Host plant use drives genetic differentiation in syntopic populations of *Maculinea alcon*

András Tartally, Andreas Kelager, Matthias A Fürst, David R Nash

The rare socially parasitic butterfly *Maculinea alcon* occurs in two forms, which are characteristic of hygric or xeric habitats, and which exploit different host plants and host ants. The status of these two forms has been the subject of considerable controversy. Populations of the two forms are usually spatially distinct, but at Răscruci in Romania both forms occurs syntopically. We examined the genetic differentiation between the two forms using eight microsatellite markers, using samples from a nearby hygric site as out group. Our results showed that while the two forms are strongly differentiated at Răscruci, it is the xeric form there that is most similar to the hygric form at Şardu, and Bayesian clustering algorithms suggest that these two populations have exchanged genes relatively recently. We found strong evidence for population substructuring, caused by high within-nest relatedness, not association with host ants use, indicating very limited dispersal of most ovipositing females. Our results are consistent with the results of larger scale phylogeographic studies that suggest that the two forms represent local ecotypes specialising on different host plants, each with a distinct flowering phenology, and is an example of a genetic barrier operating on a temporal scale rather than spatial

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Host plant use drives genetic differentiation in syntopic populations of *Maculinea alcon*

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Abstract

The rare socially parasitic butterfly *Maculinea alcon* occurs in two forms, which are characteristic of hygric or xeric habitats, and which exploit different host plants and host ants. The status of these two forms has been the subject of considerable controversy. Populations of the two forms are usually spatially distinct, but at Răscruci in Romania both forms occur syntopically. We examined the genetic differentiation between the two forms using eight microsatellite markers, using samples from a nearby hygric site, Şardu, as an outgroup. Our results showed that while the two forms are strongly differentiated at Răscruci, it is the xeric form there that is most similar to the hygric form at Şardu, and Bayesian clustering algorithms suggest that these two populations have exchanged genes relatively recently. We found strong evidence for population substructuring, caused by high within-nest relatedness, not association with host ants use, indicating very limited dispersal of most ovipositing females. Our results are consistent with the results of larger scale phylogeographic studies that suggest that the two forms represent local ecotypes specialising on different host plants, each with a distinct flowering phenology, and is an example of a temporal rather than spatial genetic barrier.

Key words: phenological separation, immigration, disruptive selection, host specificity, conservation units, *Myrmica*, *Gentiana* , *Maculinea rebeli*

Introduction

Larvae of *Maculinea* van Eecke (Lepidoptera: Lycaenidae) butterflies start their development on specific host plants. A few weeks later they are adopted into the nests of suitable *Myrmica* Latreille (Hymenoptera: Formicidae) colonies, where they act as social parasites of the ants (Thomas et al., 1989). This unusual life cycle has shaped their evolution, as different populations are strongly selected to adapt to different initial host plants and *Myrmica* species depending on their availability (Thomas et al., 1989; Witek et al., 2008).

Larvae of the *Maculinea alcon* Denis & Schiffermüller group follow a rather specialised development as, compared to other *Maculinea* species, they are not simply predators of ant brood, but are fed by *Myrmica* workers in preference to their own brood – a behaviour that has been described as a "cuckoo" strategy (Thomas & Elmes, 1998). Because they are constantly interacting with worker ants, this means that they need to adapt precisely to the local host ant species, e.g. by mimicking the odour (Akino et al., 1999; Nash et al., 2008; Thomas & Settele, 2004) and the sound (Barbero et al., 2009) of the ants, in order to be accepted by a suitable *Myrmica* colony. While the initial host plants of this group are all species of *Gentiana* L. (and in rare cases also *Gentianella* Mönch), they can occur in very different open habitats, such as lowland and mountain meadows or wet and dry swards (Munguira & Martín, 1999; Oostermeijer, Vantveer & Dennijs, 1994; Settele, Kühn & Thomas, 2005; Tartally, Koschuh & Varga, 2014). Based on these different habitat types, several forms or (sub)species of the *M. alcon* group have been described. The most widely accepted separation within this group is that the nominotypic *M. alcon* occurs on humid meadows and there is another xerophilous form which is usually referred to as *M. rebeli* Hirschke (Thomas et al., 2005; Thomas & Settele, 2004; Wynhoff, 1998). However, two papers (Habeler, 2008; Kudrna & Belicek, 2005) have made the

63 case that the latter form is most likely not synonymous with the nominotypic *M. rebeli*, which is
64 found at higher altitude, and has a unique host plant and host ant usage (Tartally, Koschuh &
65 Varga, 2014). Furthermore, recent molecular phylogenetic (Als et al., 2004; Ugelvig et al.,
66 2011b) and population genetic (Bereczki et al., 2005; Bereczki, Pecsenye & Varga, 2006;
67 Sielezniew et al., 2012) studies suggest that the hygrophilous and xerophilous forms of *M. alcon*
68 are not two distinct lineages. To avoid confusion, we will refer to the "typical" hygrophilous
69 form of *M. alcon* as '*M. alcon* H' and the xerophilous form as '*M. alcon* X' throughout the rest
70 of this manuscript.

