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Short Communication

A phylogenetic revision of the *Glaucopsyche* section (Lepidoptera: Lycaenidae), with special focus on the *Phengaris–Maculinea* clade

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ABSTRACT

Despite much research on the socially parasitic large blue butterflies (genus *Maculinea*) in the past 40 years, their relationship to their closest relatives, *Phengaris*, is controversial and the relationships among the remaining genera in the *Glaucopsyche* section are largely unresolved. The evolutionary history of this butterfly section is particularly important to understand the evolution of life history diversity connected to food-plant and host-ant associations in the larval stage. In the present study, we use a combination of four nuclear and two mitochondrial genes to reconstruct the phylogeny of the *Glaucopsyche* section, and in particular, to study the relationships among and within the *Phengaris–Maculinea* species.

We find a clear pattern between the clades recovered in the *Glaucopsyche* section phylogeny and their food-plant associations, with only the *Phengaris–Maculinea* clade utilising more than one plant family. *Maculinea* is, for the first time, recovered with strong support as a monophyletic group nested within *Phengaris*, with the closest relative being the rare genus *Caerulea*. The genus *Glaucopsyche* is polyphyletic, including the genera *Sinia* and *Iolana*. Interestingly, we find evidence for additional potential cryptic species within the highly endangered *Maculinea*, which has long been suspected from morphological, ecological and molecular studies.

1. Introduction

The European species of the large blue butterflies (genus *Maculinea* van Eecke [1915], family Lycaenidae) have, considering their rarity, been exceptionally well studied. This is primarily due to their remarkable life cycle, which depends on the dual presence of specific food-plants and host-ants. While these associations were described more than a century ago (Frohawk, 1906), only extensive research over the last 40 years has revealed their extreme complexity (Barbero et al., 2009; Nash et al., 2008; Thomas et al., 1989, 2009).

Maculinea caterpillars initially live and feed on species-specific food-plants, however, when reaching the fourth instar, they undergo a dramatic change in lifestyle. From being phytophagous, they become obligately parasitic, only surviving if adopted into the nests of certain ants of the genus *Myrmica*. In the ant nest, the caterpillars live entirely as predators of ant grubs (larvae and pupae – the "predatory" lifestyle) or on a mixed diet of ant regurgitations, unfertilized trophic eggs and ant grubs (the "cuckoo" lifestyle). Caterpillars overwinter in the ant nest, pupate there in the late spring and emerge as adult butterflies in the summer. Altogether, they spend 11–23 months in the ant nest, imposing a considerable fitness cost to the host-ant colony (for more details on *Maculinea* biology and ecology see Thomas et al. (1989)).

This specialized life cycle has attracted researchers from several fields within biology, including evolutionary biologists, eager to understand its origin and evolution (Als et al., 2004; Fiedler, 1998; Fric et al., 2007; Pech et al., 2004; Pierce et al., 2002). Ant associations are common within the butterfly family Lycaenidae, with 75% of the species with known life histories having either obligate or facultative association with ants during part of their development. The vast majority of these associations are, however, mutualistic, where the attending ants protect the caterpillars from natural enemies on the food-plant in return for nutritious secretions (Fiedler, 1991; Pierce et al., 2002). A parasitic lifestyle involving both phytophagy and carnivory, such as that found in *Maculinea* has evolved at least twice in the Lycaenidae (Pierce, 1995); in the *Phengaris–Maculinea* clade, and in the African genus *Lepidochrysops*. In *Lepidochrysops*, the parasitic lifestyle appears to be associated with significant diversification (approximately 120 species; Fiedler, 1998; Pierce, 1995; Pierce et al., 2002), a paradox since such life cycle complexity likely is an evolutionary dead end (Pierce, 1995). Diversification is less

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pronounced in *Phengaris–Maculinea*, even if additional cryptic species may exist in less intensively studied areas of their Palaearctic distribution ranges.

