

Phylogeny of *Euphydryas* Checkerspot Butterflies (Lepidoptera: Nymphalidae) Based on Mitochondrial DNA Sequence Data

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ABSTRACT We present a molecular phylogeny of butterflies belonging to the genus *Euphydryas* s.l., based on partial sequences of 3 mtDNA genes (COI, ND1, and 16S). *Euphydryas* s.l. has been divided into 4 genera in an earlier revision. The current results show 3 well-supported groups within the genus, corresponding to 3 of the 4 proposed genera. The pair-wise divergences of 1 of the sequences (COI) are on the order of 3% within the 3 groups and 7% between the groups. This level of variation leads us to suggest that the entire group should be considered to be in the genus *Euphydryas* with 3 subgenera: *Euphydryas*, *Hypodryas*, and *Eurodryas*.

KEY WORDS *Euphydryas*, molecular phylogeny, mitochondrial DNA

BUTTERFLIES BELONGING TO the genus *Euphydryas* (sensu lato) Scudder (Lepidoptera: Nymphalidae: Melitaeini) have a Holarctic distribution. Eight species are found in the Palaearctic region and 6 in the Nearctic region (Higgins 1978). Although the monophyly of this group is not in dispute, the status of the group is controversial at the moment. Higgins (1978) raised the group to tribal status and divided it into 4 genera. The monophyly of the 4 genera has been questioned, and many authors consider the group to consist of a single genus within the tribe Melitaeini (Ehrlich and Murphy 1982, Brussard et al. 1985, Scott 1986, Britten et al. 1993, Karsholt and Razowski 1996). The putative genera of Higgins (1978) are *Euphydryas* (1 species) and *Occidryas* Higgins (4 species) which are exclusively North American, *Eurodryas* Higgins (4 species) which is exclusively Palaearctic, and *Hypodryas* Higgins which has 1 North American species and 4 Palaearctic species (Fig. 1).

Euphydryas species have long been the object of studies in population biology, especially in North America (Ehrlich et al. 1975, Bowers 1983, Baughman et al. 1990, Warren 1994, Thomas and Singer 1998). Despite this, there has been no attempt to construct a phylogeny for the entire group. There have been some attempts to construct a phylogeny for the North American species using information on isozymes (Brussard et al. 1985, Britten et al. 1993), but these lack resolution. Isozymes also have been used to investigate the validity of 2 species—*E. anicia* (Doubleday & Hewitson) and *E. colon* (Edwards)—closely related to *E. chalcidona* (Doubleday & Hewitson), and the results

indicated that these 3 species should be considered as 1 (Brussard et al. 1989). Zimmermann et al. (1999) present a phylogenetic hypothesis for the European Melitaeini based on isozymes and sequences of the ND1 gene, in which they show that the European *Euphydryas* s.l. species form a monophyletic group. The 2 putative Palaearctic genera *Eurodryas* and *Hypodryas* also form monophyletic lineages.

Evolutionary and biogeographic studies of the group have been hindered by the lack of a good phylogenetic hypothesis for *Euphydryas* s.l. Because some species are highly variable morphologically, DNA sequence data potentially provide numerous additional characters that are phylogenetically informative. It is particularly interesting to investigate whether the species within the 2 regions are more related to each other than to species in the other region.

The application of molecular methods in insects and particularly in Lepidoptera has increased exponentially within the last decade (e.g., Martin and Pashley 1992, Pashley and Ke 1992, Brower 1994, Sperling and Harrison 1994, Weller et al. 1996, Brower and DeSalle 1998). Many of these studies are based on sequences from only 1 gene, usually in mitochondrial DNA. Different genes are constrained to change in different ways (e.g., protein coding versus ribosomal genes, Simon et al. 1994) and can thus be informative at different levels of systematic hierarchy. It has recently been acknowledged that combining data sets can give more reliable results (Kluge 1989, Page 1996, Brower and Egan 1997).

