Molecular systematics and phylogeny of the ‘Marbled Whites’ (Lepidoptera: Nymphalidae, Satyrinae, Melanargia Meigen)

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Abstract. We investigated genetic divergence and phylogenetic relationships amongst all known species of Palaearctic butterflies of the genus Melanargia using sequence information from three genes [mitochondrial cox1 barcode region (658 bp), ribosomal 16S rRNA (c. 518 bp), and nuclear wg (404 bp)]. Results show a lack of DNA divergence among several poorly characterized taxa, as well as deep divergences within and between others. We corroborated the molecular information with morphological and genitalic characters as well as with geographic data. We revise the taxonomy of Melanargia, and propose a new systematic scheme for the group. We revise some previous synonymies (M. lucasi meadwaldoi stat. rev., M. ines fathme stat. rev., M. ines jahandiezi stat. rev., M. meridionalis tapaishanensis stat. rev.), revise the status of some subspecies into species (M. transcaspica stat. nov., M. lucida stat. nov., M. wiskotti stat. nov.), and of several species into subspecies of other taxa (M. evartianae sadjadii stat. nov., M. larissa hylata stat. nov., M. larissa grumi stat. nov., M. larissa syriaca stat. nov., M. larissa titea stat. nov., M. lugens montana stat. nov., M. epimede ganymedus stat. nov.), revise the status of subspecies and transfer them to other species (M. larissa lorestanensis stat. nov., M. larissa iranica stat. nov., M. larissa karabagi stat. rev., M. larissa kocaki stat. nov., M. transcaspica eberti stat. nov.), and propose new synonymies (M. larissa titea = M. titea standfussi syn. nov., M. titea titania syn. nov., M. leda leda = M. leda yunnana syn. nov., M. lugens lugens = M. lugens ahyoui syn. nov., M. lugens hengshanensis = M. lugens hoenei syn. nov., M. halimedehalimede = M. halimede gratiani syn. nov., M. asiatica asiatica = M. asiatica dejeani syn. nov., M. asiatica elisa syn. nov., M. asiatica sigberti syn. nov.).

Introduction

Butterflies of the genus Melanargia Meigen comprise 24 species distributed from Europe to the far east of Russia. Commonly known as ‘Marbled Whites’ owing to their chequered black and white wing pattern, they are characterized also by a dilated vein 12 at the base of the forewing (Higgins, 1975). This phenotypic distinctiveness has warranted a monotypic tribe (Melanargiini Wheeler), but with further phylogenetic relationships with other Satyrines remaining unclear. Previous molecular studies have suggested various members of Erebiini, Maniolini or Satyrini (Martin et al., 2000; Yin et al., 2007), or the Asiatic Orsotriaena, Neotropical Cyllopsis or Afrotropical Neocoenyra (Peña et al., 2006) as closest relatives of Melanargiini. Within Melanargia, three subgenera are often recognized: Melanargia (including the taxa M. galathea, M. russiae, M. larissa, M. hylata, M. titea, M. syriaca, etc.); Argeformia (including M. arge, M. ines, M. occitanica and M. pherusa); and Halimede (including eight species confined to eastern Asia) (Oberthür & Houlbert, 1902;栖木 & 弐栄, 1907; 沈, 1940).

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1922; Verity, 1953). Disagreement over the status and placement of species within these subgenera (e.g. see Wagener, 1983) exists, compounded by electrophoretic evidence, which placed \( M. \) arge in a separate clade, sister to all \( Melanargia \) (Mensi et al., 1990).

Despite a shared uniform general wing pattern, species of \( Melanargia \) vary, particularly in the intensity of black suffusion on the upperside of the wings. Within each species, populations comprise very light and/or very dark individuals, and the geographical distribution of the lighter or darker forms shows no correlation with regional humidity or altitude (Hesselbarth et al., 1995). However, this variation seems to be heritable: in a rearing experiment, the offspring of \( Melanargia \) galathea resembled their parents (Roos, 1983).

Structural characters used traditionally in Lepidoptera provide little insight into species boundaries in \( Melanargia \). The larvae are indistinct and show wide individual variation (Jutzeler, 1994; Jutzeler & Leestmans, 1994; Nardelli & Giandolfo, 1994; Jutzeler et al., 1995, 1996; Nardelli et al., 1998). The commonly used genitalic characters, the shape and number of valval terminal teeth, do vary within some species (Higgins, 1975; Bozano, 2002). Although microscopic structure and sculpture of the eggshells can be informative (Wagener, 1983), few studies exist. The few karyotyped species show no informative variation (\( M. \) lachesis and \( M. \) galathea \( n = 24 \), Lorkovic, 1941; \( M. \) russiae \( n = 24 \), de Lesse, 1960; \( M. \) titea \( n = 23 \), Larsen, 1975; \( M. \) larissa iranica \( n = 19 \) Lorkovic, 1977 in litt., Hesselbarth et al., 1995). Similarly, the host preference in \( Melanargia \) is uninformative, as all species feed on various grasses in one family (Poaceae).