Maculinea is placed in the *Glaucopsyche* section of the tribe Polyommatini, together with the genera *Glaucopsyche* Scudder [1872], *Scolitantides* Hübner [1819], *Pseudophilotes* Beuret [1958], *Iolana* Bethune-Baker [1914], *Euphilotes* Mattoni [1978], *Philotes* Scudder [1876], *Philotiella* Mattoni [1978], *Palaeophilotes* Forster [1938], *Praephilotes* Forster [1938], *Otnjukovia* Zhdanko [1997], *Turanana* Bethune-Baker [1916], *Sinia* Forster [1940], *Micropsyche* Mattoni [1978], *Subsolanoides* Koiwaya [1989], *Caerulea* Forster [1938] and *Phengaris* Doherty [1891] (Als et al., 2004 and references therein; Eliot, 1973; Mattoni, 1977, 1979). The relationships between these genera are poorly known, and were not well resolved in a previous molecular phylogeny that included representatives of ten of the genera (Als et al., 2004). Where life histories are known, it is evident that each genus utilises food-plants from a single plant family and has facultative mutualistic associations with their host-ants – with the exception of *Maculinea* and *Phengaris* (Brower, 2008a; Fiedler, 1991, 1998; Pierce, 1995; Pierce et al., 2002). Butterflies in the Oriental genus *Phengaris*, like *Maculinea*, use food-plants from more than one plant family, and their caterpillars have a parasitic lifestyle similar to that of *Maculinea*. Previous molecular and morphological studies point to *Phengaris* as the closest genus to *Maculinea* (Als et al., 2004; Pech et al., 2004). However, the two approaches do not agree on the relative position of the two genera. While the molecular analysis recovered *Maculinea* as a monophyletic group within *Phengaris* (Als et al., 2004), the morphological analysis placed *Phengaris* as a monophyletic and derived group within *Maculinea* (Pech et al., 2004). An attempt to merge these two datasets resulted in two possibilities: *Maculinea* being paraphyletic with respect to *Phengaris* or vice versa (Fric et al., 2007).

In the present study, we aim to (i) determine the relationships between genera within the *Glaucopsyche* section, (ii) obtain better support for the relationship between *Phengaris* and *Maculinea*, and (iii) look for evidence of additional cryptic species within *Maculinea*. We do so by expanding the existing molecular dataset from Als and colleagues (2004), firstly by increasing the taxonomic representation by adding further taxa from the *Glaucopsyche* section, and secondly by sequencing additional nuclear genes and an additional fragment of the mitochondrial gene *cytochrome oxidase I*, thereby increasing the number of independent markers and the signal from the most variable marker. This approach has previously proved useful in resolving subtribe/genera relationships in insects (Brower and DeSalle, 1998; Sequeira et al., 2000).

2. Materials and methods

2.1. Taxon sampling

For the general *Glaucopsyche* section phylogeny, we added 11 taxa to the dataset collected by Als and colleagues (2004), thereby including representatives from three additional genera (*Euphilotes*, *Philotes*, and *Philotiella*; Table S1). We were unable to obtain samples of the four rare, monotypic genera *Palaeophilotes* (from Chinese Turkestan), *Praephilotes* (from Turkmenistan), *Micropsyche* (from Afghanistan) and *Subsolanoides* (from China). Two taxa (*Lampides boeticus* and *Phylaria cyara*) belonging to the *Lampides* and *Phylaria* sections within the Polyommatini (Brower, 2008b) were used to root the tree. To study additional cryptic speciation within *Maculinea*, we added 27 specimens mainly from areas where particular *Maculinea* species had not been sampled in the previous molecular study (i.e. Eastern Europe, Russia and Mongolia).

Samples previously used by Als and colleagues (2004) were available from the DNA and Tissues collection of the Museum of Comparative Zoology (MCZ) at Harvard University, while new samples were collected and stored in 96% ethanol at −20 °C prior to DNA extraction. Exceptions were *Caerulea coligena* (FH-05-M006) and *Phengaris atroguttata* (SY-02-J002), which had been dried before storage in 96% ethanol (see Tables S2 and S3 for a sample overview).

