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Maculinea alcon and *M. rebeli* (Insecta: Lepidoptera: Lycaenidae) – one or two Alcon Blues? Larval cuticular compounds and egg morphology of East Austrian populations

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Abstract

Maculinea alcon and *M. rebeli* are ant-parasitic Blue butterflies with nearly identical morphology, little genetic divergence but marked ecological separation. We approached the long-running dispute whether the two taxa have to be regarded conspecific by applying two established methods of insect systematics to East Austrian populations. (1) Gas chromatography - mass spectrometry of cuticular compounds showed that larvae carried no taxon-specific compounds prior to their adoption by host ants. Ordination procedures, however, suggested some grouping of the profiles of each taxon. (2) SEM-based morphometry of eggs likewise revealed slight differences, though single individuals frequently could not be allocated.

Our results agree with recent investigations on adult morphology, DNA sequences and allozymes and substantiate that *M. alcon* and *M. rebeli* are less differentiated than expected for full species. Causes may be incomplete allopatric speciation and / or ongoing ecological speciation.

Key words: Lepidoptera, Lycaenidae, *Maculinea rebeli*, *Maculinea alcon*, Blue butterflies, morphometry, chemotaxonomy.

Zusammenfassung

Die ameisenparasitischen Bläulinge *Maculinea alcon* und *M. rebeli* sind morphologisch nahezu identisch, weisen geringe genetische Divergenz auf, haben aber unterschiedliche ökologische Ansprüche. Die bisher ungelöste Frage der Konspezifität wurde an ostösterreichischen Populationen mit zwei in der entomologischen Systematik etablierten Methoden untersucht. (1) Gaschromatographie-Massenspektrometrie von kutikulären Substanzen zeigte, dass Larven vor ihrer Adoption durch Wirtsameisen keine taxonspezifischen Substanzen besitzen. Ordinationsverfahren ergaben dennoch eine leichte Gruppierung der Profile beider

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Taxa. (2) Die REM-gestützte morphometrische Analyse von Eiern ergab ebenfalls geringfügige Unterschiede, einzelne Eier konnten aber nicht zugeordnet werden.

Unsere Ergebnisse stimmen mit aktuellen Forschungsergebnissen zu Adultmorphologie, DNA-Sequenzen und Allozymen überein und bestätigen, dass *M. alcon* und *M. rebeli* weniger differenziert sind, als es für Arten zu erwarten wäre. Unvollständige allopatrische Speziation und / oder aktuelle ökologische Artbildung könnten die Ursache sein.

Introduction

Larvae of the Alcon Blue *Maculinea alcon* (DENIS & SCHIFFERMÜLLER, 1775) and Mountain Alcon Blue *Maculinea rebeli* (HIRSCHKE, 1904) feed upon the flower buds of their host plants for several weeks before they must be adopted by a *Myrmica* ant for further development in the subterranean nest (AKINO & al. 1999, THOMAS & al. 1998, and references therein). The caterpillars' odour similarity to host ant larvae, crucial for the adoption process (AKINO & al. 1999), is most probably due to the biosynthesis of cuticular compounds that mimic host-ant odour cues (AKINO & al. 1999, ELMES & al. 1991). Being fed trophallactically by ant workers, the "cuckoo" larvae hibernate, pupate and emerge the following summer. The preference for specific *Myrmica* hosts varies over the geographical ranges of *M. alcon* and *M. rebeli* (ALS & al. 2002, ELMES & al. 1994, STEINER & al. 2003).

Maculinea alcon was described from the Vienna region (Austria; without indication of the locality), *M. rebeli* from Mt. Hochschwab (c. 1700 m a.s.l., Styria, Austria). Originally introduced as a subspecies of *M. alcon*, the latter was elevated to species rank by BERGER (1946). As the type series of *M. alcon* was destroyed, the identity of this taxon is unclear. Some authors reported morphological differences between adults of the two species (BERGER 1946, SCHULTE 1958), others doubted or denied differential characters (BALINT 1986, BERNARDI 1947, BEURET 1949, EBERT 1961, KAABER 1964, REZBANYAI-RESER 1990, URBAHN 1964). Slight morphological differences were found in the larvae (MUNGUIRA 1987). The situation was further complicated when BERGER (1946) described lowland populations as *M. rebeli xerophila* (type male) regarding the nominate subspecies as exclusively alpine. According to BALINT (1986), BERGER erroneously attributed females of *M. alcon* to *M. rebeli xerophila*. BALINT (1991) treated *M. rebeli xerophila* as a species, MUNGUIRA & MARTÍN (1999) denied species status of *xerophila* and KUDRNA (2001) sank the taxon into the junior synonymy of *M. alcon*. It was argued that both *Maculinea alcon* and *M. rebeli* may comprise other, cryptic species (ELMES & al. 1994, ELMES & al. 2004). Presently most authors attribute species names according to the habitat: *M. alcon* in moist habitats, typically with the host plant *Gentiana pneumonanthe* L. but also with *G. asclepiadea* L. and *Gentianella germanica* (WILLD.) s.l.; *M. rebeli* in dry habitats, typically with *Gentiana cruciata* L. but also with *Gentianella germanica* s.l. and *Gentianella campestris* (L.) BÖRNER, and occasionally with *Gentiana lutea* L. (for a review: HÖTTINGER & al. 2003 and SCHLICK-STEINER & al. 2002; additionally BERECZKI & al. 2005, BERNHARD & al. 2005).

