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Multilevel studies on the two phenological forms of Large Blue (*Maculinea arion*) (Lepidoptera: Lycaenidae)

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Abstract

The main goal of our research was to study comprehensively the differences between the two phenological forms of the socially parasitic and globally threatened Large Blue (*Maculinea arion*) in the Carpathian Basin using four character sets (mitochondrial sequences, allozymes, male genitalia and wing morphometrics). Comparative analyses of distance matrices, phylogenetic trees and ordination patterns have been applied. The genetic and morphometric patterns revealed by our studies were discordant. While we experienced a significant differentiation between the 'spring' and 'summer type' of *M. arion* in both wing and genital traits, the two phenological forms did not show any genetic differentiation on two mitochondrial loci and in allozymes. At the same time, all individuals were infected by *Wolbachia*. Although certain wing traits may not represent reliable tracers of phylogeny because of the particular adaptive significance, the wing characteristics involved in our research are probably determined genetically. Additionally, the significant differentiation of male genitalia also indicates incipient prezygotic isolation arising from phenological differentiation between the 'spring and summer arion'. It is possible that all extant differences between the two forms are attributable to (1) different host-ant use, (2) incipient speciation, (3) cytoplasmic incompatibility (CI) by *Wolbachia* or the combination of these factors. In addition, discordant results indicate that the combined use of different approaches and data sets is strictly necessary to clarify systematic and evolutionary relationships.

Key words: Spring and summer arion – mtDNA variability – *Wolbachia* – geometric morphometry – Carpathian Basin

Introduction

In the past few decades, traditional morphology-based taxonomy has been increasingly replaced by DNA-based species identification. A short, standardized gene region of mtDNA (mitochondrial cytochrome c oxidase subunit I – COI) was proposed as a 'DNA barcode' for discriminating most animal species (Hebert et al. 2003; Dinca et al. 2010). The proposal to develop an identification system based on a single gene marker attracted early criticism (Ebach and Holdrege 2005). Additionally, performance tests have shown significant differences in identification success in the case of different animal groups (Hebert et al. 2004; Wiemers and Fiedler 2007). As a consequence, a great need remains for comprehensive studies and in-depth multidisciplinary assessments before any conclusion is drawn. Such a multilevel approach became necessary to study the distinctness of two phenological forms of the Large Blue (*Maculinea arion*).

The genus *Maculinea* Van Eecke, 1915¹ (Lepidoptera: Lycaenidae) is one of the most intensively studied insect groups in Europe (Settele et al. 2005). This is partly attributable to their very special social parasitic life cycle and partly to being umbrella species (Fleishman et al. 2005; Spitzer et al. 2009). Additionally, these butterflies face a serious conservation risk as their habitats have suffered severe decrease and fragmentation. Within the genus *Maculinea*, one of the most conspicuous declines was shown by the Large Blue – *Maculinea arion* (Linnaeus, 1758). This species became extinct in the Netherlands in 1964 (Tax 1989), in the UK in 1979 (Thomas 1995) and in Belgium in 1996 (although later the species was re-introduced

into the UK and it also re-colonized in Belgium) (Goffart 1997). It shows a serious retreat all over Europe, especially on the northern border of the distribution area of the species (Wynhoff 1998); therefore, Large Blues are endangered on a European scale. The species is included in Annex IV of the European Habitats Directive, and it is listed in the IUCN Red List of Threatened Species as 'near threatened' and considered as 'endangered' (EN) in the European Red List of Butterflies (Munguira and Martin 1997; Van Swaay et al. 1998, 2010).

Maculinea arion is a highly variable species morphologically. Nowadays there are at least twenty named forms listed (Verity 1940–1953; Cheshire 2011) and three subspecies are commonly recognized in Europe (Higgins and Riley 1970; Thomas 1996; Tolman and Lewington 2009): (1) *Maculinea arion arion* (Linnaeus, 1758) is the most widespread nominate form, which is described from Germany (Nürnberg); (2) *Maculinea arion ligurica* (Wagner, 1904), which is described from Liguria region of north-western Italy originally as a varietas; (3) *Maculinea arion obscura* (Christ, 1878) is a high-mountain form in the Swiss Central Alps (Zermatt, Liestal), but phenotypically similar forms also occur in high mountains of the Balkan Peninsula. In the Carpathian Basin, the former two subspecies have been reported (Varga 2010), and there are numerous differences between them. The fast-flying, smaller-sized and dark violet-blue *M. a. arion* (referred to as 'spring arion' hereafter) flies from mid-May to mid-June and prefers short-grass dry swards with cushions of early-flowering *Thymus* species (*Th. serpyllum* L., *Th. pannonicus* All. and related species), which serve as initial food plants. The slower, larger and light silvery blue *M. a. ligurica* (referred to as 'summer arion') is on the wing from the end of June to mid-August and mostly occurs at xerothermic oak forest fringes, on woodland clearings and in fen-like habitats in hilly areas. Females oviposit among flower buds of late-flowering *Thymus* species (mostly *Th. pulegioides* L.) and/or *Origanum vulgare* L. (Varga 2010; : Annotation No. R23). Although differences in food plant use also imply some difference in habitat preference, there is only a weak ecological isolation between the two forms.