2.2. Molecular protocols

DNA was extracted from single butterfly legs or one thoracic segment, using the Invisorb® Spin Tissue Mini Kit (Invitek GmbH, Westburg). Final elution volume was 60 μL and DNA aliquots were stored at −20 °C. Two mitochondrial genes, *cytochrome oxidase I* and *II* (*COI* and *COII*), and two nuclear genes *elongation factor 1 alpha* (*EF1α*) and *wingless* (*wg*) were amplified for all taxa. For the taxa included in the general *Glaucopsyche* section phylogeny, two additional, more slowly evolving nuclear genes were amplified, *histone 3* (*H3*) and the ribosomal subunit *28S*. Amplifications were carried out by standard polymerase chain reaction (PCR; see Supplementary material and Table S4 for PCR conditions and primer information). Sequencing reactions were performed in a final reaction volume of 10 μL, using BigDye™ chemistry (Applied Biosystems), 10 μM primers and 10–20 ng DNA, and PCR products were precipitated using an $MgCl_2$ protocol. Sequencing was conducted in both directions on an Applied Biosystems 3031xl automated sequencer or by a sequencing facility (Macrogen). Retrieved sequences were edited in Sequencher® (Gene Codes). The *tRNA-leu* sequence between *COI* and *COII* was not included in the analysis. Final concatenated alignments consisted of 1437 base pairs (bp) from *COI*, 654–660 bp from *COII*, 1065–1170 bp from *EF1α*, 369 bp from *wg* and for the taxa used in the *Glaucopsyche* section phylogeny also 327 bp of *H3* and 816 bp from *28S* (Tables S5 and S6).

2.3. Phylogenetic analysis

Sequences for each gene were aligned using Muscle (Edgar, 2004) and translated into amino acids in MacClade 4 (Maddison and Maddison, 2000) to check for the presence of pseudogenes (stop codons within the gene). The program Gblocks (Castresana, 2000) was used to eliminate poorly aligned positions in a reproducible manner and to concatenate the six (or four) genes, using the settings: −t = c; −v = 5000; −b1 = default; −b2 = (half the number of taxa) + 1; −b3 = 5; −b4 = 5; −b5 = all; a = save. The best fitting substitution model for each gene was found using MultiPhyl Online (Keane et al., 2007) based on the Akaike Information Criterion (AIC).

Phylogenetic analyses were performed by Bayesian Inference (BI) and Maximum Likelihood (ML), applying distinct nucleotide substitution models for each gene as specified in Table S8. MrBayes 3.1 (Ronquist and Huelsenbeck, 2003) was used for BI, using two runs with independent starting trees to assure convergence of Markov Chain Monte Carlo (MCMC) runs. One cold and three heated chains were applied, with a default temperature of 0.2. The analyses were run for 1,000,000 generations, sampling every 100 generations and discarding the first 100,000 generations. The program GARLI 1.0 (Zwickl, 2006) was used for ML, conducting eight independent, heuristic searches with default termination conditions to find the best tree. To estimate the support of each node, 1000 bootstrap permutations were performed in GARLI and a 50% majority rule consensus tree was calculated in Geneious Pro™ (Biomatters).

Support for key nodes in the best tree recovered from the combined analysis (two mitochondrial and four nuclear genes) was

investigated in further analysis using only the mitochondrial and nuclear genes respectively. For the partial data set containing several samples per species, an additional analysis was performed using the species tree approach implemented in the phylogenetic analysis software *BEAST (Heled and Drummond, 2010). Using a Bayesian model under coalescence, *BEAST jointly estimates gene trees and the species tree from multi-locus and multiple-allele sequences (Heled and Drummond, 2010). One MCMC chain was run for 30,000,000 generations, sampling every 3000 generations. The burn-in value was set 3000, and the majority rule consensus tree was generated from the remaining trees. Substitution models for the different genes were specified as Table S8, treating sequences from mitochondrial and nuclear genes as haploid and diploid respectively. Root locations were determined via molecular clock. Best trees and consensus trees with posterior probabilities (BI) and bootstrap support (ML) were visualised in FigTree 1.2 (http://tree.bio.ed.ac.uk/software/figtree/). The program MEGA 4.0.2 (Tamura et al., 2007) was used to compute the number of variable sites per gene, nucleotide frequencies, and percentage sequence divergence within the *Maculinea* species using the Kimura 2-Parameter nucleotide model.