Maculinea alcon and *M. rebeli* are model organisms for butterfly myrmecophily research (e.g. AKINO & al. 1999, FIEDLER 1998, PIERCE & al. 2002) and flagship species in butterfly conservation biology (SWAAY & WARREN 1999, THOMAS & SETTELE 2004).

Based on adult morphology and ecology, PECH & al. (2004) showed paraphyly of *M. alcon* and argued that "*M. rebeli* should possibly lose its full species status". Shortly after ALS & al. (2004) presented a DNA-based phylogeny and concluded likewise that *M. alcon* and *M. rebeli* "show little genetic divergence and are probably a single ecologically differentiated species"[1]. Most recently, BERECZKI & al. (2005) inferred from allozyme data that "the pattern of genetic differentiation does not support species status of *M. alcon* and *M. rebeli* in Central Europe". The present paper provides further data towards a sound taxonomic decision. Arguments come from two largely neglected directions: larval semiochemistry and egg morphology.

Cuticular hydrocarbons profiles (which are species-specific and genetically determined: e.g. BAGNÈRES & MORGAN 1991, PROVOST 1991) have resolved taxonomic problems in Lepidoptera and several other insect orders. The analysis of cuticular compounds also proved useful in discriminating sibling species (summarised by HOWARD 1993). Though cuticular compounds of *M. rebeli* have been studied repeatedly (AKINO & al. 1999, ELMES & al. 2002, SCHLICK-STEINER & al. 2004, SCHÖNROGGE & al. 2004), there is no such data on *M. alcon*.

The long tradition of external egg morphology in insect systematics experienced a renaissance with the advent of SEM (GARCÍA-BARROS & MARTÍN 1995). Differences were found even between closely related and otherwise most similar butterflies, including lycaenids (JUTZELER & al. 2003), although most analyses were confined to qualitative assessments. MUNGUIRA (1987) presented SEM photographs of *M. alcon* and *M. rebeli* eggs and concluded that the slight differences are rather due to individual variation than to differentiation at species level. Egg size and volume (0.05 mm^3, identical in the two species) was determined by GARCÍA-BARROS (2000). However, high-precision morphometry, as used for the study of other insect groups (e.g. ants: SEIFERT 2002), has never been applied.

For the comparison of larval cuticular compounds and egg shell morphologies we selected populations from a small area in Central Europe which comprises (or lies close to) the type localities of *M. alcon* and *M. rebeli*.

Material and methods

In 2001 and 2003, we collected egg shells and pre-adoption larvae (Blue larvae prior to adoption by ants) from gentian host plants at three sites of two *M. alcon* populations, and at ten sites of eight *M. rebeli* populations in East Austria, within an area measuring 130 km across (Table 1). At each site sampled gentian plants were 10 - 50 m apart. Plant and ant hosts were *Gentiana cruciata* / *Myrmica sabuleti* MEINERT, 1861, *M. specioides* BONDROIT, 1918 and *M. scabrinodis* NYLANDER, 1846 at *M. rebeli* sites; and *Gentiana pneumonanthe* / *Myrmica scabrinodis* at *M. alcon* sites (see HÖTTINGER & al. 2003 and SCHLICK-STEINER & al. 2002 for details).

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[1] PECH & al. (2004) provided indication that the genus name *Maculinea* VAN EECKE, 1915 may be a junior synonym of *Phengaris* DOHERTY, 1891, but refrained from a formal nomenclatorial act. Though ALS & al. (2004) confirmed the phylogenetic conclusions, they also stuck to "*Maculinea*".