71 The host plant and host ant usage of the two *M. alcon* forms are different, because different
72 gentian and *Myrmica* species are available on the hygric sites of *M. alcon* H and xeric sites of *M.*
73 *alcon* X. While *M. alcon* H starts development typically on *Gentiana pneumonanthe* L., *M. alcon*
74 X typically uses *G. cruciata* L. The development of *M. alcon* X typically continues in nests of
75 *Myrmica schencki* Viereck and *My. sabuleti* Meinert but *M. alcon* H most often uses *My. rubra*
76 L., *My. ruginodis* Nylander or *My. scabrinodis* Nylander as host ant. Furthermore, some other
77 minor or locally important host plant and host ant species have been recorded for both forms
78 (Als, Nash & Boomsma, 2002; Elmes et al., 1994; Elmes et al., 1998; Höttinger, Schlick-Steiner
79 & Steiner, 2003; Meyer-Hozak, 2000; Settele, Kühn & Thomas, 2005; Sielezniew &
80 Stankiewicz, 2004; Steiner et al., 2003; Tartally, Koschuh & Varga, 2014; Tartally et al., 2008;
81 Tartally et al., 2013; Thomas et al., 1989; Witek et al., 2008).

82 Despite these differences in the host plant and host ant usage of *M. alcon* H and *M. alcon* X,
83 phylogenetic reconstruction using morphological and ecological characters suggests that western
84 Palaearctic *M. alcon* H are closer to European *M. alcon* X than Asian *M. alcon* H (Pech et al.,
85 2004). In combination, all these results suggest local ecological but not genetic differentiations

of the two forms between hygric and xeric sites. Until recently this could only be tested by comparing sites that were separated by tens of kilometres or more, but in the last decade a site has been recorded from Răscruci (Transylvanian basin, Romania) where patches supporting *M. alcon* H and *M. alcon* X occur in a mosaic separated by tens of meters. The two forms use different host plants and mostly different host ants on this site (Tartally et al., 2008), and their flying periods are largely separated based on the phenology of their host plants (Czekes et al., 2014; Timuş et al., 2013). Our aim was to investigate the genetic differentiation between the two forms of *M. alcon* on this unique syntopic site and to relate this to these differences in host plant and host ant use.

Materials and Methods

Field methods

Two sites in Transylvania were visited in the summers of 2007 and 2009 to record host plant and host ant usage and to collect genetic samples of *M. alcon*. Host ant specificity results from 2007 have already been published in (Tartally et al., 2008). The first site is at Răscruci (46°54' N; 23°47' E; 485 m a.s.l.), which is predominantly an extensively grazed tall-grass meadow steppe with *Gentiana cruciata* (the host plant of *M. alcon* X), but also with numerous small marshy depressions with tall-forb vegetation in which *G. pneumonanthe* (the host plant of *M. alcon* H) is common (Czekes et al., 2014). This site gave the possibility to compare the host ant specificity and population genetics of *M. alcon* H and *M. alcon* X within the same site. To collect samples, two nearby patches were chosen within this mosaic site where *G. pneumonanthe* and *G. cruciata* were well separated from each other (there was a ca. 20 m wide zone without gentians). In other

parts of this site border effects (because of the potential migration of *Myrmica* colonies) or the co-occurrence of the two gentians made it difficult to find *M. alcon* larvae originating clearly from *G. pneumonanthe* or *G. cruciata*. The patch with *G. pneumonanthe* will henceforth be referred to as 'Răscruci wet' (*M. alcon* H patch), while the patch with *G. cruciata* will be referred to as 'Răscruci dry' (*M. alcon* X patch). The nearest known *M. alcon* site (a *M. alcon* H site) to Răscruci is at Şardu (46°52' N; 23°24' E; 480 m a.s.l.), which was chosen as a control site. Şardu is a tall-grass, tall-sedge marshy meadow with locally dense stands of *G. pneumonanthe*.

To obtain data on the host ant specificity and to get samples for genetic analysis, *Myrmica* nests were searched for within 2 m of randomly selected *Gentiana* host plants, which is considered to be the approximate foraging zone of worker ants of the genus *Myrmica* (Elmes et al., 1998). Searches were made no earlier than four weeks before the flying period of *M. alcon* at both sites, so that any *M. alcon* caterpillars or pupae found must have survived the winter in the ant nest, and hence have become fully integrated (Thomas et al., 2005). Nests were excavated carefully but completely, after which the ground and vegetation were restored to as close to the original conditions as possible. After counting any *M. alcon* caterpillars, pupae and exuviae, up to ⅓ of the specimens were randomly selected from among them for later genetic analysis, placed in 96% ethanol, and stored at -20 °C until DNA could be extracted. The remaining *M. alcon* were returned to the nest, so as to minimize the effect on the local population. Five to ten worker ants were also collected from each ant nest and preserved in 70% ethanol for later identification in the laboratory using keys by Seifert (1988) and Radchenko and Elmes (2010). For further details, see Tartally et al. (2008).