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¹Here, we follow the recommendation of Balletto et al. (2010) concerning the generic names *Maculinea* Van Eecke, 1915 versus *Phengaris* Doherty, 1891.

Moreover, numerous syntopic occurrences of the 'spring' and 'summer arion' have been recorded in Aggtelek Karst region (Hungary) (Tóth & Bereczki, pers. obs.). Our knowledge on the host-ant use of the two subspecies is insufficient. *Myrmica sabuleti* Meinert, 1861 is known to be the main host ant of *M. arion* in Western Europe, and it was also found as a putative host ant in some *M. arion* habitats in Hungary (Tartally 2008). In Poland, *M. arion* is characterized by multiple host-ant use, which varies geographically (Sielezniew et al. 2003, 2010a,b,c; Sielezniew and Stankiewicz 2008). Hitherto, ant-nests infected by larvae of *M. arion* have not been found in Hungary probably because of the lack of intensive surveys. The phylogenetic studies focusing on the section *Glaucopsyche* show potential cryptic species within the predatory *Maculinea*, and high divergences were also found in *M. arion* (Als et al. 2004; Fric et al. 2007; Ugelvig et al. 2011b). Consequently, the two phenological forms of *M. arion* may be candidates for putative cryptic species.

At the same time, Sielezniew (2012) and Sielezniew et al. (2012) discovered *Wolbachia* infestation in *M. arion* and *M. alcon* populations, which may have influence on phylogeny of *Maculinea* species. *Wolbachia* infections are associated with a variety of phenotypic effects on the hosts: (1) cytoplasmic incompatibility (CI); (2) male killing, the consequence of which is sex ratio distortion; (3) feminization of genetic males, which may also bias the sex ratio; and (4) parthenogenesis induction. The former three effects have been revealed in butterflies. CI, that is, when the sperm of infected males is incapable of fertilizing the eggs of uninfected females and females infected with a different *Wolbachia* strain, is the most frequently observed phenotype (Hoffmann and Turelli 1997; Werren et al. 2008). The spread of *Wolbachia* via CI can drive the spread of maternally inherited genetic elements, such as mtDNA. Specific mitochondrial genotypes may be associated with a *Wolbachia* CI strain surreptitiously. Consequently, the distribution of mtDNA variation in infected populations does not conform to the expectations of neutral theory (Narita et al. 2007; Gompert et al. 2008; Nice et al. 2009), which has been revealed not only in butterflies, but also in other invertebrate species (Turelli and Hoffmann 1991; Shoemaker et al. 2003; Shaikevich et al. 2005). *Wolbachia* may homogenize biological species for mtDNA following introgression of endoparasites, as reported in *Acrea* (Jiggins 2003) and

Drosophila (Ballard and William 2000b), causing a 'one barcode – two species' phenomenon (i.e. two biological entities have the same mtDNA sequence). On the contrary, *Wolbachia* can make one species appear as two due to the high intraspecific mtDNA diversity associated with possession of different parasite strains, as reported in *Adalia* (Hurst et al. 1999a,b), leading to a 'two barcodes – one species' phenomenon. Additionally, divergent mitochondrial sequences may suggest the presence of cryptic species falsely (Hurst and Jiggins 2005).

Although our previous results (Bereczki et al. 2011) have shown that the two putative subspecies cannot be differentiated based on allozyme loci, we intended to study the dissimilarities between the two forms in a multilevel research including DNA and morphological surveys. We also intended to explore whether the outcome of the different methods would be congruent or not. Besides, we were interested in discovering whether the influence of *Wolbachia* on mtDNA variability and selective sweep works in *M. arion* populations. Thus, the aims of our research were: (1) to reveal whether the two types of *M. arion* differentiate on the basis of mitochondrial sequences, (2) to establish the measure of the differentiation in allozymes, (3) to study the morphological differences between them on the traits of external genitalia and wings and (4) to compare the level of the genetic and morphometric differentiation.