Despite new ‘species’ of \( Melanargia \) having been described as recently as 2006 (\( M. \) sadjadii Carbonell & Naderi, 2006), considerable uncertainty remains over species diversity. The eight East Asian species, although less intensively studied, seem to be well differentiated in morphology (particularly of the male genitalia) and stable in their taxonomic status since Wagener (1956). The relationship and status of European \( M. \) galathea, \( M. \) lachesis and \( M. \) lucast has been debated (Higgins, 1969, 1975; Tilley, 1983, 1986; Wagener, 1983; Mazel, 1986), but a general consensus may exist (Bozano, 2002). The taxon \( pherusa \), usually considered a subspecies of \( M. \) occitanica (cf. Bozano, 2002), sometimes has been regarded as a good species on the grounds of subtle differences between the early stages (Stauder, 1926; Jutzeler et al., 1996), although enzyme electrophoresis showed very close affinity between the two (Mensi et al., 1990). The taxon \( meda \), described originally as a separate species, (Wagener, 1976), is now a subspecies of \( M. \) teneates based on the shape of male genitalia, the distribution ranges and the existence of intermediate populations (Bozano, 2002).

Another problem concerns the species boundaries and taxonomic relationships in a western Palearctic complex comprising several closely related ‘species’, namely \( M. \) larissa, \( M. \) hylata, \( M. \) syriaca, \( M. \) grumi and \( M. \) titea. Despite a lack of definitive diagnostic characters and a nearly complete absence of sympatry, these taxa have been treated predominantly as valid species, each with subspecies that often are used interchangeably. Past morphometric studies on this group have been inconclusive. Wagener (1983) used differences in egg morphology as evidence for the status of \( 12 \) \( Melanargia \) species, including the five members of \( M. \) larissa group. In a multivariate analysis on morphometric measurements from the male uncus and valva of 307 specimens of the five species in the \( M. \) larissa group, M. Ercolinio & V. Sbordoni (1997, unpublished) provided some evidence for species-level separation of \( M. \) grumi and \( M. \) syriaca, but demonstrated that \( M. \) larissa and \( M. \) hylata show very few, if any, differences (V. Sbordoni, personal communication).

The use of poorly defined and ‘fluid’ diagnostic characters from wing elements, egg morphology or even genitalia in \( Melanargia \) has negatively impacted the taxonomy of the group and has obstructed morphological phylogenetic studies of the genus, and a genetic analysis of \( Melanargia \) has been supported before (Hesselbarth et al., 1995). Here, for the first time, we use both mitochondrial and nuclear DNA sequences to infer the phylogeny of \( Melanargia \) and to evaluate the robustness of the latest species-level taxonomy for the genus (Bozano, 2002) (Supporting Information SI1) against molecular data and corroborating information from morphology (wing-pattern and genitalia) and geography.

**Materials and methods**

**Taxon sampling**

A total of 353 specimens from all 23 species (\textit{sensu} Bozano, 2002) and several subspecies of \( Melanargia \), as well as \( M. \) sadjadii (Carbonell & Naderi, 2006) were selected from private collections of the authors or received as donations (Supporting Information SI2). The voucher data are publicly available through the published project ‘\( Melanargia \) of the World’ (MEL) on the Barcode of Life Database (BOLD; www.barcodinglife.com). Also included in the analysis were 11 \( Melanargia \) sequences from GenBank (Martin et al., 2000: AF214589, AF214603, AF214621; Peña et al., 2006: DQ338706–8, DQ338843–5; Yin et al., 2007: EF545701–2). Outgroups for the phylogenetic analyses were chosen from previous phylogenetic studies (Martin et al., 2000; Peña et al., 2006) and sequences obtained from GenBank (Supporting Information SI2).

**Genitalia preparations**

Previously published genitalia figures were re-examined (Wagener, 1959–1961; Bozano, 2002). When molecular data suggested a need for taxonomic revision at species or subspecies level, selective dissections of single specimens of \( Melanargia \) were carried out by GCB and WtH (Supporting Information SI3). Dissected specimens were not used in the molecular analysis. The genitalia were fixed in Euparal glyc erin and photographed in lateral view with the frontal valva.
removed in order to have a better view of the inner side of the other valva. The frontal valva was embedded separately. The aedeagus sometimes had to be removed.

Molecular techniques

Two dry legs from each adult specimen were detached and stored in individual vials. The extraction of total genomic DNA, amplification and sequencing were performed in the Biodiversity Institute of Ontario using previously described protocols (Hajibabaei et al., 2005). Initially, full-length mtDNA barcode sequences (658 bp) were obtained for nearly all specimens, and, based on results from sequence similarity (neighbour-joining) analyses and the quality of DNA, a subset was selected for additional gene sequencing. Failed samples were targetted for smaller fragments of coxl (132 bp) using mini-barcode primers and protocols described previously (Meusnier et al., 2008). Ribosomal 16S rRNA and nuclear wingless (wg) genes were also obtained using primers and protocols described previously (Brower & DeSalle, 1994; Aubert et al., 1999). Amplified DNA from all specimens was sequenced in both directions for each gene, and final sequencing products were run on an ABI 3730® DNA analyser (Applied Biosystems, Foster City, CA). Complementary strands were assembled into contigs and edited manually, and primers were removed using Sequencher 4.5 (Gene Codes Corporation, Ann Arbor, MI). Sequences were aligned using CLUSTALX 2.0 (Thompson et al., 1997), evaluated by eye and converted to Nexus using SE-AL 2.0a11 (Rambaut, 2002). New sequences were deposited in GenBank, and accession numbers are given in Supporting Information SI2. coxl barcode sequences are also available publicly through the published project ‘Melanargia of the World’ (MEL) on the Barcode of Life Database (BOLD; www.barcodinglife.com).

Sequence data analysis

Neighbour-joining (NJ) trees for barcode data were constructed initially using the quicktree algorithm (Howe et al., 2002) and under the Kimura two-parameter (K2P) model (Kimura, 1980). Additional NJ and maximum parsimony (MP) analyses were conducted independently on each partition. Nucleotide base frequencies differed significantly in coxl and 16S but not in wg, and parsimony-informative characters in coxl and wg were typically in the third codon positions. The combined dataset of selected taxa had 1582 characters, of which 332 were parsimony-informative (Table 1).