We present here the most complete phylogenetic analysis of *Euphydryas* butterflies to date based on molecular data. We sequenced portions of 3 mitochondrial DNA genes with different rates of molecular evolution. Two of these genes are protein coding—cytochrome oxidase subunit I (COI) and NADH dehydrogenase 1 (ND1). The 3rd gene codes for the large ribosomal RNA subunit (16S). We specifically

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Higgins' (1981) morphological classification

Scott's (1986) classification of North American *Euphydryas*

Euphydryini

Euphydryas
phaeton

Occidryas
ancia
chalcedona
editha
colon

Hypodryas
maturna
intermedia
cynthia
iduna
gillettii

Eurodryas
aurinia
*alexandrina**
desfontainii
*orientalis**

Melitaeini

Euphydryas
phaeton
chalcedona (*ancia*, *colon*)
editha
gillettii

* Species not included in this study

Fig. 1. The taxonomic classification of *Euphydryas* s.l. according to two authors.

aim to investigate whether the genera proposed by Higgins (1978) are monophyletic in our cladistic analysis of the molecular markers. With the resulting phylogenetic hypothesis we propose a biogeographical hypothesis for *Euphydryas* s.l.

Materials and Methods

Taxon Sampling. We attempted to procure several specimens per species from as widely separated populations as possible (Table 1). We attempted to obtain specimens of all species but were not successful in getting 2 species—*E. orientalis* (Herrich-Schäffer) and *E. alexandrina* (Staudinger). These 2 species are, however, closely related to *E. aurinia* (Rottemburg)

(Higgins 1950) and we do not believe their inclusion would change our results significantly. Each specimen was treated as an operational taxonomic unit in the analyses. Three species were chosen as outgroups—*Melitaea didyma* (Esper) and *Chlosyne palla* (Boisduval) belonging to the same tribe Melitaeini (Higgins 1981), and 1 species, *Hypolimnys bolina* (F.), belonging to another closely related tribe Kallimini (Harvey 1991). The latter species was included because the relationships of the melitaeine genera are unknown.

Molecular Techniques. We extracted whole DNA from freshly frozen or dried specimens (Table 1). We were able to extract DNA from individuals up to 10 yr old that had been stored in relatively dry conditions (room temperature) but had not been relaxed for

Table 1. Collection data for specimens from which DNA was extracted

Species	Collection locality	Collection date	Condition	Collector
Outgroups				
<i>Hypolimnas bolina</i>	Ulu Gombak, Selangor, Malaysia	01 Sept. 1995	Dried	K. Fiedler
<i>Melitaea didyma</i>	Montpellier, France	25 April 1997	Frozen	A. Komonen
<i>Chlosyne palla</i>	Trail, B.C., Canada	13 July 1997	Dried	N. Kondla
Ingroups				
"Eurodryas"				
<i>aurinia</i>	Cervièeres, France	02 July 1995	Frozen	H. Descimon
<i>aurinia</i>	Tov Aimak, Mongolia	18 June 1997	Dried	Y. Marusik
<i>desfontainii</i>	Montes Universales, Spain	? May 1995	Frozen	H. Descimon
"Hypodryas"				
<i>matura</i>	Joutseno, Finland	01 July 1997	Frozen	N. Wahlberg
<i>matura</i>	Tov Aimak, Mongolia	18 June 1995	Dried	Y. Marusik
<i>intermedia</i>	Névache Judis, France	01 July 1995	Frozen	H. Descimon
<i>iduna</i>	Inari, Finland	12 July 1998	Dried	K. Karttunen
<i>cynthia</i>	Albulapass, Switzerland	27 July 1995	Frozen	H. Descimon
<i>gillettii</i>	Pondera Co., MT	15 July 1996	Dried	S. Kohler
"Euphydryas"				
<i>phaeton</i>	Anne Arundle Co., MD	10 June 1997	Dried	M. Soukup
"Occidryas"				
<i>editha</i>	Fresno Co., CA	1994	Frozen	M. Singer
<i>editha</i>	Powell Co., MT	30 May 1997	Dried	S. Kohler
<i>chalcona</i>	MariCopa Co., AZ	18 April 1998	Dried	B. Walsh
<i>chalcona</i>	Santa Barbara Co., CA	24 March 1998	Frozen	I. Hanski
<i>anicia</i>	Blaine Co., MT	20 May 1998	Dried	S. Kohler
<i>anicia</i>	Spring Mountain, ID	1997	Frozen	W. Watt
<i>colon</i>	Modoc Co., CA	15 July 1998	Dried	G. Pratt
<i>colon</i>	Trail, B.C., Canada	05 July 1997	Dried	N. Kondla