Table 1: Analysed populations, sites and samples of *Maculinea alcon* and *M. rebeli* in East Austria. GC-MS: gas chromatography - mass spectrometry of cuticular hydrocarbons of pre-adoption larvae (each sample consisted of five pooled larvae); SEM: SEM-aided egg morphometry.

	populations	sites	samples	
			GC-MS	SEM
Maculinea alcon				
	Vienna (300 m a.s.l.)	1	-	7
	Waldviertel (600 m a.s.l.)	2	5	14
Maculinea rebeli				
	Rotwald (720 m a.s.l.)	1	1	2
	Nördliches Weinviertel (250 m a.s.l.)	2	3	4
	Steinfeld (330 m a.s.l.)	2	1	2
	Leiser Berge (450 m a.s.l.)	1	-	2
	Rosaliengebirge (300 m a.s.l.)	1	2	2
	Rohrwald (380 m a.s.l.)	1	-	2
	Dunkelsteinerwald (280 m a.s.l.)	1	-	6
	Eisenwurzen (700 m a.s.l.)	1	-	3

Cuticular compounds of pre-adoption larvae

We anaesthetised pre-adoption larvae at - 20 °C and extracted cuticular compounds with n-hexane. Extracts were analysed by gas chromatography - mass spectrometry (GC-MS) (HP 6180C GCD Systems; EID; HP-5 column diameter 0.25 mm, length 30 m; carrier gas helium; 1 µl samples injected in splitless mode; column oven set at 50 °C for 5 min, programmed from 50 °C to 200 °C at 20 °C min^{-1}, then from 200 °C to 300 °C at 5 °C min^{-1} and maintained at the final temperature for 10 min). For the transfer of MS data we used the factory default settings of ChemStation (HP) with a mass range of 33 - 500 amu and a scan cycle time of 0.7 s.

As preliminary investigations had shown that some peaks in the GC-MS profiles of single larvae were too weak, we pooled five individuals from the same host plant for each sample. We extracted a total of 12 samples of pooled individuals (Table 1). Results from the seven samples of *M. rebeli* have been presented by SCHLICK-STEINER & al. (2004).

GC profiles were synchronised by retention time adjustment. MS data were used to ensure that corresponding peaks indicated the same compound. We normalised absolute intensities in the GC profiles by assigning 100 % to the highest peak of each profile. Then we constructed a master GC by compiling all of the 105 compounds detected to a hypothetical profile. Profiling of the 12 chromatograms, i.e. comparison of each profile with the master GC, was performed with the package Mass Frontier 4.0 (HighChem Ltd., Bratislava): each retention time of a peak was assigned to one dimension of the vector of each sample. We used the resulting 12 vectors (105 dimensions, relative ratio intensities of the single peaks) for all subsequent ordinations.

By combining three different methods of ordination (linear and non-linear) we tried to obtain more robust results than by applying a single method:

(1) Principal Components Analysis (PCA), using Mass Frontier 4.0.

(2) Bray-Curtis similarity index (BRAY & CURTIS 1957): We estimated the similarity of the peaks between each of the 66 possible pairs of samples using the Bray-Curtis similarity index, as described by ELMES & al. (2002). We applied non-parametric one-way analysis of ranked similarities randomisation (ANOSIM) to test the probability that the pairwise similarities within and between *M. alcon* and *M. rebeli* samples are the same. Non-metric multidimensional scaling ordination plots (MDS) should visualise the Bray-Curtis pairwise similarities. We measured the extent of any final lack of agreement by Standardised Residual Sum of Squares (STRESS). The lower the STRESS the better the MDS plot represents the original Bray-Curtis similarities. The degree to which relative abundance values are taken into account by the similarity index depends on data transformation prior to calculations, with successive transformation diminishing the degree. We conducted preliminary calculations using untransformed, square root transformed and fourth root transformed data (not shown). Based on these preliminary tests we decided in favour of fourth root transformation, as visualization of groupings in MDS plots resulted in slightly increased discriminative power with decreased STRESS, although global R values of ANOSIM for *M. alcon* and *M. rebeli* were nearly identical across transformations. Also with respect to biological inferences, the fourth root transformation is a sound compromise between no transformation and use of presence-absence data, because in the case of the *Myrmica-Maculinea* system it is not known whether mere presence or absence of a certain compound or its abundance is of functional importance to ants and butterflies (ELMES & al. 2002). Bray-Curtis similarity values, ANOSIM tests, MDS-plots and STRESS values were generated by Primer 5.2.9 (CLARKE & GORLEY 2001).