Materials and methods

Sampling

DNA studies were based on sequences of 29 field-collected specimens (Appendix 1, Fig. 1), of which nine were 'spring arion' and 15 were 'summer arion' (the identification was carried out based on the date of sampling). We had four couples of 'spring and summer arion' from the same locality (see in boldface in Appendix 1, Fig. 1). We used only one *M. arion* individual from GenBank database (identifier: SPAus – accession number: HQ918148.1) because only its sequence overlapped completely with those of our samples. *Maculinea alcon*, *M. teleius* and *M. nausithous* specimens were used as outgroups.

In our allozyme and morphometric studies, we used the same set of individuals (only males). Altogether 11 samples (143 individuals) were collected between 2002 and 2011 (Appendix 2, Fig. 1): four were 'spring type' and seven were 'summer type' *M. arion* (the identification was also

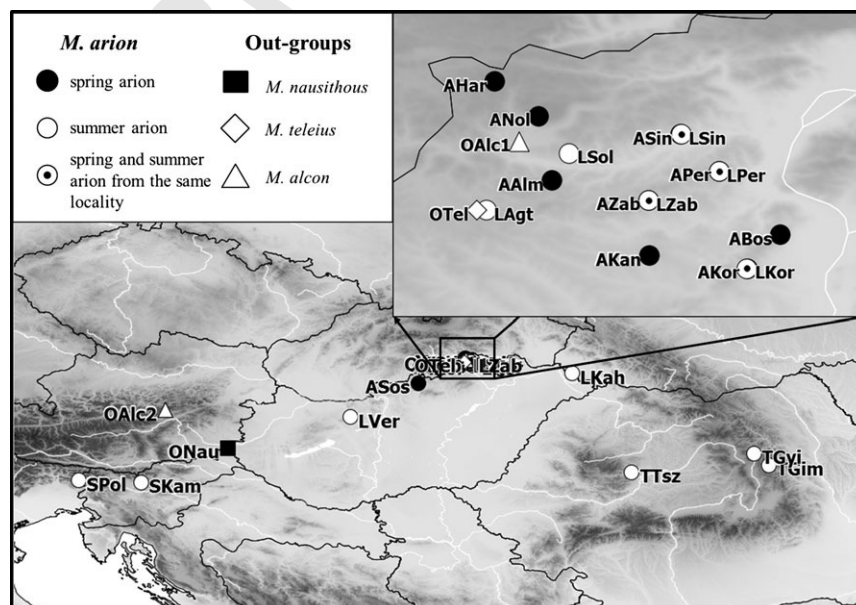


Fig. 1. Sample sites. See the abbreviations in Appendices 1 and 2

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carried out based on the sampling time). We possessed one pair of samples originated from the same syntopic population (see in boldface in Appendix 2). The samples from Kamnik and Polovnik (Slovenia) were unified because of the low number of individuals. *M. nausithous* (16 individuals) was included as outgroup. Images were collected at the end of the egg laying period and stored at -80°C until electrophoresis and morphometric analyses.

Molecular analyses

DNA studies

DNA was extracted by homogenizing either the head or thorax (Appendix 1) in 800 μl extraction buffer (Gilbert et al. 2007). The samples were incubated for 24 h at 56°C with gentle agitation and then centrifuged at 14 000 rpm for 1 min. The supernatant was washed twice with an equal volume of chloroform–isoamyl alcohol (24 : 1) to remove proteins. The DNA was precipitated by adding the mixture of 80 μl ammonium acetate (7.5 M) and an equal volume of ice-cold isopropanol and storing the samples at -20°C for 4 h. The DNA was pelleted by centrifugation at 14 000 rpm for 10 min at 4°C . After centrifugation, the supernatant was discarded and the DNA pellet was washed twice with 70% ice-cold ethanol. The pellet was air-dried for 1 h at room temperature and was redissolved in 50 μl elution buffer (10 mM Tris–HCl, pH 8.0 and 0.5 mM EDTA, pH 9.0). DNA aliquots were stored at 4°C .