131 *Host ant specificity*

132 Host ant specificity (deviation from random occurrence in nests of different *Myrmica* species)
133 was calculated based on the number of fully grown butterfly larvae, pupae and exuviae in two
134 ways: *P1* is the 2-tailed probability from a Fisher exact test of heterogeneity in infection of host
135 ant nests (as implemented at http://www.quantitativeskills.com/sisa/), and *P2* is the probability
136 from a randomization test of ant nests between species, using the software MACSAMP (Tartally et
137 al., 2008). The published (Tartally et al., 2008) and unpublished data on the host ant specificity
138 were combined for the analyses. In the case of Răscruci, host ant specificity results were
139 calculated separately for Răscruci wet and Răscruci dry and also based on the combined data
140 from both patches ('Răscruci both' below).

141

142 *DNA extraction and microsatellite analysis*

143 DNA was extracted from approximately 1-2 mm^3 of tissue from caterpillars or pupae using a
144 10% Chelex-10mM TRIS solution with 5 µl Proteinase K. Samples were incubated at 56 °C for
145 minimum 3.5 hours or overnight and boiled at 99.9 °C for 15 min. The supernatant was collected
146 and stored at 5 °C or -20 °C for short or long term storage, respectively. For each sample, nine
147 polymorphic nuclear microsatellite loci developed for *Maculinea alcon* were amplified: Macu20,
148 Macu26, Macu28, Macu29, Macu30, Macu31, Macu40, Macu44, and Macu45 (Table 1; Ugelvig
149 et al., 2011a; Ugelvig et al., 2012) using a red Taq MasterMix (Sigma-Aldrich). These primer
150 pairs were tested using standard PCR conditions: initial denaturation for 5 min at 95 °C followed
151 by 30 cycles of 30 sec at 95 °C, 30 s at locus-specific annealing temperature (see table 1) and 30
152 s extension at 72 °C finishing with elongation of 15 min at 72 °C run on a Thermo PCR PXE 0.2

153 Thermal Cycler. Total reaction volume was 10 µl of which 1 µl was template DNA. PCR
154 products were run on a 3130xl Genetic Analyzer with GeneScan 500 LIZ (Life Technologies) as
155 internal size standard and analyzed with GENEMAPPER® Software version 4.0 (Applied
156 Biosystems). Locus Macu40 could not be scored consistently (excessive stutter bands) and was
157 omitted from all further analysis. The overall proportion of missing values (alleles) in the data set
158 was 4.6 %.

159

160 *Tests for Hardy-Weinberg and linkage disequilibrium*

161 The eight microsatellite loci analysed were tested for linkage disequilibrium (genotypic
162 disequilibrium) between all pairs of loci in each sample and for deviations from Hardy-Weinberg
163 proportions using FSTAT version 2.9.3.2 (Goudet, 1995) based on 480 and 1260 permutations,
164 respectively. The software package MICRO-CHECKER version 2.2.3 (Van Oosterhout et al., 2004)
165 using 1,000 iterations and a Bonferroni corrected 95% confidence interval, was employed to test
166 for possible null-alleles.

167

168 *Population structure, genetic differentiation and relatedness*

169 We studied the genetic clustering of individual genotypes based on allelic frequencies and genetic
170 divergence employing three different Bayesian algorithms implemented in the software packages
171 BAPS version 5.2 (Corander et al., 2008b), STRUCTURE version 2.3.4 (Falush, Stephens &
172 Pritchard, 2003, 2007; Hubisz et al., 2009; Pritchard, Stephens & Donnelly, 2000) and INSTRUCT
173 version 1.0 (Gao, Williamson & Bustamante, 2007), the last of which does not assume Hardy-

Weinberg equilibrium within clusters. In each program the most likely number of genetically distinct clusters (K) was estimated for each K in the range 1 or 2 to 12, allowing for sub-structuring of samples. In BAPS, the upper bound to number of samples was set to 60 ($N_{individuals}$ = 60) with 10 repetitions, when employing the generic learning clustering method setting. Log(ml) values for individual mixture clustering were averaged over the 10 best visited partitions over 100 runs using the fixed K mode. The level of admixture was examined based on the individual clustering mixture analysis for the most likely K, allowing 1 individual to define a population but otherwise using the parameter settings recommended by Corander *et al.* (2008a): 100 iterations, 200 reference samples and 10 iterations for reference individuals. In STRUCTURE, a burn-in length of 50,000 MCMCs was used to secure approximate statistical stationarity, followed by a simulation run of 500,000 MCMCs using an admixture model with correlated allele frequencies as recommended by Pritchard, Stephens & Donnelly (2000). No location prior was used, and LnP(D) values were averaged over 20 iterations. In INSTRUCT, 10 chains were used, with a burn-in length of 50,000 MCMCs and a simulation run of 1,000,000 MCMCs, and the option was used to infer both population structure and the inbreeding coefficients for subpopulations.