spreading (N.W. and M.Z., unpublished data). Relaxing specimens probably results in invasion of tissues by fungi or bacteria that break down cells and thus DNA. DNA was extracted using a phenol-chloroform protocol. The head and thorax (frozen specimens) or 2 legs (dried specimens) were homogenized in 250 μ l insect extraction buffer (50 mM Tris-HCl pH 8.0, 25 mM NaCl, 25 mM EDTA, 0.1% SDS). Cells in the homogenate were lysed using 2 μ l Proteinase K at 60°C for 3 h. DNA was extracted with phenol-chloroform-isoamylalcohol (25:24:1). Tubes were mixed thoroughly until the solution was homogenous, centrifuged at 13,000-rpm for 5 min and the supernatant decanted with a pipette. DNA was then precipitated with 15 μ l NaCl and 425 μ l ice-cold 100% ethanol. Tubes were placed in -20°C overnight, after which they were centrifuged at 13,000-rpm for 6 min. The pellet was washed with room-temperature 70% ethanol and allowed to dry. For DNA extracted from head and thorax, the dried pellet was resuspended in 200 μ l of TE and this was diluted further to 1:100 for use in polymerase chain reaction (PCR). DNA extracted

from 2 legs was resuspended in 50 μ l of TE and this solution was used for PCR. Specimens from which DNA was extracted from legs are deposited at the Finnish Museum of Natural History at the University of Helsinki as voucher specimens. The wings of all other specimens are stored at the Laboratoire de Systématique Evolutive, University of Provence.

We sequenced 3 regions of the mitochondrial genome. These were a 600-bp region in the beginning of cytochrome oxidase I (COI) corresponding to position 1539-2139 of the *Drosophila yakuba* sequence (Clary and Wolstenholme 1985); a 490-bp region in the beginning of NADH dehydrogenase 1 (ND1) corresponding to position 12,104-12,594 of the *D. yakuba* sequence; and a \approx 490 bp region in the middle of the large (16S) ribosomal subunit corresponding to position 12,873-13,351 of the *D. yakuba* sequence. The primers used are shown in Table 2.

All fragments were amplified with PCR in a total volume of 20 μ l. The following thermal cycling protocol was used: 10 min at 95°C, 35 cycles of 1 min at 94°C, 1 min at 47°C, and 1 min 30 s at 72°C, and a final

Table 2. Primers used for amplifying mitochondrial DNA

Mitochondrial DNA gene	Primer name ^a	Primer	Source
COI	LCO1490-J-1514	5'GGTCAACAAATCATAAAGATATTGG	Folmer et al. 1994
COI	HCO2198-N-2175	5'TAAACTTCAGGGTGACCAAAAAATCA	Folmer et al. 1994
ND1	3264-J-12095	5'ATCAAAGGACGCTCGATTAGTTTC	Aubert et al. 1996
ND1	1957-N-12567	5'CGTAAAGTCCTAGGTTATATTTCAGATTCC	Aubert et al. 1996
16S	LR-J-12887	5'CCGGTTTGACGCTCAGATCA	Simon et al. 1994
16S	LR-N-13398	5'GCCCTGTTTATCAAAAACAT	Simon et al. 1994

^a The last number in the primer name refers to the position of the 3' end of the primer in the *D. yakuba* sequence (Clary and Wolstenholme 1985) sensu Simon et al. (1994).