A neighbour-joining tree of barcode haplotypes is given in Fig. 1, with bootstrap and Bremer values given for supported nodes. Species-level identification of several samples was revisited by re-examination of vouchers following barcode results. Some identification errors were corrected, including one specimen of M. larissa identified originally as M. russiae (DNAwithmel 026), and one specimen of M. teneates identified originally as M. evartianae (DNAwithmel 041). Phylogenetic hypotheses for each of the three genes, as well as for the combined dataset, are presented in Fig. 3. The ML analysis for the combined data (Fig. 3d) recovers three previously proposed subgenera, Melanargia, Argeformia and Halimede.

Clade 1: subgenus Argeformia

Our results do not support M. arge as sister to all Melanargia, as suggested previously (Mensi et al., 1990). Instead,
Fig. 1. Neighbour-joining tree of cox1 barcodes of *Melanargia* (including GenBank sequences) examined in this study. Numbers in parentheses represent number of individuals in each cluster; numbers on branches are bootstrap values from maximum parsimony analysis followed by Bremer support values. The four-letter codes correspond to species names. The 'larissa' and 'halimede' haplogroups each represent multiple species, with the complete haplotype complements presented in Fig. 2. Haplotypes in bold are those used in the phylogenetic analysis (Fig. 3).
M. lucasi appears to be closely related to M. occitanica, which together with M. ines forms a sister-clade to all other Melanargia (Fig. 3d). Specimens of arge from Puglia and Campania (Italy) show limited (cox1, wg) or no (16S) genetic divergence (Supporting Information SI4).

We found a deep split in the M. ines clade separating the African specimens (haplotype INES4) from those in mainland Europe (Fig. 1). This gap was more pronounced in the cox1 barcodes (4.4%) than in 16S (1.4%) or wg (1.3%) genes. Examination of genitalia showed some structural differences between these two lineages: in Spanish populations, the distal tip of the valva is rounded and there are four teeth of similar size arranged in a row, whereas in the population from High Atlas in Morocco there are five teeth on the valva and the ventral tooth is much larger than the others (Fig. 4a, b). Average genetic variation within these two clades was low, with the exception of 16S among African populations (1.8%), where individuals of the previously synonymized taxon M. ines ssp. jahandiezi (n = 3) clustered very distant from other ines (Fig. 3b). Their genitalia also showed differences, with several smaller teeth instead of a single fifth tooth at the lower edge of the valva (Fig. 4c).

As with M. ines, in M. occitanica we observed two distinct lineages, one consisting of specimens of the nominal subspecies from France and Spain (OCCI5–6) and a second comprising ssp. pherusa from Italy (OCI4) together with all African populations (OCCI1–3). The genitalia of specimens in these two groups are very similar: the distal tip of the valvae in both populations has three or four teeth arranged on a common base (Fig. 4d, e). Within the African clade, the taxon pherusa stands out with a notably longer branch length (Fig. 1).

Clade 2: subgenus Melanargia

Our results show unambiguously that the taxon lucasi is a well-differentiated species, distinct from M. galathea and M. lachesis (Figs 1, 3). The genitalia of M. galathea, M. lachesis and M. lucasi are more similar to one another than to other species in the subgenus, although M. lucasi has a smaller uncus and a broader triangular valva, which ends in five or six teeth (as opposed to seven to eight in M. galathea and M. lachesis) on a common base at the pointed distal end (Fig. 4f, g). The genetic variation within the Moroccan populations is small; however, our single specimen from Tunisia (LUCAL3) is notably divergent. The genitalia of the Tunisian M. lucasi are very similar to those of the Moroccan specimens both in form and number of valval teeth, although the wing patterns show certain differences (cf. Tennent, 1996).

We observed consistent genetic difference between M. galathea and M. lachesis (cox1: 0.70 ± 0.10%; 16S: 0.34 ± 0.22%; wg: 2.59 ± 0.64%). The average intra-specific variation in these two species was also higher in wg than in mitochondrial genes, a phenomenon that warrants further scrutiny.

In all reconstructions, M. russiae was divided into two distinct groups: one that included specimens from north (ssp. eberti, TRAN1) and north-eastern (ssp. transcaspica, TRAN2) Iran, and another comprising all other populations from Europe to Central Asia (RUSS1–9). In the second M. russiae clade, two specimens of ssp. cleanthe (RUSS9) were somewhat distant from others; however, despite a wide geographic coverage, variation within the remaining russiae was relatively small. Similarly, M. parce divided into two distinct groups, one of the nominal subspecies (PARC) and a second comprising ssp. lucida (LUCI1). In our phylogenetic reconstructions, the taxon lucida was always sister to all (except for wg, for which no lucida sequences could be obtained); the nominal M. parce were closely related to the NNE Iran M. russiae (sssp. transcaspica and eberti), and these together were sister to all other M. russiae. The genitalia in this group show clear differences that coincide with their genetic divergence: specimens of lucida have about 20 small teeth on the distal end of the valva, but in the nominal M. parce there are only about eight to nine claw-like teeth on the end of the valva, in a somewhat different arrangement. In nominal M. russiae, these claw-like teeth number about 11, and the ventral claw is the largest, whereas the taxon eberti has about 7 shorter teeth, which are less bent than in M. russiae, and the taxon transcaspica has about 16 teeth, which in size and form are similar to those of eberti. The aedeagus in M. russiae is shorter than in the taxa eberti and transcaspica (Fig. 4k–p).