The I subunit of the cytochrome c oxidase gene (COI), which is commonly used in barcoding animal life (Hebert et al. 2003; Wiemers and Fiedler 2007), offers an adequate tool to test whether the morphological and ecological differences between the two forms of *M. arion* manifest at the DNA level. We therefore sequenced this section of the mitochondrial genome together with subunit II (COII) to obtain insight into the phylogeny of taxa at the species level. These two mitochondrial genes were amplified by four modified universal primer pairs (COI: HybLCO and HybHCO, HybJerry and HybPat of Wahlberg and Wheat (2008); COII: Georges and Phyllis, Strom and BtLys of Monteiro and Pierce (2001)). Primers were modified at their 5'-end to include the universal sequencing primer T4 promoter. Amplification from 1 μl of DNA extracts was carried out in 25 μl final reaction volumes containing $10\times$ PCR buffer, 2 mM MgCl_2 , 0.2 mM dNTPs, 0.02 units per μl of Taq DNA polymerase (Dream Taq Green, Fermentas) and 0.2 μM of each primer. Amplification was carried out in an ABI Veriti thermal cycler programmed for: initial denaturation for 3 min at 94°C ; 35 cycles of 30 s at 94°C , 30 s at the locus-specific annealing temperature of $54/54/52/56^{\circ}\text{C}$ (in the order of primer pairs, see above), 1 min at 72°C ; final elongation of 10 min at 72°C . The success of PCR amplification was checked by running 2 μl of product on 1% agarose gels stained with ethidium bromide. PCR products were sequenced by commercial service provider Macrogen Inc. (South Korea). Sequences were edited and revised manually by CHROMAS LITE v. 2.01 and aligned by MEGA v. 4.0 (Tamura et al. 2007).

The presence of *Wolbachia* was checked by the amplification of the 16S ribosomal region with *Wolbachia*-specific primers W-Spec of Werren and Windsor (2000) following their guidelines of amplification. Infected accessions (Appendix 1) were tested further with the strain-specific ftsZ primers and PCR conditions of Werren et al. (1995) and Sasaki et al. (2002).

Based on the concatenated COI and COII sequences, phylogenetic tree reconstruction methods of three different search criteria were applied to demonstrate phylogenetic relationship between the 'spring' and 'summer arion'. Heuristic searches were run in PAUP v. 4.0b10* (Swofford 2003) under both maximum parsimony (MP) and maximum-likelihood (ML) criterion separately, while Bayesian phylogenetic relationships were assessed in MRBAYES v. 3.2.1 (Ronquist et al. 2012). The MP search used TBR search algorithm holding 10 trees at each iteration step with 'MulTree' option in effect, while 'steepest descent' not in effect. The ML search utilized an evolutionary model of TVM+I as selected by MODELTEST v. 3.7 (Posada and Crandall 1998) with default settings. For the Bayesian analysis, we specified the evolutionary model HKY+H+G as selected by MRMODELTEST v. 2.3 (Nylander 2004), and two separate runs were conducted for 2 million generation samplings every 1000th generation. Resulting probability files were checked by TRACER v. 1.5 (Drummond and Rambaut 2007) for convergence and effective sample sizes of the runs, then were combined and a maximum clade credibility tree was computed after discarding first 25% of trees as 'burn-in'. All analyses were run on Biportal (Kumar et al. 2009).

Allozyme studies

Allozyme polymorphism was studied at 12 loci by vertical polyacrylamide gel electrophoresis. Thoraxes homogenized in 300 μl of extraction buffer were used to study *Gpdh*, *G6pgdh*, *Hk*, *Idh*, *Mdh*, *Pgi*, *Pgm* and *Sod*. Abdomens homogenized in 200 μl of extraction buffer were used to analyse *Acon*, *AcpH*, *Aox* and *Est*. The extraction buffer, the electrophoresis buffer systems and running conditions, together with the staining solutions, were applied as described in Bereczki et al. (2005). Genotypes of the individuals were scored according to their enzyme pattern.

Since our previous research (Bereczki et al. 2011) has involved a detailed study about the allozyme variability of *M. arion*, the recent study has been based on a reduced data set. Therefore, this paper only includes the analyses that have been carried out in parallel with the morphometric surveys. Allele frequencies were used to estimate Nei's genetic distances (Nei 1975), and an UPGMA dendrogram (Sneath and Sokal 1973) was constructed on the basis of the distance matrix using PAST v. 2.17 (Hammer et al. 2001). Pairwise F_{ST} values were also established by GENALEX v. 6.4 (Peakall and Smouse 2006).