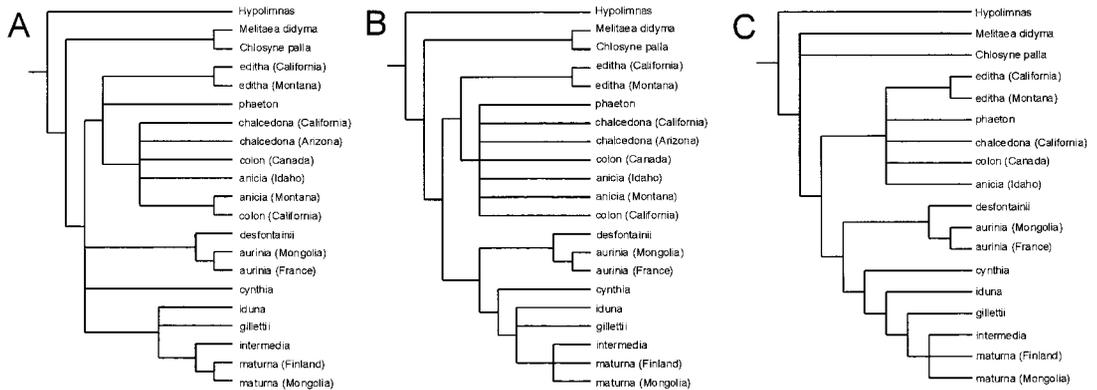


Fig. 2. (A) Strict consensus tree of 26 trees found in a parsimony analysis of the COI data set (length = 286 steps, CI = 0.67, RI = 0.72). (B) Strict consensus tree of 8 trees found in a parsimony analysis of the 16S data set (length = 188 steps, CI = 0.82, RI = 0.86). (C) Strict consensus tree of 8 trees found in a parsimony analysis of the ND1 data set (length = 229 steps, CI = 0.75, RI = 0.72).

divergence suggests that the *Euphydryas* group as a whole is relatively young.

The species pair *E. matura* and *E. intermedia* present an interesting case. The pair of *E. matura* have exactly the same sequence over 1,614 characters (bp + indels) of the combined data set. The protein coding sequences (COI and ND1) of *E. intermedia* differ from the *E. matura* sequence by only 1 bp in the COI gene. However, the supposedly more conservative 16S sequence has 7 differences between the 2 species. Our *E. intermedia* sample comes from a highly isolated population in the European Alps. We were unable to acquire a specimen from eastern Asia, which is the main area of distribution for this species. It would be interesting to see whether individuals in these populations have the same haplotype as their European counterpart. The homogeneity of the *E. matura* sequence is rather surprising because all other widely distributed melitaeine species show variation on the order of 10 bp in the COI gene when comparing individuals from widely separated populations (N.W., unpublished data).

All 3 gene sequences yield similar cladograms, but with different resolutions (Fig 2). The number of equally parsimonious trees given by the different data sets are 26, 8, 8, and 5 for COI, 16S, ND1, and combined data, respectively. The incongruence length difference of the 3 data sets is 0 and the test value of $\alpha = 1.000$ shows that the different data sets do not increase homoplasy at all when combined (see Farris et al. 1995). Most of the variation is within the *E. chalcedona* clade, which remains unresolved (Fig. 3). Fig. 3 gives a strict consensus tree for the 5 trees from the combined data set, with the associated Bremer support and bootstrap values. Because all data sets gave similar results and there was no incongruence in the strict consensus trees, we henceforth consider only the results of the combined data set. The 5 most parsimonious trees given by the combined data set are 703 steps long (CI = 0.74, RI = 0.76), and there are 210

trees between 1 and 3 steps longer than the most parsimonious trees.

The clades corresponding to the Nearctic *Euphydryas* (except *E. gillettii*), “*Hypodryas*” (including *E. Gillettii*), and “*Eurodryas*” have strong support when measured either with Bremer’s support indices or