The genetic divergences between M. sadjadii (EVAR1) and M. evartianae (EVAR2), and between nominal M. teneates (TENE1, 3) and M. teneates ssp. meda (TENE2) were relatively small. The genitalia of taxa teneates and meda have minor differences. The aedeagus of teneates is somewhat larger and has a bigger diameter; the valvae are triangular with about 10 small, straight teeth on the distal tip. In meda, there are up to 15 even smaller teeth on the valva. Specimens of M. teneates from Gilan (TENE3) are divergent from others, but the genitalia are similar and no other reliable morphological character corroborated this pattern (Fig. 4q–s).

Among the 152 specimens in the M. larissa group (LAR1) (including M. hylata, M. larissa, M. syriaca, M. grumi and M. titea) and throughout the genes examined, with a few exceptions we found no significant genetic differentiation among populations of these taxa, and the exceptions were not fully congruent with the current taxonomic arrangements (Figs 1–3). Among the 128 full-length cox1 barcode sequences included in the haplotype analysis, the most common haplotype shared between M. hylata, M. larissa, M. grumi, M. syriaca and M. titea (LAR101) occurred in 31 specimens (Fig. 2). The taxon wiskotti (WISK, n = 7), normally recognized as a subspecies of M. titea, was notably divergent from others (Figs 1–3). Individuals of wiskotti are larger than nominal M. titea but their genitalia are about the same size; there are 8 to 10 teeth with a common base on the end of valvae, which are only slightly bent. In the nominal M. titea, these teeth are arranged more regularly, in rows, whereas they are arranged irregularly on a common base in the taxon wiskotti (Fig. 4t–x). Other distinct clusters included populations of M. larissa from Greece (ssp. larissa, LARI24–29) and central Turkey (ssp. taurica + massageta, LARI31–34), as well as M. hylata from southern Iran (ssp. iranica, LARI17–22).
Fig. 2. cox1 haplotype networks for the (a) *larissa* and (b) *halimede* haplogroups. Numbers within or next to circles are the haplotype number. Small solid circles represent missing haplotypes, and lines between circles correspond to a one-step mutational change. Circle size is proportional to haplotype frequency, where the smallest numbered circles in each map represent one specimen. Sequences shorter than 658 bp and those with ambiguous bases have been excluded. Original identifications are used in shading both diagrams.
An additional three specimens from southern Fars (LARI23) always clustered separately, close to the taxon *iranica* (Fig. 2).

**Clade 3: subgenus Halimede**

Among the eight species in this group, in our mitochondrial and combined gene trees, *M. leda* was sister to others with moderate support (Figs 1, 3). In the *wg* tree, it fell within other species without support. This small species has very distinct genitalia with many apical teeth on the valva. The other seven species in this group, much like the *M. larissa* haplogroup, demonstrated a polyphyletic pattern with small intra- and inter-specific molecular divergence. Most species are phenotypically very similar and cannot be identified reliably without examination of genitalia. The male genitalia of these seven species were prepared and studied (GCB, not presented...
here), and, in corroboration with molecular results, initial identifications for several specimens were re-evaluated.

In our NJ trees from cox1 and 16S sequences as well as in the cox1 haplotype analysis, specimens collected from the same locality and on the same day often showed haplotype variation, whereas more distant species [e.g. M. ganymedes (Qinghai) and M. asiatica (Sichuan)] shared the same haplotype (HAL101) (Fig. 2). Specimens of M. meridionalis ssp.
tapaishanensis from Shanxi (HALI15) consistently clustered separately from other species, including from other M. meridionalis. This taxon is morphologically very well characterized by the extreme dark suffusion on the wings.

**Discussion**

**Unexpected deep divergence**

The large gaps observed between the North African and south European populations of *M. ines* and *M. occitanica* suggest a prolonged period of lack of genetic exchange caused by an early split in the range of the ancestral stock. The last contact between Europe and Africa occurred at the end of Miocene (7–5.3 Ma) (Sammartin, 2003), when a temporary closure of the water corridors between Africa and the Iberian Peninsula permitted biotic exchange between the two continents (Krijgsman, 2002). During this period, known as the ‘Messinian Salinity Crisis’, not only was Spain connected to Morocco, but Tunisia was connected to the Italian mainland via Sicily as well as to the islands of Sardinia and Corsica (Steininger & Rögl, 1996; De Jong, 1998). The barrier was restored when Gibraltar re-opened by the beginning of the Pliocene (5 Ma). This short period of connection between the two continents has been suggested as a plausible explanation for vicariance between the African/Iberian lineages of many organisms, including butterflies (e.g. Zerynthis ruminia, Nazari & Sperling, 2008; members of the genus Elphistonia, Leestmans, 2005). Based on our findings, African populations of *M. ines* and *M. occitanica* should be recognized as (at least) distinct subspecies from their counterparts in mainland Europe. We revive previously synonymized ssp. jahandiei (stat. rev.) for the High Atlas populations of *M. ines*, and ssp. fathine (stat. rev.) applicable to the remaining African populations of this species.