(3) Self-Organizing Maps (SOM, KOHONEN 2001): SOM are a neural network algorithm based on unsupervised training. While some linear methods are unsupervised, too, the non-linear SOM are known to especially well visualise patterns behind complex data sets. No statistical test is attached to SOM and the results are influenced by the order of samples in data input (KOHONEN 2001). Nevertheless, SOM are a powerful tool for knowledge discovery in databases and data mining (KOHONEN 2001) and were also successfully applied in GC-MS data processing (STEINER & al. 2002, SCHLICK-STEINER & al. 2004). Basic decisions concern the size of the output grid (number of neurons) and the number of training cycles. We ran SOM analyses with Mass Frontier 4.0 and used a hexagonal output grid (each hexagon represents a neuron) of 6 x 6 neurons, since in preliminary tests this grid-size proved a reasonable compromise between maximum discrimination and loss of data connectivity. Training cycles were stopped by the program when the analysis was saturated (at 3000 cycles). Samples located in the same or in a neighbouring neuron are more similar than samples ordinated in distant neurons.

Egg morphometry

Larvae of *M. alcon* and *M. rebeli* hatch through the base of the eggs. We compared the surface morphology of empty egg shells by measuring six characters from digital SEM images (magn. x400) of air-dried eggs (21 *M. alcon* from three sites, 23 *M. rebeli* from ten sites; Table 1) in an axial micropyle-centered view (Fig. 1). Distance measures were taken from the flat screen images at 100 % resolution (18.5 x 13.9 cm; 50 µm = 31.4 mm) by a caliper with an accuracy of 0.1 mm (on the recommendation of SEIFERT 2002).

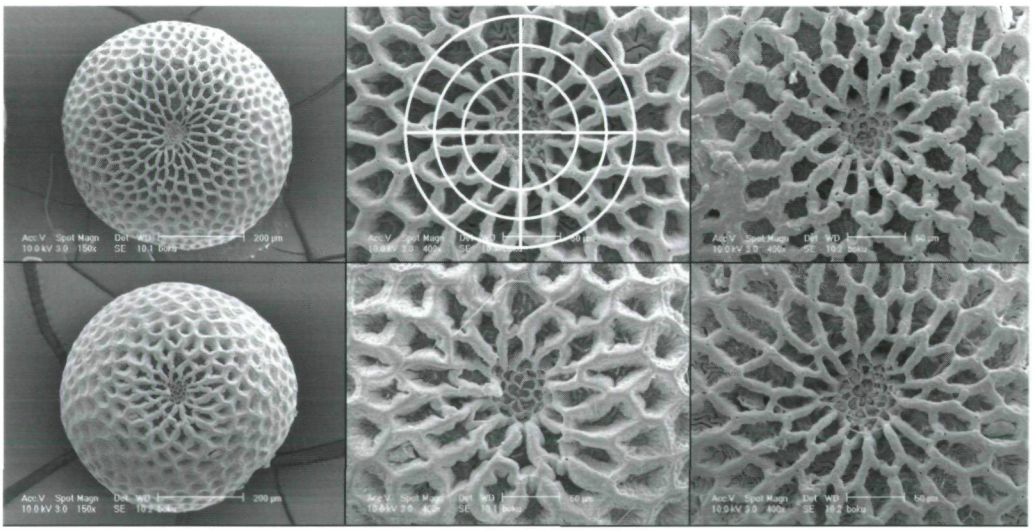

Fig. 1: SEM photographs of eggs of *Maculinea alcon* (above) and *M. rebeli* (below) in polar (micropyle) view. Left: typical eggs (150×); middle: polar region of typical eggs (400×) with circles for morphometric analyses (100, 150 and 200 µm in diameter); right: polar region of atypical eggs (400×).

Morphometric characters (Fig. 1):

nR100, nR150, nR200 – number of longitudinal ribs crossed by a micropyle-centred circle of 100 µm / 150 µm / 200 µm in diameter. When the circle crossed a node, the number of fusing ribs immediately centrewards of the node was taken.

R100, R150, R200 [µm] – width of longitudinal ribs crossed by the circles. The shortest distance between the margins of an intact rib was measured. For exactly radial ribs the measuring points were the intersections with the circle; in all other cases the measuring length was positioned in such a way that the circle crossed its mid point. When a circle crossed a node, we took the width of the fusing ribs immediately centrewards.

Data were analysed with Levene's test for homogeneity of variances, ANOVA, MANOVA and discriminant analysis using SAS 8.2.

Results

Cuticular compounds of pre-adoption larvae

The chromatograms of *Maculinea alcon* and *M. rebeli* pre-adoption larvae varied among single sites of populations as well as across populations in qualitative and quantitative characters of the profiles. This variation was more pronounced in *M. alcon* (Fig. 2a - c) than in *M. rebeli* (Fig. 2d - e). We defined as high-intensity peaks (STEINER & al. 2004) those 23 peaks that were evident by visual inspection. No high-intensity peak occurred exclusively in one of the two taxa. Single profiles never displayed each of the 23 compounds, but all compounds were found in the collectives of both *M. alcon* and *M. rebeli*.