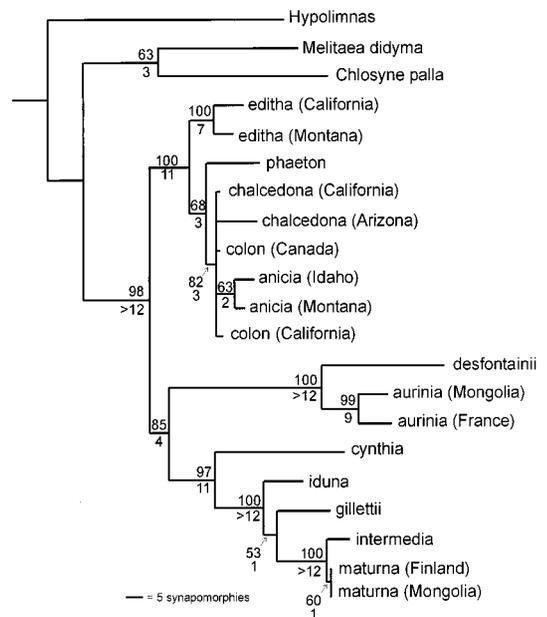


Fig. 3. Strict consensus tree of 5 trees found in a parsimony analysis of the combined data set. Branch lengths are proportional to the number of synapomorphies (unique base changes) assigned to that branch. Numbers above the branches give bootstrap values for the nodes to the right of the number. Numbers below the branches give Bremer support values for nodes to the right of the number. More than 12 means that >12 steps are required to make the clade above that node polyparaphyletic.

bootstrap. The monophyly of the Palaearctic species is fairly well supported, with 4 extra steps necessary to make the group paraphyletic (Fig. 3). Most internal branches in the 3 clades are well supported, though the *E. chalcedona* clade remains unresolved. The positions of *E. gillettii* as the sister species to *E. maturna-E. intermedia* has little support. We prefer to consider this node to be unresolved. Very similar sequence divergences between *E. iduna*, *E. gillettii*, and the *E. maturna-E. intermedia* clade suggests that this might be a true trichotomy. The trichotomy could have come about through allopatric speciation, with a glacial maximum splitting the ancestral population into 3 populations: a Nearctic population (*E. gillettii*), a Palaearctic arctic population (*E. iduna*), and a Palaearctic temperate population (*E. maturna* and *E. intermedia*).

Discussion

We found that 3 of the 4 putative genera in the *Euphydryas* s.l. correspond to 3 distinct clades in our molecular phylogenetic hypothesis. One clade consists of only Nearctic species that have formerly been included in the genera *Occidryas* and *Euphydryas*. Our phylogenetic hypothesis differs from previously published hypotheses in that *E. phaeon* is the sister species to the *E. chalcedona* group, whereas traditionally *E. editha* has been viewed as the sister species to this group (Higgins 1978, Brussard et al. 1985, Britten et al. 1993). Our phylogenetic hypothesis shows that the genus *Occidryas* is invalid taxonomically and that the widely held tradition of using *Euphydryas* as the genus for this group has been correct.

Our small sample of the *E. chalcedona* group shows that the relationships in this group may be as complex using mtDNA as when using isozymes (Brussard et al. 1989). A more detailed study is needed to elucidate the phylogenetic patterns within the group. It is entirely possible that the relationships within the group are even more complex than suggested by Brussard et al. (1989), as often 2 "subspecies" may apparently co-exist without interbreeding (Brussard et al. 1989; M. Singer, personal communication).

The other 2 distinct clades in our phylogenetic hypothesis correspond to Higgins' (1978) *Eurodryas* and *Hypodryas*. The 2 missing species, *E. orientalis* and *E. alexandrina*, are certainly in the *E. aurinia* clade and indeed have previously been considered subspecies of *E. aurinia* (Higgins 1950). It would be interesting to reconstruct the relationships of species within this group. One species, *E. aurinia*, is spread throughout the Palaearctic and is divided into many subspecies (Higgins 1950). The other 3 species have highly restricted distributions, with *E. desfontainii* found in southern Spain and northern Morocco, *E. orientalis* in Asia Minor, and *E. alexandrina* in the mountains of Central Asia (Higgins 1950). *Euphydryas desfontainii* is well diverged from *E. aurinia* with respect to the average sequence divergences of *Euphydryas* s.l. (Table 4). Higgins (1950) affiliates *E. orientalis* with *E.*

desfontainii and *E. alexandrina* with *E. aurinia* based on morphological characters.