The situation in *M. occitanica* accords with the current taxonomic arrangement for the species (Bozano, 2002). Our re-arrangement does not support a separate specific status for the taxon *pherusa*, as suggested earlier (e.g. Mensi et al., 1990; Nardelli & Giandolfo, 1994). In Africa, *M. occitanica* shows an east–west clinal gradient, in which Moroccan specimens are very similar to the nominal subspecies from the Iberian Peninsula, whereas in Algeria the wing pattern elements approach the pattern of *pherusa*. The relict distribution of *pherusa* in Sicily, and its close affiliation with the African lineage of *M. occitanica* (albeit with a notable distance), suggests a more recent split in the range of the species through either a vicariance or a dispersal event between Sicily and Africa. The Sicilian and Tunisian coasts were much closer during the Pleistocene as a result of low sea levels (Stöck et al., 2008). To cross from Tunisia to Sicily, these butterflies would have had to fly in a more or less west–east direction, which could have been facilitated by the west–east winds predominant in most of Europe and outside the tropics from 35° to 70° latitude. Divergence within the past million years from an African sister-lineage has been demonstrated also in the Sardinian Burnet moth * Zygaena ornata* (Naumann et al., 1984), Chalcides lizards (Giovannotti et al., 2007) and discoglossid frogs (Zangari et al., 2006) from Sicily and Sardinia, and in Sicilian Green toads in the genus *Bufo* (Stock et al., 2008).

The taxon *lucasi* generally has been thought to be a sister-species or a subspecies of *M. galathea* (Higgins & Riley, 1970; Tennent, 1996). Habel et al. (2008) report a lack of divergence between the African *M. galathea* (i.e. *M. lucasi*) and those in southern Europe. Our results do not support their findings: The taxon *lucasi* (type locality: Bejaia, Algeria) seems to be quite distinct from *M. galathea* (Figs 1, 3), and *M. galathea* str. does not exist in North Africa (Tennent, 1996; Bozano, 2002). Within our Moroccan specimens of *M. lucasi* we observed minimal variation, but the only included Tunisian specimen (LUCA3) diverged (Fig. 1). The genitalia of the Tunisian *M. lucasi* (ssp. *lucasi*) are very similar to those of the Moroccan specimens both in form and number of valval teeth, but the wing patterns show differences. We revive the oldest available name for Moroccan populations, ssp. meadowaldoi, for the Moroccan populations of *M. lucasi* (stat. rev.).

*Melanargia russiae* and *M. parce* collectively cover a wide geographic range extending from Western Europe to Central Asia. The paraphyly of the gene trees and the notable split of each of these species into two distinct lineages suggest undetected cryptic speciation events. Among the material examined in our study, the *M. russiae* from NNE Iran (ssp. *eberti* and *transcaspica*) is distant genetically from others throughout the range of the species and clusters near the nominal *M. parce*. There is no known sympathy between the taxa *eberti* and *transcaspica* and either *M. russiae* or *M. parce* (Tshikolovets, 1998; Nazari, 2003). The genetic distance between the nominal *M. parce* and the taxon *lucida* is also notable. Both *parce* and *lucida* are distributed in Tian shan, Pamir, S Uzbekistan and NW Tajikistan (Ghisar and Western Alai range) with considerable overlap, although *lucida* occurs more in the southern parts of these mountain ranges (Tshikolovets, 2000, 2003, 2005). Male genitalia features corroborate the four distinct lineages within the *russiae/parce* group demonstrated by molecular evidence (Figs 1, 3). We consider this evidence sufficient to revise the taxonomy of this group by recognizing: (i) *Melanargia lucida* stat. nov.; (ii) *M. parce*; (iii) *M. transcaspica* stat. nov. (with subspecies *transcaspica* and *eberti* stat. nov.); and (iv) *M. russiae* (with ssp. *russiae*, *cleanthe*, and *japygia*). Our molecular results do not show notable differentiation between some recognized subspecies (e.g. ssp. *russiae* and *japygia*), but for now we accept them as valid subspecies based on their geographic isolation and phenotypic differences.

**Lack of divergence when it is expected**

A lack of genetic divergence between otherwise morphologically distinct biological entities is by no means unprecedented (for example see Sturmbauer & Meyer, 1992; Kerr et al., 2007; Wiemers & Fiedler, 2007). Such discordance between variation in morphology and that of genetic markers is explained best through a number of alternative, but not necessarily mutually

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exclusive, mechanisms that result in distorted gene or species genealogies, including paralogous pseudogenes, hybridization, incomplete lineage sorting, vertically transmitted symbionts, or simply inadequate phylogenetic signal (Funk & Omland, 2003; Hurst & Jiggins, 2005). Alternatively, they may reflect poor taxonomy, in which a variable species has been over-split (Kerr et al., 2007; Descimon & Mallet, 2008).

Two species-groups in our study, namely the larissa and the halimede haplogroups, showed very limited genetic divergence throughout the selected genes, which did not reflect the current species taxonomy. Although inadequate phylogenetic signal cannot be ruled out, lack of molecular divergence in this case is not restricted to mitochondrial genes, as patterns observed through cox1 and 16S rRNA were present also in the nuclear wg gene. Selection is unlikely to be operating on a variety of molecular markers such that effectively both fast-evolving mitochondrial and slow-evolving nuclear genes would show a lack of differentiation between these species. Examination of faster-evolving markers (e.g. internal transcribed spacer (ITS) or microsatellite data) may provide resolution to the phylogenetic relationships in the larissa and halimede complexes.

The shared pattern of low molecular divergence in nuclear and mitochondrial genes and the lack of documentation of male–female bias in Melanargia populations rule out the possibility that our results were affected by the presence of vertically transmitted mitochondrial bacteria. We also observed no signs of pseudogene DNA contamination (polymerase chain reaction ghost bands, base substitutions at conserved sites, sequence ambiguities between forward and reverse strands, or in-frame stop codons) in any of our processed samples.