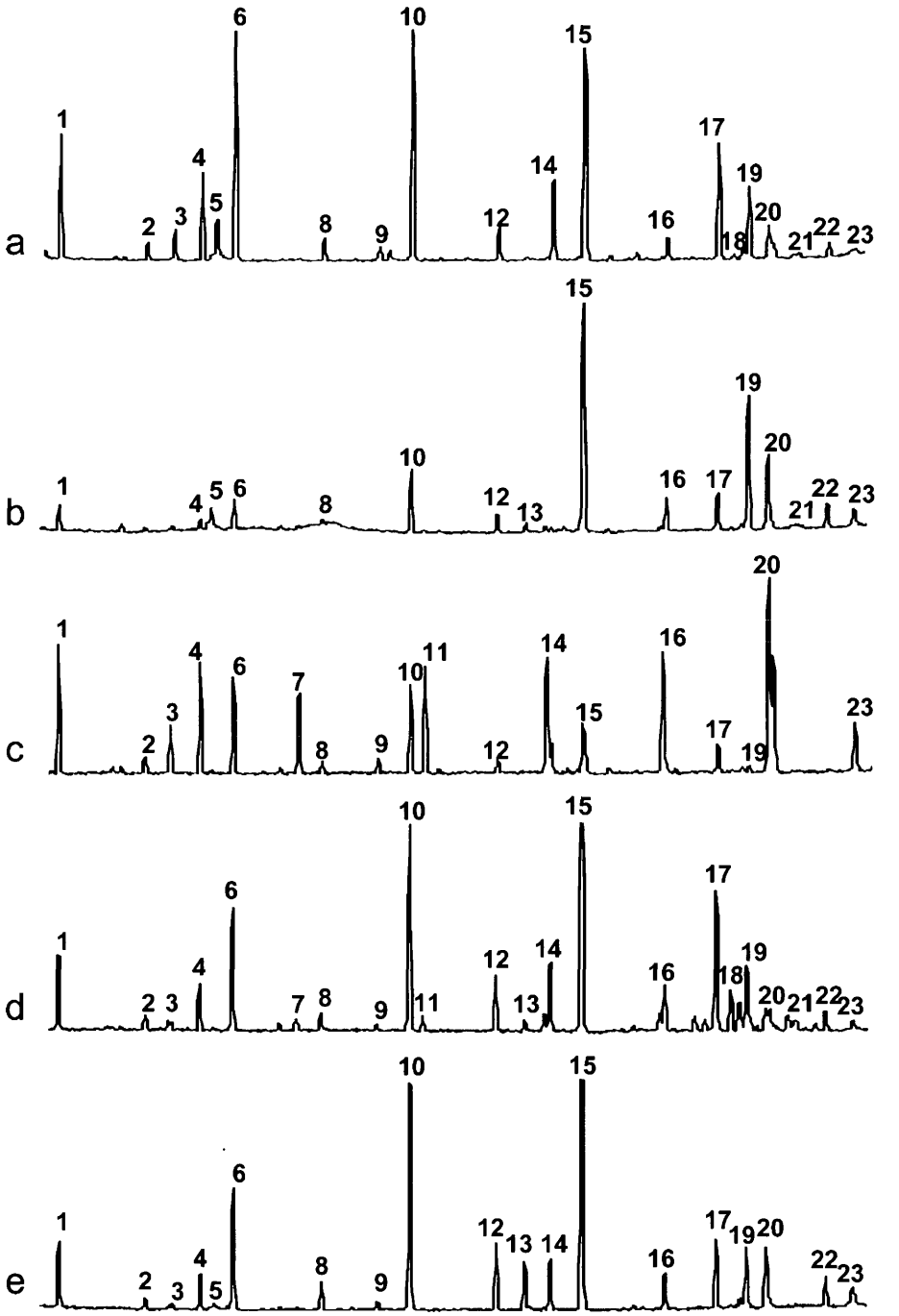

Fig. 2: Typical partial chromatograms of *Maculinea alcon* (a - c) and *M. rebeli* (d, e) pre-adoption larvae. High-intensity peaks are numbered consecutively.