3. Results

3.1. Phylogeny of the Glaucopsyche section

The eight independent ML runs recovered the same topology (−InL = 19735.1) (Fig. 1), which was in agreement with BI, yielding high posterior probabilities and ML consensus bootstrap support for most splits. Only one minor disagreement was found between the two methods: The BI consensus tree placed *Philotes sonorensis* at the base of the *Glaucopsyche* clade with low posterior probability (0.58), whereas this node was unresolved in the ML consensus tree, which placed both *P. sonorensis* and *Scoliantides orion* as sisters to the *Glaucopsyche* clade (trees not shown).

The combined analysis recovered three of the described genera within the *Glaucopsyche* section (*Philotiella*, *Euphilotes* and *Pseudophilotes*) as monophyletic with good support (Fig. 1). Conversely, *Otnjukovia* and *Sinia* were placed within *Turanana* and *Glaucopsyche*, respectively, and the two species *P. bavius* and *G. piasus* were highly diverged from the rest of their respective genera. *Maculinea* was recovered as a monophyletic group with high bootstrap support, rendering the genus *Phengaris* paraphy-

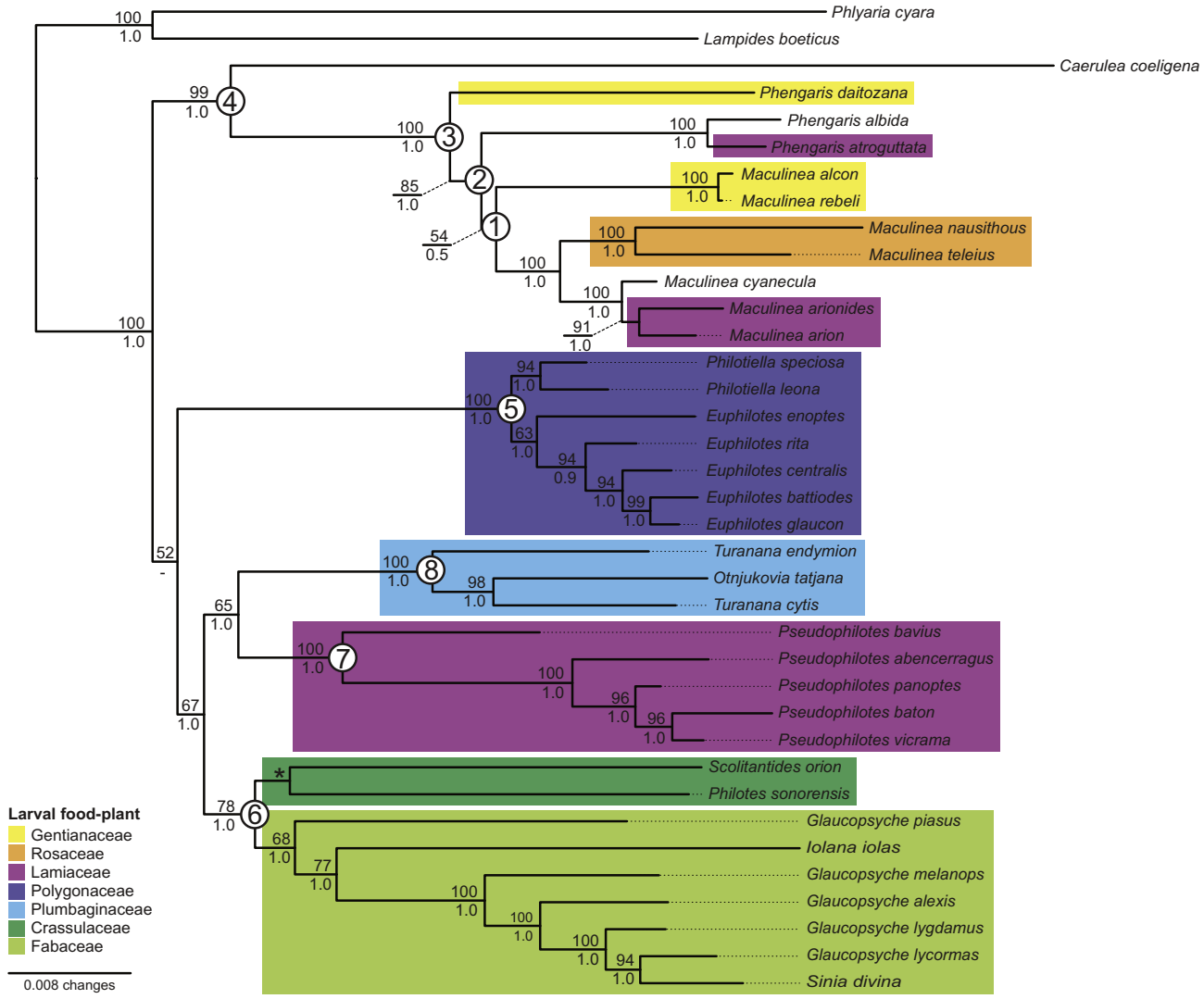

Fig. 1. Phylogeny of the *Glaucopsyche* section. Best maximum likelihood topology from the analysis of the combined dataset. Numbers above the branches are Bayesian posterior probabilities, and below branches ML bootstrap support. An asterisk indicates a node not recovered in either the BI or ML consensus tree. The Bayesian analysis groups *Scoliantides orion* together with the *Glaucopsyche* clade whereas the node is unresolved in the ML analysis. Colours indicate the family of the larval food-plant based on (Brower, 2008a; Fiedler, 1998) and circled numbers assign key nodes, whose support was analysed under different configuration and partitioning of the dataset (Table 1).

