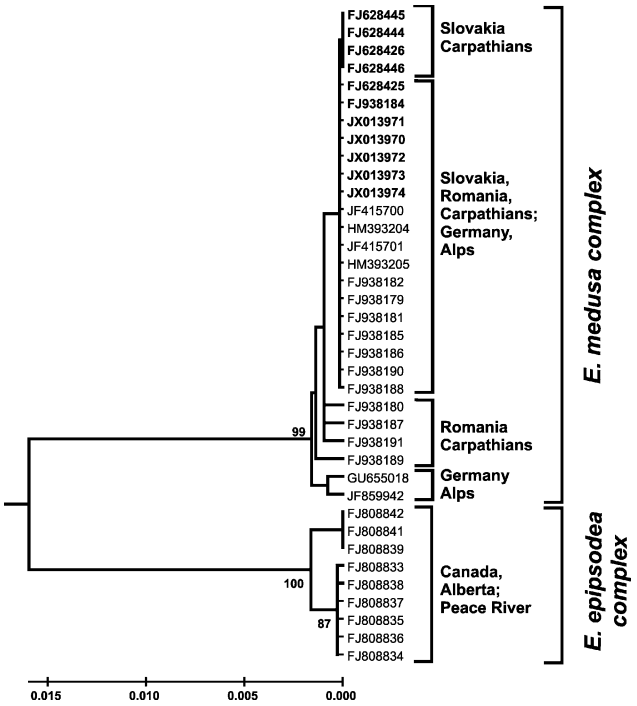

Fig. 1. Phylogenetic relatedness of *Erebia medusa* collected from locations in the Western Carpathians and those from the Romanian Carpathians and German Alps, and *E. epipsodea* from Alberta and Peace River in Canada. The phylogenetic tree inferred from the mitochondrial COI gene was constructed using the NJ algorithm. Numbers at nodes indicate bootstrap values after 1000 repetitions. The scale at the bottom of the figure indicates the number of nucleotide substitutions per site. The sequences obtained in this study are shown in bold.

bers: FJ628444–FJ628446, FJ628426, FJ628425 and JX013970–JX013974 for COI gene sequences and GU001957–GU001960 for NADH subunit 1 sequences.

For the phylogenetic analysis of COI gene sequences, the sequences obtained in our study together with those of *E. medusa* that originated from specimens collected in the Southern Carpathians (Dinca et al., 2010), Germany (Hammouti et al., 2009), *E. epipsodea* from North America (Bromilow & Sperling, 2011) and those of *E. oeme, E. theano, E. brimo, E. pandrose* and *E. pawloskii* available in the GenBank database were used. For the phylogenetic analysis of NADH dehydrogenase subunit 1 gene sequences, the sequences obtained in our study together with the ND1 sequences of *Erebia* obtained by Martin et al. (2000) (GenBank entries AF229937, AF229944, AF229953–AF229955, AF229962–AF229964, AF229968, AF229972) were used.

The sequences were aligned using the ClustalW algorithm implemented in MEGA 5 software (Tamura et al., 2007) and phylogenetic trees were constructed using all phylogeny reconstruction methods available in this software: Neighbor Joining (NJ), Maximum Likelihood (ML), Minimum Evolution (ME) and Maximum Parsimony (MP). Bootstrap analysis with 1000 iterations was used to assess the statistical strength of the branch positions.

RESULTS AND DISCUSSION

During our study of the molecular variability in COI sequences of 10 individuals of *E. medusa* from the Slovakian Carpathians were obtained. Sequence analysis