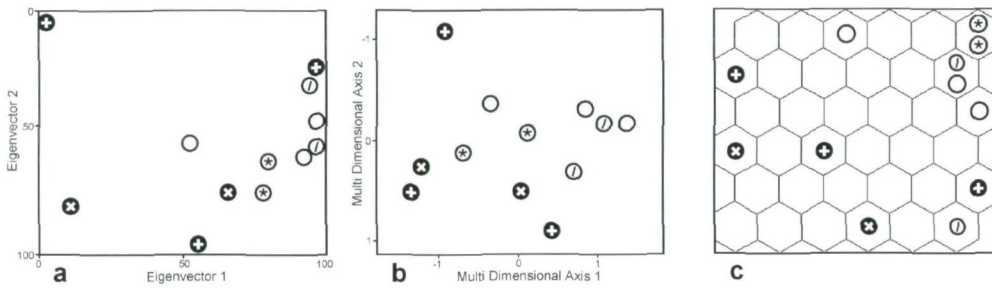

Fig. 3: Ordination of 12 samples of *Maculinea alcon* (black) and *M. rebeli* (white), based on the peaks of 105 compounds in the chromatograms. (a) PCA of the profiled chromatograms, based on the relative quantities of compounds; (b) MDS ordination of the Bray-Curtis similarity values for the relative peak intensities, based on fourth root transformations (STRESS = 0.07); (c) results of a SOM analysis mapped on a hexagonal output grid (6 × 6 neurons, each represented by a hexagon). Samples located in the same or in a neighbouring hexagon are more similar than samples ordinated in distant hexagons. Samples indicated by ✖, +, ★, / were collected at the same site, respectively.

The three ordination methods produced similar though slightly differing results. The first two principal components of the PCA of the raw data revealed signs of differences between *M. alcon* and *M. rebeli*, but separation was incomplete (Fig. 3a). The MDS ordination based on the Bray-Curtis similarity values of fourth root transformed raw data gave a good representation of all the measured similarities (STRESS = 0.07) and a slightly better separation (Fig. 3b). An ANOSIM test for species grouping (*M. alcon* vs. *M. rebeli*) revealed very weak grouping (global R = 0.353, P = 1.8 %). The ANOSIM test for different collection sites revealed no geographic grouping (global R = -0.017, P = 48.7 %). SOM separated the samples most distinctly, albeit incompletely (Fig. 3c). All ordination methods suggested considerable variation within the two butterfly taxa, as shown by empty SOM-neurons between the samples. Variation within *M. alcon* appeared more pronounced. All ordinations indicated that within-site variation partly exceeded between-site variation (Fig. 3).

Egg morphometry

A qualitative comparison of SEM photographs suggested that *M. alcon* eggs have more but relatively narrow longitudinal ribs in the polar region around the micropyle. Atypical eggs (Fig. 1), however, seemed to close the gap between the two taxa. The morphometric analyses confirmed the latter impression and indicated overlap in all of the six characters (Table 2), especially in those close to the micropyle (nR100, R100). For the rib width characters R100, R150 and R200, with a varying number of values per egg, we took the arithmetic means because Levene's test for homogeneity showed homogeneous variances. The MANOVA test including all six morphometrics revealed significant separation of *M. alcon* and *M. rebeli*. An ANOVA of the six single characters showed significant separation only in the cases of nR200, R150, and R200. R150 was

Table 2: Morphometric comparison of the egg sculpture of *Maculinea alcon* and *M. rebeli*. Bold: arithmetic mean ± standard deviation; in [ ]: minimum and maximum values. Abbreviations of morphometric characters in the text.

	M. alcon	*M. rebeli*
	n = 21	n = 23
nR100	**17.6 ± 2.0** [14; 20]	**16.8 ± 1.7** [14; 20]
nR150	**20.1 ± 2.6** [15; 27]	**19.3 ± 1.7** [15; 22]
nR200	**23.8 ± 2.8** [19; 29]	**21.9 ± 2.3** [19; 27]
R100	**9.2 ± 1.0** [7.5; 11.1]	**9.7 ± 0.8** [8.4; 11.2]
R150	**9.1 ± 1.2** [7.6; 11.6]	**11.0 ± 1.9** [8.3; 15.3]
R200	**11.6 ± 1.7** [9.2; 15.3]	**14.1 ± 2.8** [10.4; 20.8]

the most powerful differential character, followed by R200 (Table 2, Fig. 4). A discriminant analysis based on all six characters produced a high error rate: just 85.7 % of *M. alcon* eggs and 60.9 % of *M. rebeli* eggs were correctly classified.