For STRUCTURE the most likely value of K (number of clusters) was estimated using the ΔK method of Evanno, Regnaut & Goudet (2005). For BAPS, the most likely value of K was based on the maximum log-likelihood. For INSTRUCT, the most likely value of K was chosen based on the deviance information criterion, but the ΔK method of Evanno, Regnaut & Goudet (2005) was also applied for comparison.

For more detailed population differentiation, samples were explored individually as well as in four different partitions: (a) pre-defined populations (POP: Răscruci dry, Răscruci wet and Şardu) which also relates to host plant use (Răscruci wet and Şardu: *G. pneumonanthe*. Răscruci dry: *G.*

197 *cruciate*), (b) host ant use (ANT: *Myrmica scabrinodis*, *My. sabuleti*, *My. schenki* and *My.*
198 *vandeli*), (c) within POP nests (POPNEST: specific nest ID within POP), and (d) year of sampling
199 (YEAR: 2007 and 2009), the latter to test for temporal differences.

200 We studied the overall population differentiation between pre-defined populations (POP)
201 calculating Weir and Cockerham's θ (1984) using FSTAT version 2.9.3.2 based on 1,000
202 permutations. As the values of θ and F_{ST} is affected by the allelic diversity at the marker loci
203 applied, we further calculated the standardized G'_{ST} (Hedrick, 2005, equation 4b), and the
204 estimator D_{EST} (Jost, 2008, equation 12) as alternative quantifications of genetic differentiation,
205 making comparisons with studies based on other marker loci possible (Heller & Siegismund,
206 2009). Nei's G_{ST} (Nei, 1973) and Jost's D_{EST} for pairs of POP samples were calculated using the
207 package DEMEtics (Gerlach et al., 2010) using R version 2.15.3 (R Development Core Team,
208 2013) and *P*-values were based on 1,000 bootstrap resamplings. Hierarchical AMOVA (Analysis
209 of Molecular Variance: Excoffier, Smouse & Quattro, 1992) was calculated for POP, ANT,
210 POPNEST, and separately for POP and YEAR using GENODIVE version 2.0b27 (Meirmans, 2006)
211 with 9,999 permutations to estimate the variance components and their statistical significance.
212 Individual-based Principal Coordinate Analysis (PCOA) with standardized covariances was
213 employed to obtain a multivariate ordination of individual samples based on pairwise genetic
214 distances, as implemented in the software GENALEX version 6.501 (Peakall & Smouse, 2012).
215 The PCoA were explored for POPNEST within ANT within POP across YEAR using nested
216 MANOVA based on the sum of the variances of the different coordinates, as implemented in
217 JMP 11.02 (SAS Institute).

218 Relatedness (*r*) was estimated between pairs of samples within pre-defined populations
219 following Queller and Goodnight (1989) as implemented in GENODIVE. Differences in pairwise

220 relatedness between samples from the same nest and from different nests were compared using a
221 stratified Mantel test in GENODIVE, based on 9999 permutations, and separately within
222 individual populations.

223

224 **Results**

225 *Host ant specificity*

226 A total of 135 *Myrmica* ant nests were found within 2 m of the host gentian plants on the two
227 sites and 90 *Maculinea* larvae, pupae and exuviae were found in 26 infested nests (Table 2; 87
228 nests and 56 *Maculinea* have already been published from these in Tartally et al. (2008)).
229 Altogether four *Myrmica* species were found and only *My. scabrinodis* was present at all sites,
230 and was the most abundant ant species (59% of all ant colonies found). This species was used as
231 a host in all three populations. Only a single *M. alcon* X was found in a nest of *My. scabrinodis*
232 despite the dominance of this ant on the dry patch and its frequent usage by *M. alcon* H on the
233 nearby humid patch (Fisher's exact test, $P = 0.032$). The much greater exploitation rates of *My.*
234 *sabuleti* and *My. schencki* led to significant overall host ant specificity on the Răscruci dry site
235 (Table 2).

236

237 *Hardy-Weinberg and linkage disequilibrium*

238 Measures of genetic diversity and *F*-statistics generated by FSTAT for each locus are listed in
239 Table S1 in the supporting information. Analysis with MICRO-CHECKER revealed that Macu29

had a highly significant ($P < 0.001$) excess of homozygotes consistent with the presence of a relatively high proportion of null-alleles, and was therefore excluded from further analysis. All other loci showed no significant deviations from Hardy-Weinberg proportions. Tests for linkage disequilibrium revealed only a few sporadic significant results showing no overall pattern (Table S2), so all loci were retained in further analysis, which was thus based on 7 polymorphic loci.