Inter-specific hybridization and gene introgression have been considered to be rare in animals (Mayr, 1963; Mallet, 2005). Cases of gene introgression as a result of hybridization have been demonstrated in various insects (Kawakami et al., 2007; Linnen & Farrell, 2007; Trewick, 2007), including Lepidoptera (Sperling, 1990; Mallet et al., 2007; Schmidt & Sperling, 2008). For hybridization to be a plausible scenario in cases of low inter-specific divergence, certain requirements (including incomplete reproductive isolating barriers, and a potential for cross-species contact in space and time) must be met. In Melanargia, with the exception of subtle inter-specific differences in the shape of the valva and the form and number of its terminal teeth, the general structure of genitalia is conserved. Historically, these traits are commonly used in characterization of Melanargia species, whereas other potentially informative character sources (e.g. female genitalia or the inverted male vesica) are largely untested in Nymphalidae. Among Satyrinae butterflies, the valvae in general are unimportant in copulation (in Maniola; Goulson, 1993). They therefore do not function as a mechanical pre-zygotic isolating barrier, and hence variation in the shape of the valvae may not be subject to selection. The existence of an incomplete reproductive isolating mechanism suggests that gene flow may be common in Melanargia, particularly among closely related species in the larissa and halimede complexes in which species share similar ecological preferences and occupy a continuous geographic range, occasionally occurring in sympathy and synchrony. Despite the presence of intermediate individuals in the areas of sympathy, for example in the larissa complex in Icel and Pülümür (Turkey) (WtH, personal observation) or between M. galathea and M. lachesis in the Pyrenees (Mazel, 1986), no clear evidence for introgression or continuous gene exchange has ever been documented, and in the absence of breeding experiments to assess the amount and nature of reproductive isolation we cannot discount the possibility that significant gene flow or reticulation is responsible for the observed phylogenetic patterns.

Incomplete lineage sorting has been documented in young species in which divergence is too recent for lineage sorting to have occurred (Cunningham et al., 1992; Zink & Dittman, 1993; Seutin et al., 1995). It posits that gene flow is occurring or has occurred very recently, and that the rates of molecular evolution are lower than the rates of morphological evolution. Geographic proximity is not a prerequisite for incomplete lineage sorting among closely related species, in which ongoing hybridization and subsequent introgression are a more likely explanation for observed polyphyly. Considering an mtDNA mutation rate of 2.3% per million years in butterflies (Brower, 1994; Ho et al., 2005; but see Gratton et al., 2008), both the larissa and halimede haplogroups are very young (mean cox1 divergence in larissa group: 0.94%; in halimede group: 0.91%), and it seems plausible that their lineages have yet to be completely sorted.

The wide geographical variation in phenotype (wing pattern and genitalia) and the failure of taxa in larissa and halimede complexes to retain their phenotypic integrity when in sympathy strongly argue for a scenario of single, morphologically hyper-variable species. Considering the effect that climatic factors can have on phenotypes (Hesselbarth et al., 1995; Nice & Shapiro, 1999), perhaps phenotypic plasticity caused by environmental factors and local microclimate has been responsible for at least part of the variation observed in the wing pattern or genitalia in Melanargia.

The five species in the larissa group cover a very wide geographic area (>3000 km), and, although their ranges are for the most part extremely close to one another, very few cases of sympatry have been reported: an examination of distribution records in Turkey (Hesselbarth et al., 1995) showed only ten localities, among thousands, where two (and never more) of these taxa occur sympatrically. The pattern observed in the reconstructed trees in our study largely contradicts the current taxonomic understanding of this group. The increase in geographical distance among specimens examined from this group seems to be positively correlated with their cox1 distances (Pearson’s $R^2 = 0.189$) (Supporting Information S15). Taking into account their widespread and continuous distribution, the high degree of individual variability, and the paucity of diagnostic morphological or genetic characterization between these taxa, conceivably many of these purported taxa represent continuous populations of a single species. The patchy distribution pattern of dark (the taxa syriaca, karabagi, kocaki) and light (M. grumi, various populations of M. larissa) forms might be the result of continuous isolation followed by dispersal during ice ages and intermittent warm periods. Within the distribution

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area of the taxa in the larissa complex, several glacial refugia are well known (e.g. Levantine and Zag-valley), and changes of flora during ice ages and warmer times in this area have been documented (Kosswig, 1955; Por, 1987; van Zeist & Bottema, 1991; Hesselbarth et al., 1995).

All four subspecies of *M. titea* (sspp. *titea*, *titania*, *standfussi* and *wiskotti*) have similar genitalia, with valval teeth that are always grouped on a common base (Bozano, 2002). The nominal *M. titea* is allopatric with the larissa complex and shows little or no molecular differentiation. *Melanargia titea* ssp. *titania* occurs sympatrically with ssp. *standfussi* (as well as with *M. syriaca*) in Hatay (Turkey) and cannot be differentiated from it. *Melanargia titea* *standfussi* also flies sympatrically with the very similar *M. gruni* in many parts of southern Turkey (Gaziantep, Adyaman, Urfa, etc., Hesselbarth et al., 1995) and shows no molecular differentiation from other taxa in the larissa complex (Figs 1, 2). The taxon *wiskotti* occurs sympatrically with *M. larissa* in Icel and Adana (Turkey), with no known intermediates. Populations of *Melanargia titea* from Antakya/Hatay (Turkey), however, show characteristics intermediate with *titea* (WH, personal observation). *cox1* barcodes place *wiskotti* outside the variation in the larissa complex and closer to *galathea* and *lachesis* (Fig. 1); this is, however, not supported by 16S, and no wg sequences could be obtained for *wiskotti*. Taking into account their geographical distribution, characters from wing pattern and genitalia, as well as our molecular results, we recognize *M. wiskotti* (stat. nov.) as a distinct species, downgrade the nominal *M. titea* to a subspecies of *larissa* (stat. nov.), and downgrade taxa *standfussi* and *titania* to junior subjective synonyms of the *M. larissa titea* (stat. nov.).