Morphometric analyses

Prior to electrophoresis, wings and the terminal segments of the abdomen were cut. Wings were fixed on transparency films and photographed by Sony DSC-H2 digital camera. Landmark-based geometric morphometric approach was used to quantify the variation in the shape of wings and the pattern of black spots on the underside. We recorded 17 landmarks on fore-wing and 16 on hind-wing (only one side was measured in both cases) by TPSDIG v. 2.1 (Fig. 2a). Procrustes generalized least squares (GLS) method was applied to get the superimposed coordinates for the statistical analyses.

The preparation procedure of male external genitalia was slightly modified after Robinson (1976). The sclerotized genitalia were separated from the body tissues by keeping the terminal segments of the abdomen in 15% KOH overnight, followed by heating for 30 min at 75°C before preparation. The genitalia were cleaned and dehydrated with 96% ethanol and mounted in Euparal fixative (mounting medium) on microscope glass. Slides were digitalized by combining an Olympus camera and a Nikon 102 stereomicroscope. Since we found only few real landmarks on valvae, we recorded a close curve on them using TPSDIG v. 2.1 (Fig. 3a). For the analysis of the outlines, elliptic Fourier analysis (Giardina and Kuhl 1977; Kuhl and Giardina 1982) was used. The algorithm fits Fourier series on x- and y-coordinates as functions of the curvilinear abscissa (Claude 2008). The statistical analyses of the two morphometric approaches were similar.

Repeatability was calculated by the following formula: $ME = S^2_{\text{within}} / (S^2_{\text{within}} + S^2_{\text{among}}) \times 100$ (Lessells and Boag 1987), where S^2_{within} is the within-measurement component of variance and S^2_{among} is the among-measurement component. ANOVA was used to calculate these values (Bailey and Byrnes 1990; Yezerinac et al. 1992) in R programme package (R Development Core Team 2010). Principal component analyses (PCAs) were applied to reduce the number of variables. The scores of the PC axes that could explain more than 1% of the total variance were used in canonical variates analysis (CVA) and multivariate analysis of variance (MANOVA) (see: Dapporto et al. 2009, 2011; Dinca et al. 2011). Jack-knifed grouping and Wilks' lambda were applied to quantify the validity of the visible pattern.

UPGMA tree was constructed based on Mahalanobis distances. The differences in centroid sizes (the square root of the summed squared distances of each landmark from the centre of the form) were analysed by univariate ANOVA. All morphometric analyses were carried out using PAST 2.17 (Hammer et al. 2001).

Results

Molecular studies

Four bits of mitochondrial (mt) cytochrome c sequences were concatenated into one 'contig' sequence for all studied taxa containing full mtCOI and partial mtCOII sequences. Final concatenated sequences were aligned without the need of introducing gaps into a matrix of 2217 base pairs (bp), of which 1361 bp

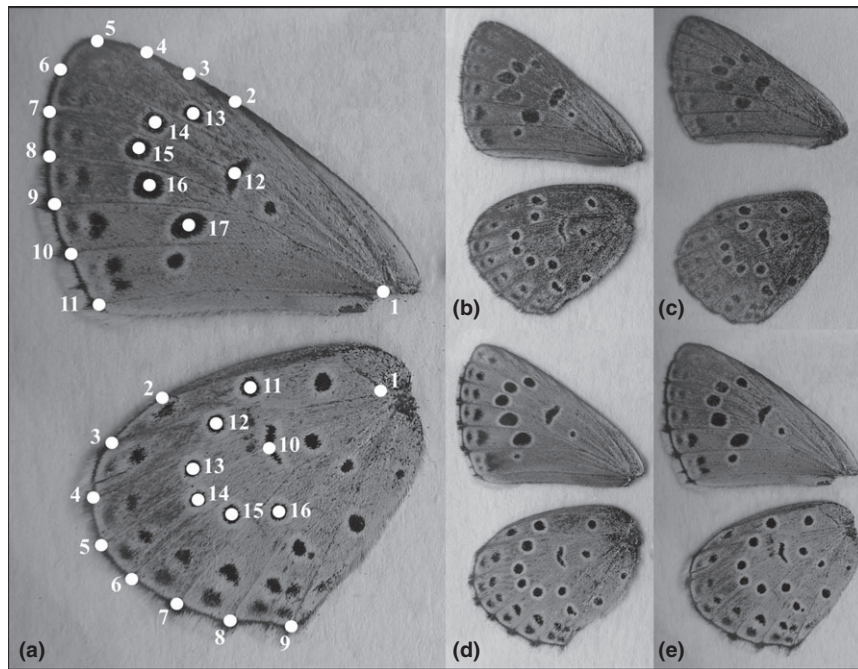


Fig. 2. Morphometric analyses. (a) landmarks on the underside of wings, (b) 'spring-type' wings from AZab; (c) 'spring-type' wings from AHAr; (d) 'summer-type' wings from LZab; (e) 'summer-type' wings from LSin. See the abbreviations in Appendix 2