Population structure

STRUCTURE analysis revealed rather invariable log-likelihood values for partitioning of the data into genetic clusters, but the highest change in log-probability value was for K = 2 (Figure 1), with lower maxima at K = 4 and K = 10 (Figures S1, S2). There was a clear overall distinction between samples from Răscruci wet in one genetic group and Răscruci dry and Şardu in another group. Levels of admixture between genetic clusters were generally low, but four individuals from Răscruci wet showed high affinity to the Răscruci dry-Şardu group, irrespective of the value of *K*. One individual found in *My. sabuleti* nest at Răscruci dry (sample code: DA14) appeared genetically similar to Răscruci wet. For values of K higher than 2 there was no additional partitioning between the pre-defined populations, but some substructure in Răscruci wet became apparent, with two partitions that were relatively dissimilar.

BAPS analysis showed similar log-likelihood values for K = 8-11, where K = 9 and K = 10 were found to be equally probable. The cluster membership of samples were explored for K = 2-5 and K = 9-11 and, for the lower Ks, revealed a high degree of similarity with the result from the STRUCTURE analysis (Figure 1). For the higher *K*s, allowing a much greater substructuring, nests within designated populations seemed to explain much of the cluster membership, particularly so

for Răscruci wet site, which also appeared more genetically homogenous as they were generally assigned to the same two clusters (Figure S2).

INSTRUCT revealed the same general pattern as STRUCTURE and BAPS (Figures 1, S1, S2), with the most likely estimate based on the deviance information criterion being K = 10 (Figure S2). Estimated within-subpopulation inbreeding coefficients were generally low at low K (F_{IS} = 0.025 for K = 2), but as expected, were higher at higher values of K (F_{IS} = 0.033-0.495 for K = 10), reflecting greater population sub-structure.

Genetic differentiation

We found significant overall genetic differentiation between pre-defined populations (θ = 0.090 D_{EST} = 0.215; Table S1). Pairwise genetic differentiation measures G_{ST} and D_{EST} were significant for all population comparisons after Bonferroni adjustment (P<0.003), with Răscruci dry and Şardu having the smallest genetic difference (G_{ST} = 0.033 and D_{EST} = 0.151), and Răscruci wet vs Răscruci dry or Şardu showing similar differentiation (G_{ST} = 0.048 and D_{EST} = 0.255; G_{ST} = 0.055 and D_{EST} = 0.221, respectively).

Hierarchical AMOVA revealed that 82.7% of genetic variance was between individuals, 11.1% was between ant nests (P < 0.001), 1.9% between ant species (P = 0.111) and 4.3% between pre-defined populations (P < 0.001). Samples from different years explained only 0.12% of the genetic variance in a separate AMOVA (P = 0.354). The Principal Coordinate Analysis retained a total of six principal coordinates with eigenvalues greater than 1, which together explained 52% of the variance in genetic distance. These showed a similar result to the AMOVA where

YEAR samples (2007 and 2009) overlapped completely in genetic ordination space ($F_{1,36} = 8.91 \times 10^{-16}$, $P = 0.999$), while samples from Răscruci wet were separated from those from Răscruci dry and Şardu (Figure 2; $F_{2,36} = 6.08$, $P = 0.005$). We found a pronounced structuring of samples when examining nests within pre-defined populations (POPNEST; $F_{18,36} = 3.59$, $P < 0.001$), with samples from the same nest clustering together, but there was no consistent clustering of samples from the nests of the same host ant species ($F_{3,36} = 0.887$, $P = 0.457$).

Relatedness

Overall pairwise relatedness of individuals sampled from the same nest (0.15) was significantly higher than that of those sampled from different nests within the same site (-0.006; Stratified Mantel test: $r^2 = 0.083$, $P < 0.0001$). Looking at individual sites, the same pattern was found at both M. alcon H sites (Răscruci wet: within-nest relatedness = 0.17, between nests = -0.015, $r^2 = 0.200$, $P < 0.0001$; Şardu: within-nest = 0.103, between nests = -0.012, $r^2 = 0.106$, $P = 0.008$), but relatedness was not significantly different within and between nests at Răscruci dry (within-nest = -0.004, between nests = 0.005, $r^2 = 0.0003$, $P = 0.428$).

Discussion

This study gives the first comparison of the host ant specificity and genetic composition of *M. alcon* H and *M. alcon* X within the same site.