Discussion

In various insect orders, cuticular compound bouquets turned out to be species-specific (BAGNÈRES & MORGAN 1991, PAGE & al. 1990, RABOUDI & al. 2005) and hereditary (DAHBI & al. 1998, PROVOST 1991). Closely related species frequently produce different substances (e.g. PAGE & al. 1997). It is therefore remarkable that no specific peaks occurred in the GC-MS profiles of *M. alcon* and *M. rebeli* pre-adoption larvae, and that the presence or absence of single substances varied within and across *M. alcon* and *M. rebeli* (Fig. 2). All three ordination methods suggested weak clustering concerning species affiliation (Fig. 3); even SOM indicated incomplete separation. Nevertheless the grouping was robust: non-transformed and transformed data yielded similar results in Bray-Curtis tests (data not shown, see: Material and methods). A similar situation has been interpreted in favour of the conspecificity of pine cone beetles (HAVERTY & al. 1989), but the current case appears more intricate. Compared to other insects, cuticular compounds of the pre-adoption larvae of ant-associated Blues might evolve under different selective pressures because they serve chemical mimicry (AKINO & al. 1999). SCHLICK-STEINER & al. (2004) have shown for pre-adoption larvae of Central European *M. rebeli* that the bouquet contains substances of several potential host ant species simultaneously: multi host-mimicry via aggregate-odour. The strategy of *M. alcon* may be the same. To our present knowledge, *M. alcon* and *M. rebeli* usually parasitise different host ants (probably a consequence of different ant species residing in the particular habitat), but at least *Myrmica scabrinodis* hosts both of the two Blues in Central Europe (HÖTTINGER & al. 2003, STEINER & al. 2003, TARTALLY & CSŐSZ 2004). In the light of this host use the similarity of cuticular compound profiles does not prove conspecificity

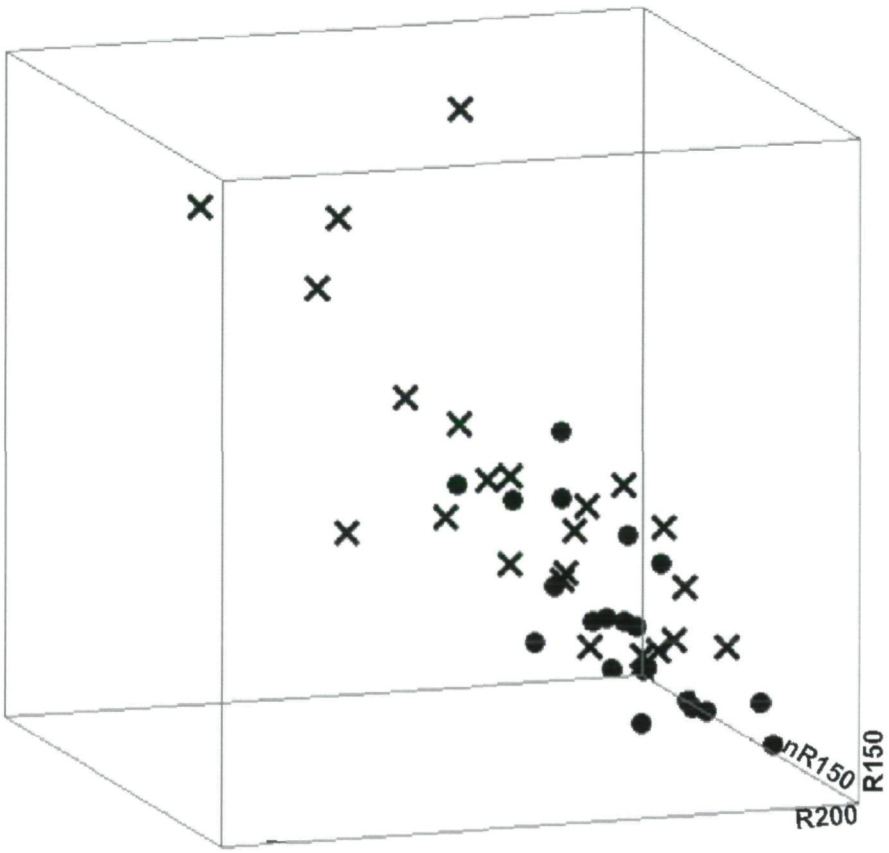

Fig. 4: Three-dimensional plotting of the egg morphometric characters R150, R200, nR150. ● = *Maculinea alcon*, ✖ = *M. rebeli*.

of *M. alcon* and *M. rebeli*, but might be a plesiomorphic trait conserved in two full species, or the result of convergent adaptation to *Myrmica* hosts.