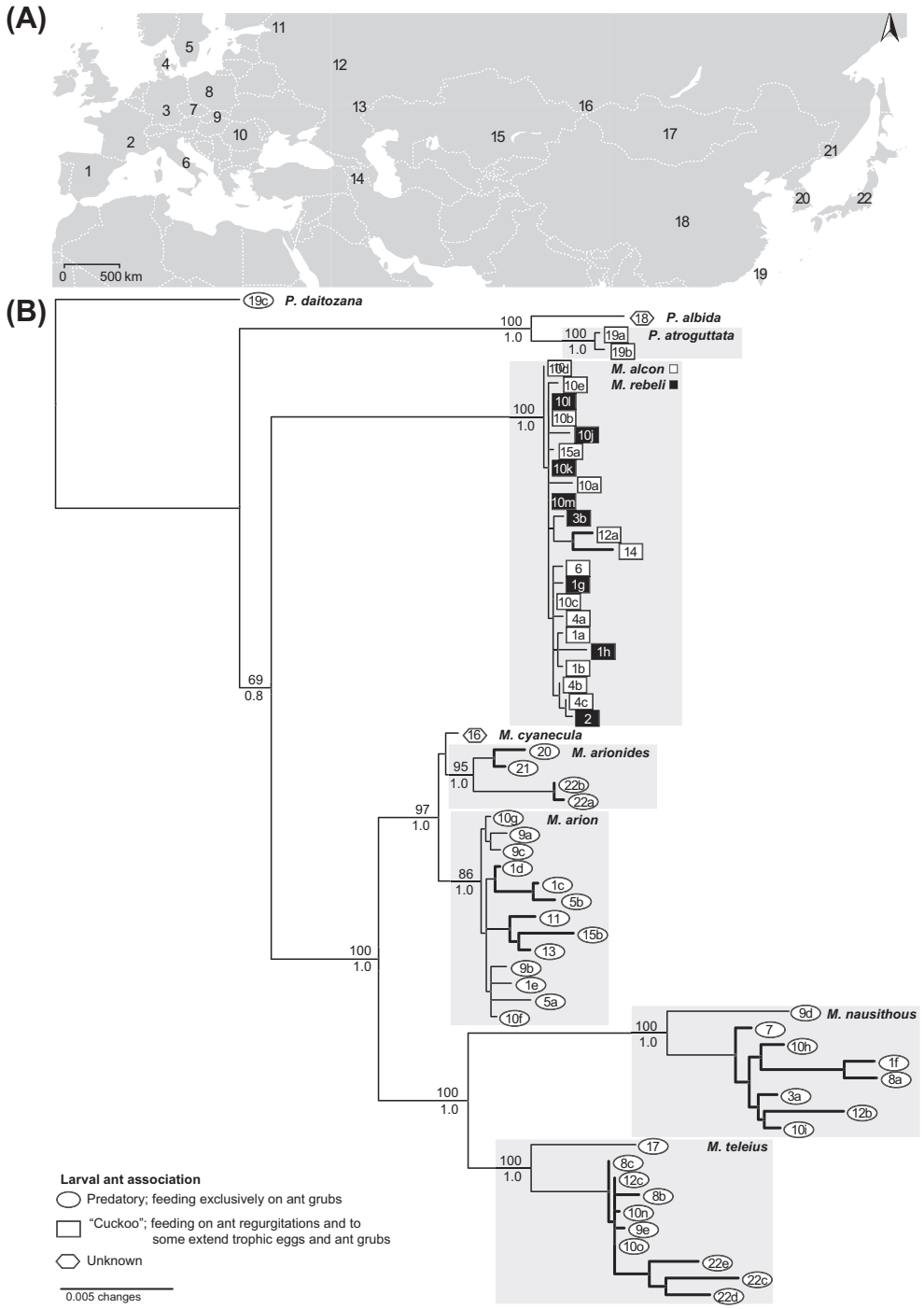

Fig. 2. Phylogeny and geographic distribution of the *Phengaris–Maculinea* clade. (A) Geographic location of collection sites of *Maculinea* and *Phengaris* specimens; numbers refer to labelling in Table S3. (B) Best maximum likelihood topology from the analysis of the combined dataset. Symbols refer to the larval lifestyle of the caterpillar inside its host-ant nest, and numbers therein indicate map location. Bayesian posterior probabilities are given above the branches and ML bootstrap support below. Thick lines indicate nodes within species that were supported by bootstrap >50.

letic (Fig. 2). The sister genus to the *Phengaris–Maculinea* clade was *Caerulea*.

3.2. Relationships within Maculinea

The eight independent ML runs resulted in three different topologies. That with the best likelihood score (−lnL = 19735.1) is shown in Fig. 2. Both BI and ML consensus trees had a similar topology, as did the species tree from *BEAST (Fig. S1). *Maculinea* formed a monophyletic clade and five of the species *M. arion*, *M. arionides*, *M. cyanecula*, *M. nausithous* and *M. teleius* were recovered as good species with high bootstrap support. Conversely, the division of *M. alcon* and *M. rebeli* as two species was not supported, despite a considerable increase in the number of samples analysed (five *M. rebeli* and seven *M. alcon* samples were added from a locality where they occur in sympatry (Table S3)).

Large structuring and relatively high *COI* sequence divergence (Table 2; Hebert et al., 2003) was found within four of the "predatory" *Maculinea* species. The existence of a Japanese cluster within *M. teleius* was supported by one further sample in this study, confirming the distinct morphology of *M. teleius* specimens from this region (See Supplementary Fig. S2; Pech et al., 2004; Nash,

Table 1
Support for key nodes using different sets of genetic markers. Node numbers refer to labelling in Fig. 1. A "–" designates that the specific node was not recovered.