The host ant specificity found in this study confirm the earlier results of Tartally et al. (2008) that these populations use the typical host ants found in other Central European studies (Höttinger, Schlick-Steiner & Steiner, 2003; Sielezniew & Stankiewicz, 2004; Steiner et al., 2003; Tartally et al., 2008; Witek et al., 2008). *M. alcon* H was found exclusively with *My. scabrinodis* at Răscruci wet and also with *My. vandeli* at Şardu, but *M. alcon* X was found mainly with *My. sabuleti* and *My. schencki* at Răscruci dry. Interestingly only one *M. alcon* X was found with *My. scabrinodis*, despite this *Myrmica* species being the most numerous at Răscruci dry (Table 2) and being the main host of *M. alcon* X in two other sites in the Carpathian-Basin (Tartally et al., 2008). *My. scabrinodis* usage could therefore be a potential link between the *M. alcon* H and *M. alcon* X populations at Răscruci (and probably in some other regions), but *M. alcon* X shows a clear separation from the *M. alcon* H in the proportional usage of this host ant. The background of this separation in the host ant specificity of *M. alcon* H and *M. alcon* X at Răscruci is not clear, but could reflect the dynamic arms race between the different genetic lineages of *M. alcon* and local host ants (Nash et al., 2008).

Our genetic results (Figures 2, 3) show strong genetic differentiation between *M. alcon* H and *M. alcon* X at Răscruci, indicating no or very limited gene flow between these two groups. This is likely due to separation in time rather than space because of the different phenology of the host plants, which results in largely non-overlapping flying seasons of *M. alcon* H and *M. alcon* X (Timuş et al., 2013). This may be reinforced by lowered fitness of any hybrid individuals that would emerge during the approximately 2-week gap when neither host plant is suitable for oviposition. The higher within-nest than between-nest relatedness between individuals of *M. alcon* H is consistent with observations of limited dispersal of ovipositing females (Körösi et al., 2008) which is likely to lead to substantial within-population substructure between nests, as

found here. The lack of this relationship for *M. alcon* X from the Răscruci dry site is also consistent with the difference in oviposition strategy and mobility of butterflies from this population and those from the Răscruci wet site (Czekes et al., 2014; Timuş et al., 2013).

The lowest level of between-population differentiation, on the other hand, was between *M. alcon* X from Răscruci and *M. alcon* H from Şardu, and Bayesian population assignment suggests that these are so similar that they have almost certainly been part of a single population. This supports pervious findings of no overall phylogenetic differentiation between the two forms of *M. alcon* (Als et al., 2004; Fric et al., 2007; Ugelvig et al., 2011b), and that the two forms tend to be more genetically similar locally than either is to more distant populations that use the same host plant (Bereczki et al., 2005; Bereczki, Pecsenye & Varga, 2006; Pecsenye et al., 2007). Genetic analysis of several Polish and Lithuanian *M. alcon* populations using microsatellite markers (Sielezniew et al., 2012) gave similar results to ours (Figures 1, 2) in that there was no clear pattern reflecting genetic division into two ecotypes. They also found that the *M. alcon* X ecotype was less polymorphic, and its populations were much more differentiated than those of the *M. alcon* H ecotype. Their data also suggest that *M. alcon* H populations form a single clade but *M. alcon* X can be split into more clades, suggesting that *M. alcon* H is an ancestral form and that *M. alcon* X represents a group of independently evolved *M. alcon* H populations that have switched to use dryer habitats with the locally available *Gentiana* and *Myrmica* species. They propose that the background of this pattern may be independent specialisations on different host ant species, since in their study clades of *M. alcon* X largely reflected host ant use. However, we find no evidence of genetic differentiation associated with host-ant usage at Răscruci or Şardu (Figures 2, 3), and no difference in genetic diversity in populations of the two ecotypes. Due to the relatively large distances and potential barriers between Răscruci and Şardu it is unlikely that

348 there has been recent gene-flow between the two sites, which suggests that Răscruci was likely
349 colonized at least twice from two different gene pools, and that the ancestors of the Răscruci wet
350 population are no longer locally extant.