The range of nominal *M. syriaca* overlaps with that of *M. titea* (sspp. *standfussi* and *titania*) in Hatay (Turkey), although the two are easily distinguished by phenotype. According to Hesselbarth et al. (1995), the range of *M. syriaca kocaki* is located within the range of the larissa complex with no sympatry. However, in Püllümür (Tunceli, Turkey), dark *kocaki*-like individuals fly together with larissa-like individuals and with others that look intermediate. *Melanargia syriaca karabagi* and *M. hylata* are also sympatric in north of Çıldır-Dag (Hakkari, Turkey) and West Azerbaijan (Iran), and many intermediates cannot be assigned reliably to either species. We consider this evidence to revise the status of the taxon *syriaca* and recognize it as part of the larger subspecific variation in the larissa complex (stat. nov.). Although subspecies *karabagi* and *kocaki* could at best be regarded as synonyms of *M. larissa*, we maintain the current taxonomy until further evidence becomes available.

*Melanargia gruni* is a light-coloured member of this complex and is indeed very similar to *M. titea* *standfussi* as well as to other members of the larissa complex. It flies in sympatry with *M. hylata* in Bitlis (Turkey). Through most of its range, *M. gruni* can be separated from *titea* only by the lack of coloration in the basal area of the hindwings in all populations of *M. titea*. Neither the nominal *M. gruni* nor the newly described ssp. *lorestanensis* shows any molecular differentiation from other members of the larissa complex. We recognize the taxa *grumi* and *lorestanensis* as subspecies of *M. larissa* (stat. nov.).

In our *cox1* trees and haplotype diagrams (Figs 1, 2), some populations of *M. larissa* and *M. hylata* (e.g. *M. larissa larissa* Greece, LARI24–29; *M. larissa taurica* + *massageta* Turkey, LARI31–34; and *M. hylata iranica* S. Iran, LARI17–22) were characterized better than others in the larissa complex. However, through most of their range *M. larissa* and *M. hylata* were genetically indistinguishable from one another. Here we considered cases of sympathy, characters from genitalia, and the results of our DNA analysis to recognize the taxa *hylata* and *iranica* as valid subspecies of *M. larissa* (stat. nov.). The taxa *massageta* and *taurica* are geographically isolated: *massageta* occurs in northern and central Turkey (Bursa to Georgia and Armenia), whereas *taurica* is restricted to western Taurus (SW Turkey). Although not differentiated in our molecular trees, they are discernible phenotypically (Bozano, 2002), and therefore we maintain their status as valid and separate subspecies of *M. larissa*.

Our results support a separate specific status for *M. lachesis*. Apart from notable differences in external morphol-
we synonymize the taxon yunnana with the nominal M. leda (syn. nov.).

Melanargia montana and M. lugens have similar genitalia, with large valvae and distal teeth, which are variable in number. They are distributed from south Sichuan through central China to Zhejiang and occur at low altitudes in subtropical habitats. They are allopatric, with intermediate populations showing intermediate characters. Considering the small genetic distance between M. lugens and M. montana, and as suggested earlier by Wagener (1959–1961) and Bozano (2002), we regard these two taxa as extremes of clinal variation, ranging from the very dark lugens phenotype found in the south-eastern limit of the range to the extremely light montana phenotype found in the south-western limit. We recognize M. lugens with the nominal subspecies M. lugens lugens (= abyoui syn. nov.) including the dark populations from SE China, M. lugens hengshanensis (= hoenei syn. nov.) including the intermediate populations from central China, Hunan to Shaanxi, and M. lugens montana (stat. nov.), including the light populations from SW China.

Among the remaining five species in the halimede complex, two genitalic groups can be determined: (i) valvae relatively large, usually more than six distal teeth (M. asiatica and M. halimede); (ii) valvae smaller, usually fewer than six distal teeth (M. ganymedes, M. epimede and M. meridionalis) (figured in Bozano, 2002). The ranges of the two species in the first group (M. asiatica and M. halimede) overlap only in south Gansu (China), although this is likely not a true sympatry as the taxon gratiani flies at higher elevations than M. asiatica. They are genetically similar (cox1: 0.3–1.1%), but, although closely related and highly variable, are separable mostly by smaller size and the pointed forewing apex of M. halimede, compared to the larger size and the rounded forewing apex of asiatica. Therefore, we regard M. asiatica as a good species, with a south-western range from north Sichuan to north Yunnan and north Burma. However, considering their distribution pattern and the general lack of morphological or molecular differentiation, we recognize no subspecies within M. asiatica.

The three species in the second group (M. ganymedes, M. epimede and M. meridionalis) also have a northern range, from Qinghai and south Gansu to the far east of Russia and Korea, and with isolated populations in eastern China. They show very little or no sympathy, and their genitalia and wing elements (with the exception of a very different development of the dark markings) are rather similar. Melanargia ganymedes is very closely related to the allopatric M. epimede, has similar genitalia and a similar (but lighter) wing pattern, and little genetic divergence (cox1: 0.15–1.88%). We therefore consider the taxon ganymedes a subspecies of M. epimede (stat. nov.).