Moreover, epigenetic modifications of cuticular compound profiles by environmental factors (GAMBOA & al. 1991, WOODROW & al. 2000) could cause variation within as well as between *M. alcon* and *M. rebeli*. The absence of geographic grouping in the ANOSIM of Bray-Curtis values and the fact that samples from the same locality are often less similar than samples from different localities (Fig. 3) leads us to suppose genetic rather than environmentally induced variation within each taxon. This view is corroborated by the genetic variation in neutral DNA markers found within a *M. alcon* population by ALS & al. (2004). A possible influence of different host plants (*G. pneumonanthe* in *M. alcon*, *G. cruciata* in *M. rebeli*) as a source of cuticular compound variation between *M. alcon* and *M. rebeli* must not be ruled out, however. Sequestration of host plant compounds by phytophagous insects is well documented, also for lycaenid butterflies (GEUDER & al. 1997), but the few studies on the influence of host plants on cuticular

compounds yielded conflicting results. Variation within and between species of *Drosophila* larvae was mainly caused by host plants (STENNETT & ETGES 1997) and variation between populations of a grasshopper was explained by different vegetation types (BUCKLEY & al. 2003). On the other hand, aphid species from the same host plant had clearly differing profiles (RABOUDI & al. 2005) and conspecific aphids from different host plants had identical profiles (SUNNUCKS & al. 1997). In the present case this ambiguity is of minor significance, since the profiles of *M. alcon* and *M. rebeli* should become even more similar if host plant effects on cuticular compounds were deducted. Anyway, an outgroup comparison – the analysis of cuticular compounds of other *Maculinea* species – would facilitate assessing the systematic relevance of this characteristic.

Egg shell sculpture exhibits substantial similarity of *M. alcon* and *M. rebeli*. Morphometric characters differ on average but overlap largely, in line with the assessment of MUNGUIRA (1987) that eggs of *M. alcon* and *M. rebeli* do not differ consistently. The eggs are evidently more similar than those of other investigated highly similar butterfly species (e.g. JUTZELER & al. 2003). Epigenetic factors such as the influence of the host plant or habitat climate should not be disregarded. Climatic factors have recently been shown to affect absolute egg size of insects, including butterflies and moths (e.g. FISCHER & al. 2004, TORRES-VILA & RODRÍGUEZ-MOLINA 2002), and postdepositional changes may be influenced by humidity (WOLF & REID 2004). As to the characters used in the present study, at least the number of ribs cannot be modified after deposition. Since external egg morphology reflects the shape of the follicular epithelium in the ovarioles (DOWNEY & ALLYN 1980), we assume that the differences in egg sculpture reveal a certain genetic differentiation between *M. alcon* and *M. rebeli*.

Although the two approaches – cuticular compounds and egg morphology – have intrinsic weaknesses, they led independently (and thus more convincingly: SCHLICK-STEINER & al. 2003, WIENS 1998) to consistent results. Considering the concordant results from adult morphology and ecology (PECH & al. 2004), DNA sequences (ALS & al. 2004) and allozymes (BERECZKI & al. 2005), we are inclined to regard the variation within our data as intraspecific variation. But speciation is usually a gradual process rather than a sudden event (AVISE & WALKER 2000), and species delimitation is subject to ongoing discussion (see SITES & MARSHALL 2003 for review). Moreover, a marker does not necessarily evolve at the same tempo as the species does, and the pace may vary across species as well as across markers (cf. HEBERT & al. 2003). Last but not least it is for reasons of nature conservation policy that we refrain from synonymizing the two taxa (cf. THOMAS & SETTELE 2004).

However well investigated a particular species taxon may be, the species category is a construct of mainly operational value (AVISE & WALKER 2000, MALLET 2001). Research should look beyond the one-or-two-species question and test hypotheses on the evolution of *M. alcon* and *M. rebeli*: (1) Incomplete allopatric speciation during glacial periods and subsequent occasional hybridisation of *M. alcon* and *M. rebeli* in situations when the two butterflies occur within the migration distance of single individuals (up to 3 km: MUNGUIRA & MARTÍN 1999). Such interbreeding could occur despite a general inclination to assortative mating during ecological divergence (cf. EMELIANOV & al. 2003). (2) Ongoing ecological speciation (SCHLUTER 2001) which started after the last glacial period. Both theoretical arguments (e.g. DOEBELI & DIECKMANN 2003) and case studies

(JOHNSON & al. 1996, SATO & al. 1999) support fast and incomplete speciation within confined areas. Ecological speciation could have been triggered by a sudden host plant shift, as proposed for other phytophagous insects (BERLOCHER 1998, DRÈS & MALLET 2001). A gradual differentiation of the host plant spectrum is also conceivable. Starting from a euryecious host plant, one part of the Blues may have specialised on gentian species in dry habitats, another part on wetland gentians. A common host plant could have been *Gentianella germanica* s.l., as this gentian occurs syntopically with the current main host plants of the two butterflies (J. Greimler pers. comm.) and is occasionally used by both *M. alcon* and *M. rebeli*. The plausibility of these hypotheses can only be assessed in the light of phylogeographic and population genetic data on butterflies and gentians.

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