Our results on the relationships of species in the *E. maturna* clade present an interesting biogeographical hypothesis. *Euphydryas cynthia* is the most basal species and is found in 2 populations, 1 in the Alps and the other in the mountains of the Balkans. The true nature of the *E. iduna*, *E. gillettii*, and *E. maturna-E. intermedia* trichotomy requires further study. If the trichotomy is real, then the ancestral population would have been split into 3 populations during the same glacial period. *Euphydryas cynthia* populations might have been isolated from the ancestral population during an earlier glacial period.

The species pair *E. maturna* and *E. intermedia* may have diverged fairly recently because there is only 1 difference over the protein coding sequence data set between these 2 species. This difference, a transition of T to C at position 25 in the COI sequence, is unique to *E. maturna*. The 2 species have a largely parapatric distribution, *E. maturna* in the western Palaearctic and *E. intermedia* in the eastern Palaearctic, with some overlap at the border (Higgins 1950). In addition, to this, *E. intermedia* has a highly isolated population in the European Alps, where it occurs at higher elevations than *E. maturna*. The homogeneity of the 2 *E. maturna* sequences is corroborated by additional COI sequences of 1 individual from France and 2 additional individuals from Mongolia (N.W., unpublished data). All sequences are the same, suggesting to us that *E. maturna* underwent a severe population bottleneck during the latest glacial period. This implies that the species may have survived a glacial period in only 1 refuge from where it has spread to its present distribution. This hypothesis needs to be tested by sampling more *E. maturna* populations.

The biogeography of *Euphydryas* s.l. is not clear without an appropriate outgroup analysis. Two alternative hypotheses that are equally parsimonious can explain Fig. 4. Either the group has originated in the Nearctic with the Palaearctic being colonized once and the Nearctic being recolonized once, or the group has originated in the Palaearctic with the Nearctic being colonized twice. The only unambiguous colonization event is that of *E. gillettii*, which has colonized the Nearctic from the Palaearctic.

The current phylogenetic hypothesis can tell us something about the evolution of ecological characters in *Euphydryas* s.l., such as host plant use. As with most butterflies, the species of *Euphydryas* are monophagous or oligophagous. All recorded host plants belong to the class Asteridae (Table 5). The Nearctic *Euphydryas* s.s. species feed mainly on plants in the Scrophulariaceae and Plantaginaceae (a recent article suggests that Plantaginaceae is in fact within Scrophulariaceae [Olmstead and Reeves 1995]). Species in the *E. maturna* clade are more diverse: the more basal species, *E. cynthia* and *E. iduna*, feed on similar host plants (*Plantago* and *Pedicularis*) in alpine and arctic habitats, respectively. The other 3 species in the clade feed mainly on shrubby or tree-like plants belonging to the families Caprifoliaceae and Oleaceae,

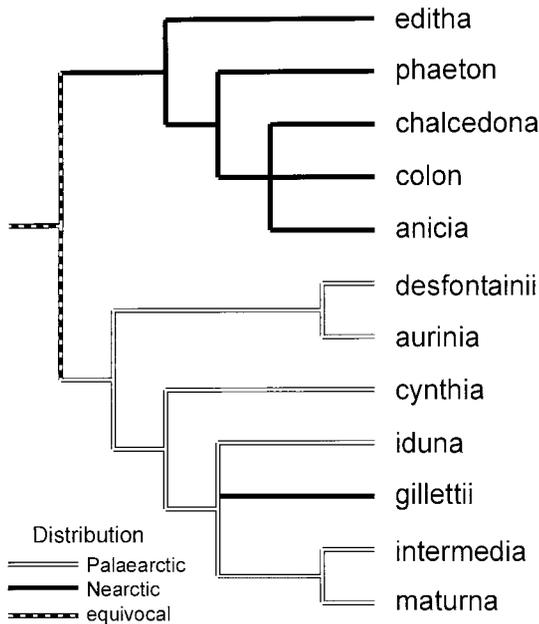


Fig. 4. Maximum parsimony reconstruction of the distribution of the genus *Euphydryas* based on the tree given in Fig. 3. The branch between *E. iduna* and *E. gillettii* has been collapsed because of low support.