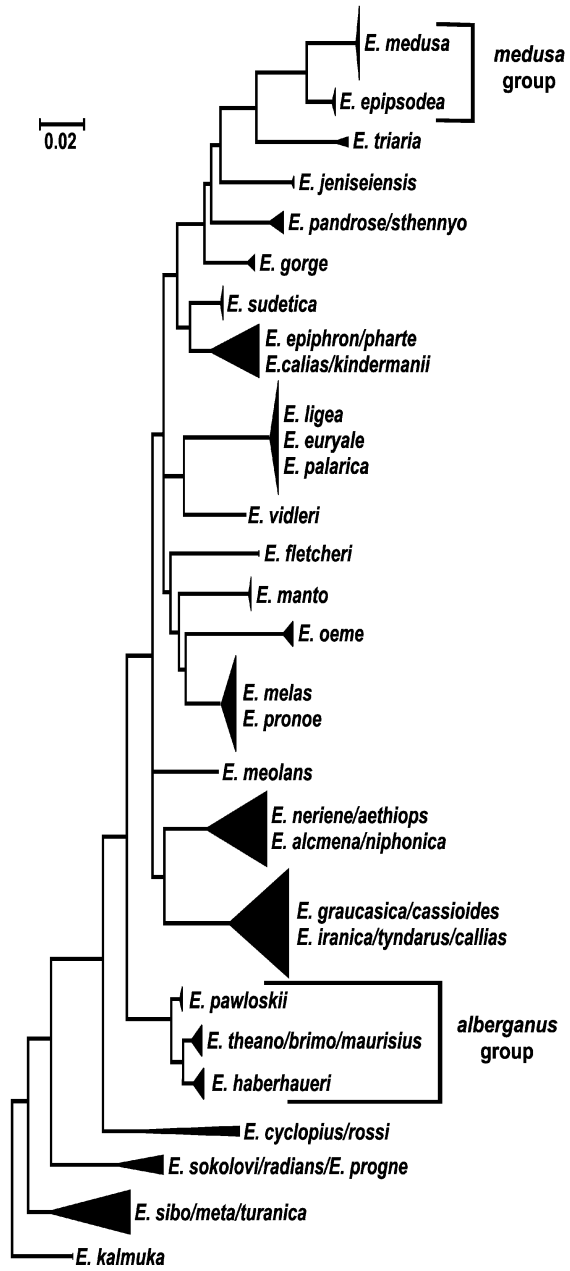

Fig. 2. Schematic unrooted Maximum Likelihood tree showing the phylogenetic relatedness of species of *Erebia* based on COI sequences. The height of each triangle is proportional to the number of sequences in the node and the width to the genetic diversity within the node.

clearly showed that these sequences belong to *E. medusa* with similarity values over 99% at nucleotide sequence level. Substantial diversity was observed within *E. medusa* sequences and several groups of isolates detected within the *E. medusa* complex (Fig. 1). Most of the *E. medusa* COI sequences are practically identical despite different geographical origins. However, in all the areas included in this study, Slovakia, Romania and Germany, the populations there differ genetically from the dominant *E. medusa* genotype. Such genetic differentiation among mountain ranges is well documented for other species of the genus *Erebia*, e.g. *E. euryale* (Schmitt & Hau-

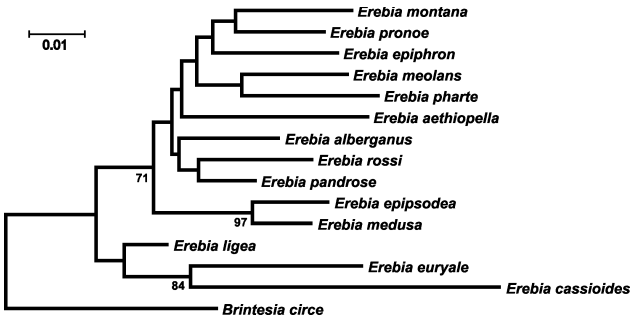

Fig. 3. Maximum Parsimony tree showing phylogenetic relatedness of species of *Erebia* based on ND1 sequences. The ND1 sequence of *Brintesia circe* (AF229946) was used as an outgroup. Numbers at nodes indicate bootstrap values after 1000 repetitions. Only bootstrap values over 70 are shown.

brich, 2008). The pattern of variability recorded in *E. medusa* differs from the several genetic lineages known for this well-studied species of butterfly (Schmitt & Seitz, 2001). Similarly, Schmitt & Müller (2007), based on an allozyme analysis, report that different lineages are present in the Alps and eastern Central Europe (Slovakia, Hungary). Hammouti (2006) proposed the formation of several major lineages in a phylogeographic scenario of *E. medusa* distribution in Europe, which resulted in two secondary sub-lineages in the area of Germany and a further two sub-lineages in the Czech Republic, with expected continuous loss of genetic diversity during the postglacial westward expansion.

Surprisingly, comparisons of all available *Erebia* COI sequences clearly indicated a close phylogenetic relationship between European *E. medusa* and American *E. epipsodea*. Phylogenetic trees using several phylogeny reconstruction methods available in MEGA5 software (Neighbor-Joining, Maximum Parsimony, Minimum Evolution, Maximum Likelihood) placed *E. medusa* and *E. epipsodea* in the same branch (Fig. 2, data documented using Maximum Likelihood method) with strong statistical support. For the Neighbor-Joining and Minimum Evolution methods, bootstrap values of 100 were recorded. The Maximum Parsimony method placed *E. epipsodea* and *E. medusa* in the same branch but was unable to resolve the relatedness of *E. epipsodea* and *E. medusa*. No phylogeny reconstruction method placed *E. epipsodea* together with *E. theano* or any other species of the *Erebia alberganus* group, which grouped together in a distant part of the tree. While the average number of base substitutions per site between *E. medusa* and *E. epipsodea* was 0.011 ± 0.002, the divergence between *E. epipsodea* and *E. theano* was four times greater (0.044 ± 0.006), while mean evolutionary diversity within species was 0.002 ± 0.001.