351 Regardless of its origin, it is clear that the *M. alcon* X population at Răscruci is ecologically
352 highly differentiated from the local *M. alcon* H populations in terms of its host plant and host ant
353 use, as well as in its behaviour (Czekes et al., 2014; Timuş et al., 2013). As such, while it may
354 not represent an evolutionarily significant unit in conservation terms, it should still be regarded
355 as a functional conservation unit (Maes et al., 2004). The site at Răscruci represents the only
356 known area where both forms of *M. alcon* occur syntopically, and so is of particular value to
357 research on speciation, and has great potential for examining adaptation at non-neutral genetic
358 markers. This is enhanced by the occurrence of two other *Maculinea* species on the same site; *M.*
359 *teleius* Bergsträsser (Tartally & Varga, 2008) and *M. nausithous kijevensis* Sheljuzhko (Rákosy
360 et al., 2010; Tartally & Varga, 2008), as well as the *Myrmica* parasites *Microdon myrmicae*
361 Schönrogge et al. (Diptera: Syrphidae; Bonelli et al., 2011) and *Rickia wasmannii* Cavara
362 (Ascomycota: Laboulbeniales; Tartally, Szűcs & Ebsen, 2007). The *Maculinea* spp. parasitoid
363 *Ichneumon eumerus* Wesmael and *I. balteatus* Wesmael (Hymenoptera: Ichneumonidae) are also
364 present (Tartally, 2008; Timuş, Constantineanu & Rákosy, 2013). Most of these species are also
365 found at Şardu (except M. alcon X and M. nausithous, Tartally, 2008). It should be emphasized
366 that all of these species can be found in the nests of, and ultimately depend on, *My. scabrinodis*
367 (as well as other *Myrmica* species, see Witek, Barbero & Markó (2014) for a review), providing
368 a unique opportunity to examine a complex set of parasitic interactions revolving around a single
369 keystone ant species.

370

Conclusion

Our analysis of *Maculinea alcon* from a unique site where both the xerophilous and hygrophilous forms of this butterfly are found within tens of meters of each other has demonstrated strong genetic differentiation between the two forms. However, the xerophilous form was not significantly differentiated from the next closest known population of the hygrophilous form. This supports other recent work suggesting that the hygrophilous and xerophilous forms are not separate species or even subspecies, and that the name *M. rebeli* has frequently been applied to the xerophilous form incorrectly. There is some overlap in host ant species use between the two forms, so the most likely proximate reason for the local genetic differentiation found is differences in host plant phenology. We suggest that the two different forms of *M. alcon* should therefore be regarded as functional rather than evolutionary significant conservation units.

Acknowledgements

We thank Tibor-Csaba Vizauer, László Rákosy and Zoltán Varga for assistance in the field and Shukriya Barzinci, Maria Mikkelsen and Sylvia Mathiasen for assistance in the laboratory. Zoltán Varga and Enikő Tóth commented on the manuscript before submission.

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Table 1(on next page)

Details of microsatellites used in this study

SR = product size range (base pairs), T_A = Annealing Temperature (ºC), N_a = Number of alleles. Ref. = Reference source (U11 = Ugelvig et al. 2011a, U12 = Ugelvig et al. 2012, New = This study).

1

Primer	SSR motif	Primer sequences 5' - 3'	SR	T_A	Dye Group	N_a	Genbank accession	Ref.
Macu20	$(CT)_n(AT)_n(CT)_n$	F: TGGCCCGATTTCCTCTAAAC R: TGCGTGTTTATTTTCATTTTAACAG	92-122	57	Fam 1	9	HM535963	U12
Macu26	$(CA)_n$	F: CTCCCGGGATAGCATTGAC R: CATTGTCGCGGTCGTAATTC	92-128	57	Ned 2	7	HM535964	U12
Macu28	$(CA)_n(CGCA)_n(CA)_n$	F: TTTTAATCAAAATCGGTTCATCC R: TCAACCACAAAGCAAGTGAGTC	195-223	57	Fam2	12	XXXXXX	New
Macu29	$(TC)_n$	F: AAACGCGCTTATGGCTAAAC R: CGGTATGTCCCGTTACATCG	81-143	57	Vic 3	15	XXXXXX	New
Macu30	$(TG)_n$	F: GACGCGCTGTTATGTATTGC R: CGTCTAGCGTGACCGTAACA	93-109	57	Pet 4	5	HM586096	U11
Macu31	$(GTA)_n(GTC)_n(GTA)_n$	F: GTTCTGTCCCCCGAACTAGG R: AAACCTGGGATTGGTTAAAAAC	110-173	62	Ned 5	5	HM586097	U11
Macu40	$(CA)_n(GA)_n(CA)_n(GA)_n(CA)_n(GA)_n(CA)_n$	F: CCGTTTGGGAGATACGATGT R: CGCGTGTGCGTATATGTGAT	110-220	57	Pet 1	-	XXXXXX	New
Macu44	$(AC)_n$	F: ATAAGTCAGCACGTCAAAGCTG R: TGCAAATACTCCGAATAAATAACTG	170-220	57	Ned 3	10	HM535965	U12
Macu45	$(AC)_n(GC)_n(AC)_n$	F: TGTGTGACTGCGGTTCTTATC R: TGTAATCGCAGGAGAGATGTG	145-217	57	Vic 4	20	HM535966	U12

2

Table 2 (on next page)

Details of sampled *Myrmica* nests

The number of nests found within 2 m of gentians at each site, their infection with *M. alcon* H or *M. alcon* X, the number of samples used for genetics and statistical tests of host ant specificity within each site: *P1* = probability from Fisher exact test and *P2* = probability from a randomization test of ant nests between species. Significant *P*-values ($P < 0.05$) are marked in bold.