usually growing in clearings of temperate forests. Host plant use by the *E. maturna* clade does not support the trichotomy described above, rather it suggests that *E. gillettii* is closer to *E. maturna*-*E. intermedia* than *E.*

Table 5. Plant families on which *Euphydryas* s.l. larvae have been recorded

Species	Commonly used host plant families ^a	Rarely used host plant families ^b
<i>E. editha</i>	Scrophulariaceae, Plantaginaceae	
<i>E. phaeton</i>	Scrophulariaceae	Lamiaceae, Plantaginaceae
<i>E. chalcedona</i>	Scrophulariaceae	Caprifoliaceae, Buddlejaceae, Orobanchaceae, Lamiaceae
<i>E. anicia</i>	Scrophulariaceae	
<i>E. colon</i>	Scrophulariaceae	
<i>E. aurinia</i>	Dipsacaceae	Gentianaceae, Caprifoliaceae
<i>E. desfontainii</i>	Dipsacaceae	
<i>E. cynthia</i>	Plantaginaceae	
<i>E. iduna</i>	Scrophulariaceae	
<i>E. gillettii</i>	Caprifoliaceae	Valerianaceae
<i>E. intermedia</i>	Caprifoliaceae	
<i>E. maturna</i>	Oleaceae, Scrophulariaceae	Caprifoliaceae

Sources: (Scott 1986, Tolman 1997, Wahlberg 1998).

^a Plant families which are used by the majority of the butterfly species populations.

^b Plant families used by single populations of the butterfly species or are used occasionally alongside plants in the major host plant families.

iduna. Finally, species in the *E. aurinia* clade are mainly associated with Dipsacaceae, generally in open landscapes.

All the plant families mentioned in Table 5 are united by the presence of a group of secondary defense chemicals known as iridoids (Jensen et al. 1975). Iridoids are divided into 2 major groups: iridoid glycosides and seco-iridoids. Both are known to be extremely bitter tasting and can have emetic effects on vertebrates (e.g., Bowers 1981). Bowers (1981, 1983) and Bowers and Puttick (1986) have shown that iridoid glycosides are necessary feeding stimulants for Nearctic *Euphydryas* larvae and that the larvae are able to sequester these compounds for use in their own chemical defense. *Euphydryas* larvae are considered aposematic and they are unpalatable to birds (Bowers 1981).

The sequestrability of iridoid glycosides and seco-iridoids apparently are different. Only iridoid glycosides have been recorded to be sequestered in all the Nearctic species (Bowers 1983, Bowers and Williams 1995) and in 1 Palearctic species, *E. cynthia* (Franke et al. 1987). The picture is somewhat complicated by the fact that some of the plant families have only (or mainly) seco-iridoids. These are Dipsacaceae, Caprifoliaceae, Gentianaceae, and Oleaceae. The 1 species that has been studied (*E. gillettii*) was unable to sequester seco-iridoids, though the larvae did sequester iridoid glycosides when feeding on a less-used host plant (Bowers and Williams 1995). The related species *E. maturna* is known to mainly use herbacious Scrophulariaceae (which have only iridoid glycosides) in Finland, whereas in the southern part of its range in Europe, it feeds on species of *Fraxinus* (Oleaceae, which contain mainly seco-iridoids) (Wahlberg 1998). The sequestering abilities of these different populations is unknown as yet. Species in the *E. aurinia* clade appear to have specialized on plants containing only seco-iridoids (Table 5). The sequestering abilities of these species has not been studied.

Our results support 3 of the 4 genera proposed by Higgins in his 1978 revision of the group; the polyphyletic genus *Occidryas* should be abandoned immediately. However, the sequence divergences between the species are fairly low, even between the 3 main clades. The group as a whole is also morphologically distinct from other melitaeine genera and is overall a compact, well-defined group. These 2 reasons, along with a wish to reduce taxonomic inflation, leads us to recommend strongly that the genus *Euphydryas* be used for the entire group, including *Hypodryas* and *Eurodryas*. The genera *Hypodryas* and *Eurodryas* should be relegated to subgenus status because they describe monophyletic groups within *Euphydryas*.

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