To confirm that *E. epipsodea* and *E. medusa* are related all available *Erebia* spp. ND1 sequences together with the *E. medusa* ND1 sequence from our previous study (M. Šemeláková et al., unpubl.) were analysed. Despite the relatively low number of sequences analyzed (14), all evolutionary history reconstruction methods placed *E. medusa* and *E. epipsodea* in a different branch from *E. alberganus* (Fig. 3, data documented for the Maximum Parsimony method). Similar bootstrap values were obtained for Minimum Evolution (96), Maximum Likelihood (96) and Neighbor-Joining (95) methods, confirming the close phylogenetic relatedness of *E. medusa* and *E. epipsodea*.

E. medusa and *E. epipsodea* were originally placed in different *Erebia* groups (Warren 1936). According to Belik (2000) the detailed analysis of the copulatory organs and wing colouration of *E. epipsodea* and *E. medusa* support a close relationship between these species. In his morphological study of *E. medusa* and *E. epipsodea* only specimens from a relatively small area in Siberia (Chita region) were compared. In our analysis samples from Central Europe were collected and the COI sequences obtained were striking similar to those of *E. medusa* throughout its eurosiberian distribution area. The data clearly reflect the close relationship between *E. medusa* and the American *E. epipsodea*. *E. epipsodea* is the closest relative of *E. medusa* based on analyses of both COI and ND1 sequences. *E. epipsodea*, despite being geographically very distant, is thus a member of the medusa group. On the other hand *E. medusa* and *E. oeme*, which are visually very similar, are genetically distant. The comparison of *Erebia* COI sequences confirmed the already documented phylogenetic relatedness of several visually-different species e.g. *E. ligea* and *E. euryale* (Martin et al., 2000). Similarly, our data on the COI gene sequences of *E. oeme* indicate that it is not closely related to *E. medusa* (Fig. 2) despite their being morphologically very similar, which makes the correct identification of *E. oeme* very difficult. Average difference in diversity between *E. medusa* and *E. oeme* is 0.027 ± 0.004, which is at least two times greater than that between *E. medusa* and *E. epipsodea*, indicating that molecular methods are the best way to discriminate between these two sibling species (Dinca et al., 2010).

We assume that *E. epipsodea* evolved by exclusion from the original lineage of *E. medusa*, i.e. *E. epipsodea* is a filial of *E. medusa*. During some of the interglacial periods, when northern parts of Eurasia were covered by tundra, *E. medusa* was probably widespread in all habitats from Scandinavia to the Bering Strait. According to several studies (Elias & Brigham-Grette, 2007), Asia was connected with North America when due to the quantity of water locked up in glaciers there was a reduction in sea level and a land bridge across the Bering Strait between these continents. Thus individuals of *E. medusa* were able to pass over this bridge into North America. The contact between the populations of *E. medusa* was broken when the sea level increased and inundated this terrestrial bridge. Due to its geographical isolation a new species of *E. epipsodea* evolved in America. *E. medusa* migrated from Asia to Europe with the expansion of the forests and persisted in forest-free areas. The process of colonization probably occurred from the East to the West Palearctic (Hammouti et al., 2009). *E. medusa* is a highly-variable species, as demonstrated by its many subspecies, or by other species which have developed from it, e.g. *E.*

polaris in Northern Europe. *E. medusa* variability was confirmed by our COI sequence analysis, which detected several geographical specific lineages within this species. A similar colonization scenario possibly also accounts for the evolution of other North American species, for example, from *E. danabensis*, which is wide-spread in the eastern area of Asia, *E. phellea* and *E. lafontainei* evolved and now occur only in North America (Troubridge & Philips, 1982). It is supposed that the North American species evolved during different periods and as a result of several migrations of the original species via the Bering Strait. Although *E. medusa* prefers unforested areas at low altitudes, its presence up to 2000 m indicates it can occur at higher altitudes. The population of *E. medusa uralensis* Staudinger, 1871 was probably preserved as a remnant of the original expansion of *E. medusa* at the border of Europe and Asia, as compared to *E. epipsodea* by Warren (1936). Unfortunately no COI sequences are available for the latter species, thus further analysis and higher numbers of available COI sequences will be needed to solve the phylogenetic relatedness and possible evolutionary routes in the *Erebia* genus.

ACKNOWLEDGEMENTS. This study was funded by VEGA agency project no. 1/0477/10 and 1/1025/12. The authors are grateful to A.J. Billingham for proof reading the manuscript.

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Received May 31, 2012; revised and accepted November 20, 2012