Melanargia meridionalis flies sympatrically with M. ganymedes in southern Gansu, and, despite a similar adult size and number of distal teeth in the genitalia, the valvae shape differs from that of both M. ganymedes and M. epimede (Wagener, 1959–1961; Bozano, 2002). In our molecular analyses, M. meridionalis tapaishanensis stat. rev. (synonymized previously with nominal M. meridionalis) consistently separated from M. ganymedes and M. epimede (cox1: 0.3–2.18%). Therefore we retain M. meridionalis as a separate species.

Conclusion

Our phylogenetic reconstruction for Melanargia confirmed previously recognized sub-generic classification, but highlighted several cases of low or no genetic divergence between traditionally recognized species, or deep divergences within taxa assumed to be homogenous across their range. We considered molecular evidence alongside available information from morphology (including wing patterns and genitalia) as well as distributional data to revise the taxonomy of Melanargia (Supporting Information S1). We propose the following taxonomic arrangement for the genus (for further synonymy see Bozano, 2002). The status of asterisked taxa has not been evaluated by sequence data.

Genus Melanargia Meigen, 1829

Subgenus Melanargia Meigen, 1829

1. Melanargia galathea (Linnaeus, 1758)
   a. Melanargia galathea ssp. galathea (Linnaeus, 1758)
   b. Melanargia galathea ssp. satnia Frusthoffer, 1917
   c. Melanargia galathea ssp. maqualenae Reichl, 1975
d. Melanargia galathea ssp. syracusana Zeller, 1847*

2. Melanargia lucasi (Rambur, 1858)
   a. Melanargia lucasi ssp. lucasi (Rambur, 1858)
   b. Melanargia lucasi ssp. meadwaldoi Rothschild, 1917

3. Melanargia lachesis (Hübner, 1790)

4. Melanargia evartianae Wagener, 1976
   a. Melanargia evartianae ssp. evartianae Wagener, 1976
   b. Melanargia evartianae ssp. sadjadii Carbonell & Naderi, 2006

5. Melanargia teneates (Ménétriés, 1832)
   a. Melanargia teneates ssp. teneates (Ménétriés, 1832)
   b. Melanargia teneates ssp. meda (Grum-Grshimailo, 1895)

6. Melanargia larissa (Geyer, 1828)
   a. Melanargia larissa ssp. larissa (Geyer, 1828)
   b. Melanargia larissa ssp. hylata (Ménétriés, 1832) stat. nov.
   c. Melanargia larissa ssp. grumi Standfuss, 1892
   d. Melanargia larissa ssp. loestanensis Carbonell & Naderi, 2007
   e. Melanargia larissa ssp. iranica Seitz, 1907
   f. Melanargia larissa ssp. taurica (Rober, 1896)
g. Melanargia larissa ssp. massageta Staudinger, 1901

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i. Melanargia larissa ssp. kocaki Wagener, 1983 stat. nov.
j. Melanargia larissa ssp. syriaca (Oberthür, 1894) stat. nov.
k. Melanargia larissa ssp. titea (Klug, 1832) stat. nov. (= titania Calberla, 1891 syn. nov. = standfussi Wagener, 1983 syn. nov.)

7. Melanargia wiskotii Rober, 1896 stat. nov.
8. Melanargia russiae (Esper, 1793)
a. Melanargia russiae ssp. russiae (Esper, 1793)
b. Melanargia russiae ssp. cleanthe (Boisduval, 1833)
c. Melanargia russiae ssp. japygia (Cyrillo, 1877)
9. Melanargia transcaspica (Staudinger, 1901) stat. nov.
a. Melanargia transcaspica ssp. transcaspica (Staudinger, 1901) stat. nov.
b. Melanargia transcaspica ssp. eberti Wagener, 1975 stat. nov.

10. Melanargia parce Staudinger, 1882
11. Melanargia lucida (Staudinger, 1886) stat. nov.

Subgenus Argeformia Verity, 1953

12. Melanargia occitanica (Esper, 1793)
a. Melanargia occitanica ssp. occitanica (Esper, 1793)
b. Melanargia occitanica ssp. pelagia (Oberthür, 1911)
c. Melanargia occitanica ssp. pherusa (Boisduval, 1832)
13. Melanargia ines (Hoffmannsegg, 1804)
a. Melanargia ines ssp. ines (Hoffmannsegg, 1804)
b. Melanargia ines ssp. fathme Wagner, 1913 stat. rev.

14. Melanargia arge (Sulzer, 1776)

Subgenus Halimede (Oberthür & Houlbert, 1922)

15. Melanargia leda (Leech, 1891)
a. Melanargia leda ssp. leda (Leech, 1891) (= yunnana Oberthür, 1891 syn. nov.)
b. Melanargia leda ssp. melli Wagener, 1961*
16. Melanargia halimede (Ménétriers, 1858)
a. Melanargia halimede ssp. halimede (Ménétriers, 1858) (= gratiani Wagener, 1961 syn. nov.)
b. Melanargia halimede ssp. coreana Okamoto, 1926*
17. Melanargia meridionalis (C. & R. Felder, 1862)
a. Melanargia meridionalis ssp. meridionalis (C. & R. Felder, 1862)

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Supporting Information

Additional supporting information may be found in the online version of this article under the DOI reference: DOI 10.1111/j.1365-3113.2009.00493.x

SI1. Comparison of classification of Melanargia according to Bozano (2002) and that proposed in this study.

SI2. Specimens used in molecular analysis, their collection data, and GenBank accession numbers for cox1 barcodes, 16S and wg genes.

SI3. Specimens used for genitalia preparations.

SI4. Uncorrected p distances of cox1, 16S and wg genes, respectively.

SI5. Geographic versus genetic distances in three genes among specimens in the larissa and halimede complexes.

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