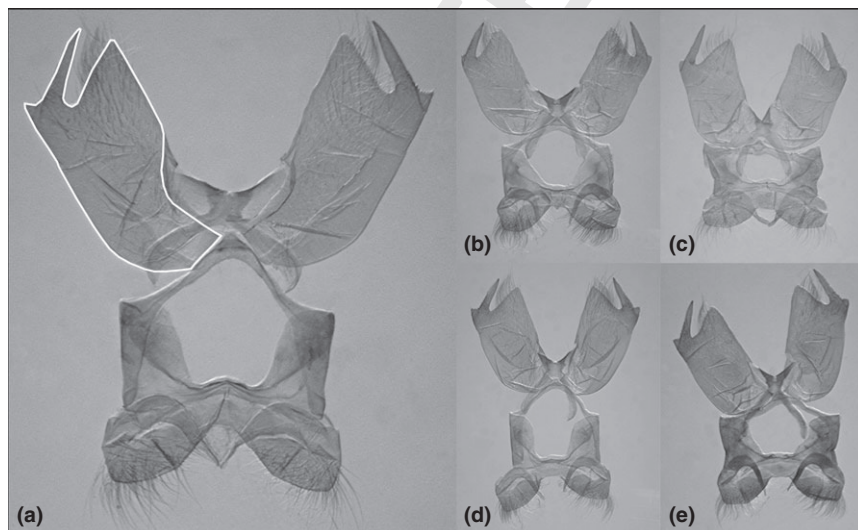


Fig. 3. Morphometric analyses. (a) closed curve on valva of genitalia; (b) 'spring-type' genitalia from AZab; (c) 'spring-type' genitalia from AHAr; (d) 'summer-type' genitalia from LZab; (e) 'summer-type' genitalia from LSin. See the abbreviations in Appendix 2

were from COI and 856 bp were from COII. Altogether, there were 10 variable positions in the alignment of *M. arion* sequences and each was parsimony informative. All three different phylogenetic tree reconstruction methods yielded the same topology of phylogenetic trees (Fig. 4a,b). The 'spring' and the 'summer type' of *M. arion* were not differentiated on the basis of these mitochondrial sequences. Moreover, the target sequences did not show any geographical pattern. Only the two Slovenian individuals from Kamnik have been found on a well-supported sub-branch (bootstrap ML: 80%; bootstrap MP: 85%; Bayesian posterior probability: 0.99) although it should be noted that the differentiation of these specimens is attributable to altogether six mutations on 2217 sites. Outgroups were clearly separated from *M. arion* individuals. This lack of resolution within *M. arion* can

be explained by infection with *Wolbachia*, which was proven by appropriate tests. All *M. arion* individuals were infected by *Wolbachia* supergroup A (Appendix 1), and *M. alcon* specimens were infected with B supergroup. *M. nausithous* was uninfected and we got a weak band indicating the infection by B supergroup in *M. teleius*.

The results of our previous allozyme study (Berezcki et al. 2011) have been confirmed by the recent analysis based on a reduced data set. The two types of *M. arion* were not separable at the enzyme level. All *M. arion* samples were located in the same branch of the dendrogram and they separated clearly from the outgroup (Fig. 4c). The samples from the same locality (AZab and LZab) did not cluster together. Pairwise F_{ST} values indicated small differences among populations even between the