1

Site	*Maculinea*	*Myrmica*	No. nests	No. with *Maculinea*	*P1*	No. of *Maculinea*	Range	*P2*	No. genetic samples
Răscruci dry	*alcon* X	*sabuleti*	10	5	**0.004**	17	1-8	**0.002**	13
		schencki	6	2		18	1-15		5
		scabrinodis	23	1		1			1
Răscruci wet	*alcon* H	*scabrinodis*	31	9	-	30	1-7	-	28
Răscruci both	both	as above			0.078			**0.021**	
Şardu	*alcon* H	*vandeli*	27	2	0.147	9	2-7	0.495	2
		scabrinodis	38	7		15	1-4		11

2

Figure 1(on next page)

Bayesian clustering of samples.

Comparison of genetic clustering of samples into two groups using the Bayesian clustering programs Structure, BAPS and InStruct. Each column represents an individual, and is divided according to its probability of membership of cluster 1 (orange) or 2 (blue).

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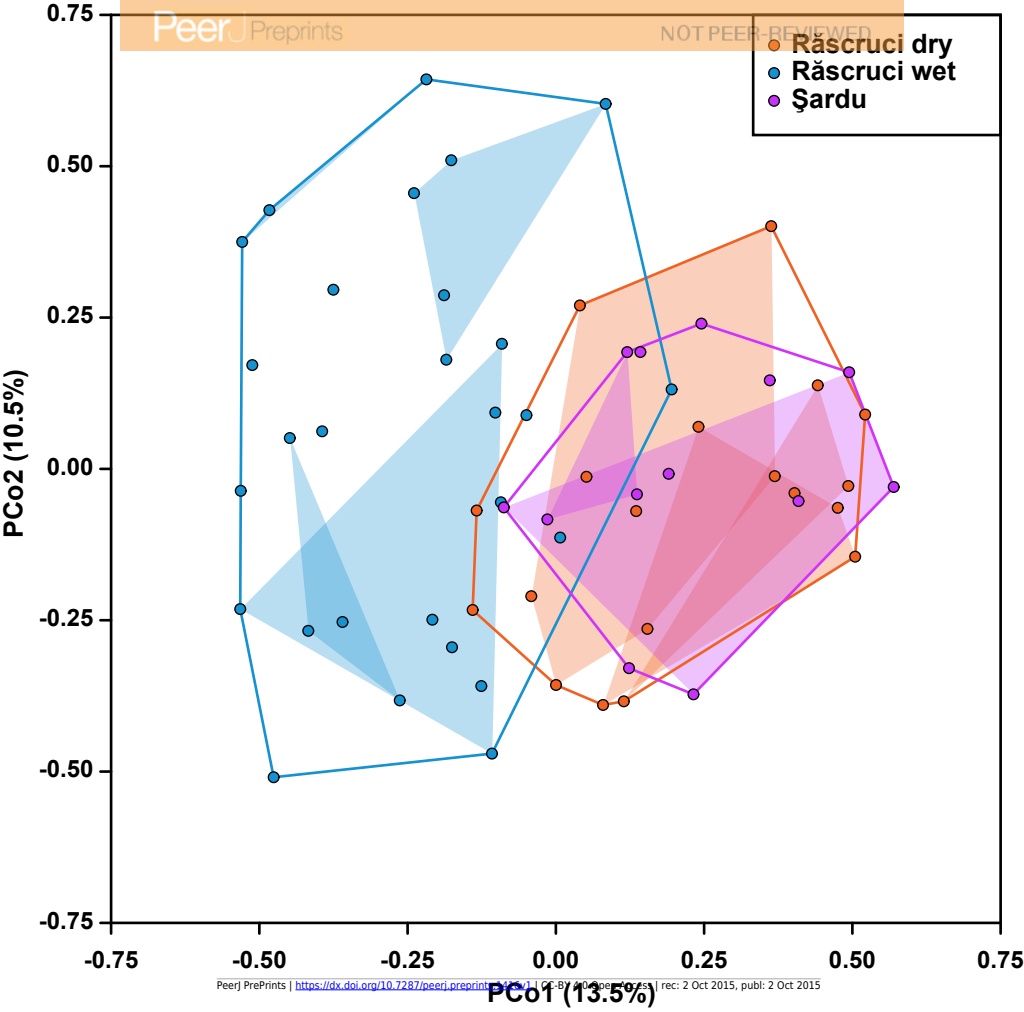
Figure 2(on next page)

Ordination of samples based on principal coordinate analysis.

Each symbol represents an individual, coloured according to its pre-defined population (blue = Răscruci wet, orange = Răscruci dry, purple = Şardu). Coloured lines are convex hulls enclosing all samples from each pre-defined population, while coloured regions are convex hulls enclosing samples collected from the same nest.



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