Genetic markers	Node	BI post. prob.	ML bootstrap
Complete	1	0.48	54
COI, *COII*, *EF1α*, *H3*, *wg*, *28S*	2	0.99	85
	3	1.00	100
	4	1.00	99
	5	1.00	100
	6	1.00	78
	7	1.00	100
	8	1.00	100
Nuclear genes	1	0.56	55
EF1α, *H3*, *wg*, *28S*	2	1.00	90
	3	1.00	100
	4	1.00	100
	5	1.00	–
	6	–	<50
	7	0.93	82
	8	1.00	100
Mitochondrial genes	1	–	<50
COI, *COII*	2	–	<50
	3	–	97
	4	1.00	<50
	5	1.00	62
	6	0.95	<50
	7	1.00	97
	8	1.00	98

Table 2
Sequence divergence in the *COI* gene within *Maculinea* species.

Taxa	Lifestyle	# Populations	% Divergence	Standard error
Maculinea alcon	Cuckoo	14	0.16	0.07
Maculinea rebeli	Cuckoo	8	0.18	0.12
Maculinea alcon + rebeli	Cuckoo	22	0.10	0.03
Maculinea arion	Predatory	13	0.28	0.07
Maculinea arionides	Predatory	4	1.03	0.21
Maculinea cyanecula	?	1	NA	NA
Maculinea nausithous	Predatory	8	1.36	0.19
Maculinea teleius	Predatory	10	1.08	0.15

unpublished data). Moreover, a sample from Mongolia was very distinct from the rest of the *M. teleius* samples. It is a female with uniformly brown wing upperside (see Supplementary Fig. S3) collected ovipositing on *Sanguisorba officinalis*. The exact taxonomical assignment of this specimen is difficult, but we broadly assign it to *M. teleius*, suggesting that further cryptic species may indeed be present within this complex (Als et al., 2004; Pech et al., 2004). For *M. arion*, a cluster of samples with purely eastern origin remained after adding four new samples, while a second cluster was recovered among localities as distantly placed as Sweden and Spain. In *M. nausithous* no clear geographic pattern was observed, but the species displayed the highest *COI* sequence divergence despite its relatively smaller distribution range. In particular, one Slovakian population was very divergent from the rest (Fig. 2).

4. Discussion

4.1. Food-plant patterns within the Glaucopsyche section

The distinction of two subtribes (Scolitantiditi and Glaucopsychiti) within the *Glaucopsyche* section as proposed by Hesselbarth et al. (1995) is not supported by the present study. Instead, five well-supported clades are recovered, of which four show high concordance to the food-plant families utilised by the different genera (Fig. 1). *Philotiella* and *Euphilotes*, both feeding on Polygonaceae, are sister genera, as are *Turanana* and *Otnjukovia* (Plumbaginaceae) and *Glaucopsyche*, *Iolas* and *Sinia* (Fabaceae). The sister genera *Scolitantides* and *Philotes* also share a food-plant family (Crassulaceae), but the relationship is not well supported. The overall pattern supports the suggestion of Fiedler (1991) that taxon-specific preferences for particular food resources exist in this group, as in most butterfly groups (Ehrlich and Raven, 1964). The exception is the *Phengaris–Maculinea* clade, which utilizes food-plants of several distantly related plant families. Importantly, the apparent taxonomic diversity of food-plants within the *Glaucopsyche* section does not reflect a relaxed food-plant choice at the species level. Monophagy (one food-plant species or genus) or stenoligophagy (one food-plant family) is the rule (except *P. abencerragus*, which utilizes both Lamiaceae and Fabaceae (Fiedler, 1991). Strong ant-associations are thus not related to oligophagy in this section as found elsewhere (Pierce, 1987; Pierce and Elgar, 1985), which likely reflects that although most of these butterfly genera are heavily tended by ants, their ant associations are not obligate (except *Phengaris–Maculinea*) (Fiedler, 1991). The low support at the basal nodes unfortunately makes it impossible to draw conclusions about the ancestral food-plant family of the section as a whole.

4.2. Relationship among genera within the Glaucopsyche section

There is generally good concordance between the described genera and the clades recovered in the phylogeny, although some reorganization may be suggested. The placement of *Otnjukovia* within *Turanana* suggests that *Otnjukovia* should be a junior subjective synonym, and close relationship between these taxa has been acknowledged previously (Brower, 2008a). Similarly *Sinia* (or at least *Shijimiaeoides*, junior subjective synomym for which *divina* is the type species) should be considered a synonym of *Glaucopsyche*. Given the divergence values, we consider that *Iolana* is a good genus (sister to *Glaucopsyche*), and that *G. piasus* should be considered representative of a different genus. This result agrees with morphological studies, which also recovered *G. piasus* as divergent from the rest of *Glaucopsyche* (Pech et al., 2004). In a similar manner, *P. bavius* is sister and highly diverged from the rest of *Pseudophilotes*, and could be considered representative of a different genus. The divergence of the sister clades *Philotiella* and *Euphilotes*, in contrast, is very low. The two genera, which were described simultaneously (Mattoni [1978]), are the only Polygonaceae feeders, and may represent a single genus. As in previous analysis, *Phengaris* is the closest relative of *Maculinea*, while the sister genus to this clade is *Caerulea*, which was also expected based on morphology (Fiedler, 1998). Unfortunately, nothing is known about the immature life cycle of either of the two rare *Caerulea* species, *C. coeligena* and *C. coelestis* (Als et al., 2004; Fiedler, 1998).

4.3. Relationship between Phengaris and Maculinea

Maculinea is recovered as a monophyletic clade sister to *P. albida* and *P. atroguttata* with good support for the first time, rendering *Phengaris* paraphyletic. This corroborates the relationship between these two genera suggested, with low support, by the previous molecular study (Als et al., 2004), but does not support previous morphological studies (Pech et al., 2004). It is worth noting that the species tree approach implemented in *BEAST did not provide support for the relationship among *Phengaris* and *Maculinea*. This method takes into account coalescence theory and thus is ideal for datasets containing multiple sequences per species. Thus, we cannot exclude that low basal supports are due to small sample sizes of the three *Phengaris* species (*P. daitozana* = 1, *P. albida* = 1, *P. atroguttata* = 2; Table S3). A proposal for the continued use of the name *Maculinea* over *Phengaris* has been published (Balletto et al.,

2010), but see (Fric et al., 2010) for counter arguments. We recommend that the nomenclatural debate is delayed until irrefutable evidence is provided.

4.4. Relationships within Maculinea

The relationships among *Maculinea* species found in this study largely support those of Als et al. (2004). The increased resolution due to additional genetic markers in this study recovered *M. cyanecula* as a good species, placed as the sister to *M. arion* and *M. arionides*. It did not, however, lead to a distinction between the two closely related "cuckoo" species, *M. alcon* and *M. rebeli*, which rather represent two ecological forms of the same species, as previously suggested (Bereczki et al., 2005). The additional samples of "predatory" *Maculinea* species (*M. teleius*, *M. nausithous* and *M. arion*) revealed further genetic structuring within these species, suggesting potential cryptic speciation. A distinct Japanese cluster and highly divergent Mongolian population exist in *M. teleius*, similarly to *M. arionides*, where the Japanese subspecies *M. a. takamukui* is diagnosable in both molecular and morphological analysis (Fric et al., 2007). In *M. nausithous*, one population from Slovakia was highly divergent, although geographically close to other study populations. Recently a new subspecies of *M. nausithous* has been described from the Transylvanian basin in Romania (*M. n. kijevensis*) based on different host-ant usage (Rákosy et al., 2010), but molecular analysis shows that it is only slightly diverged (Dincă et al., 2011). This particular region of Europe also shows higher haplotype diversity of *M. arion*, which coincides with a large number of ecological or morphological subspecies described from this area (e.g. Varga-Sipos and Varga, 2005). This pattern of genetically distinct lineages in the central/southern parts of Europe may reflect different glacial refugia as has been found for several species of animals and plants (Taberlet et al., 1998), and may prove particularly important for future conservation of these highly endangered butterflies.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2011.05.016.

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