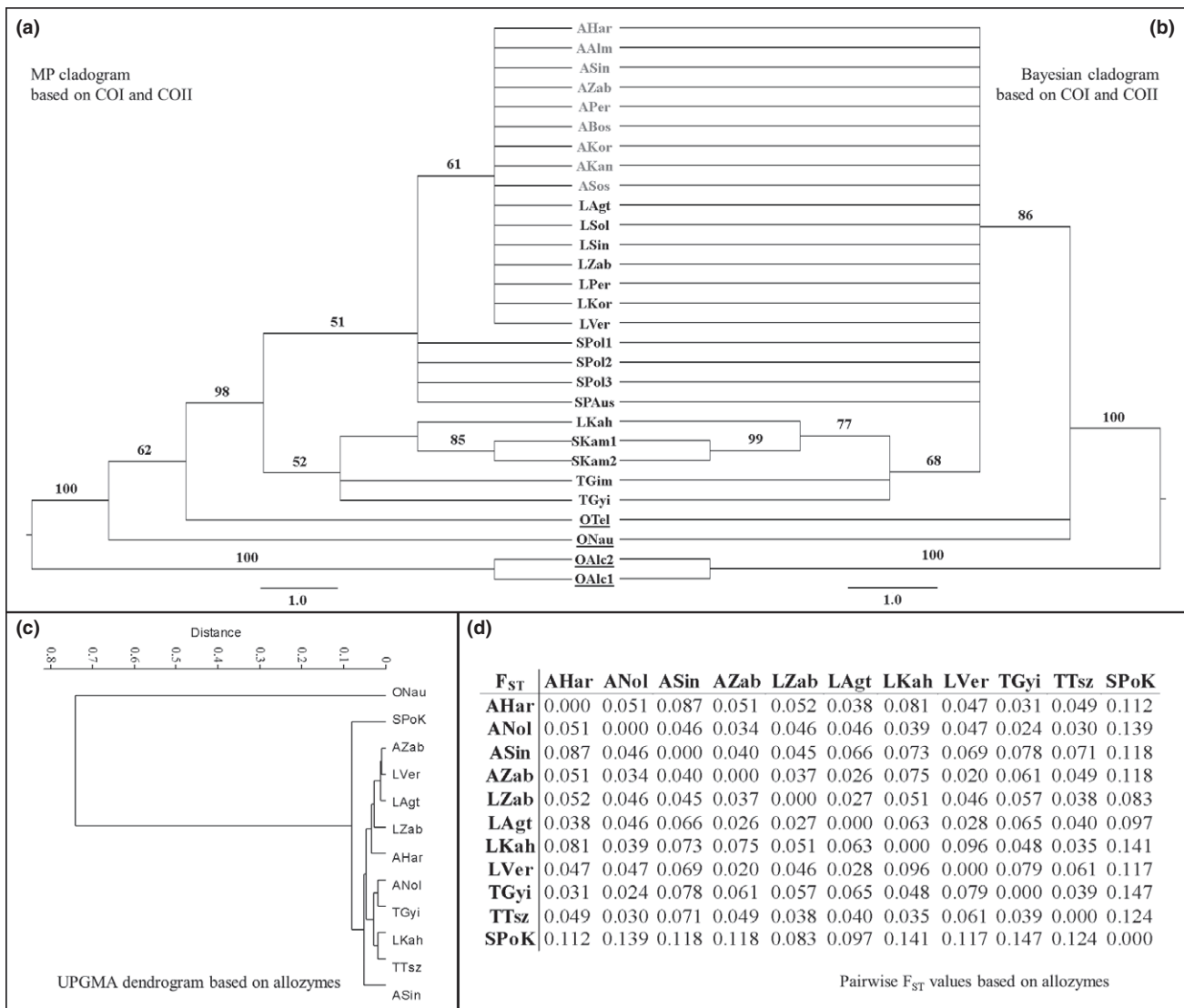


Fig. 4. The results of molecular studies. (a) Maximum parsimony cladogram based on the concatenated COI and COII sequences with bootstraps. See the abbreviations in Appendix 1. (b) Bayesian cladogram based on the concatenated COI and COII sequences with clade credibility values. See the abbreviations in Appendix 1. (c) UPGMA dendrogram based on allozymes using Nei's genetic distances. See the abbreviations in Appendix 2. (d) Pairwise F_{ST} values calculated on the basis of allozymes. See the abbreviations in Appendix 2.

samples which are located in different geographical regions at long distances apart. The separation of the Slovenian sample from all the other ones was the highest extent (Fig. 4d).

Morphometric studies

The repeatability of measurements was satisfactory ($ME < 5\%$) in most cases. The error was $< 9\%$ in the case of each hind-wing and genitalia coordinate. We excluded the landmarks of fore-wing from our analyses that have more than 20% measurement error.

The results of our morphometric survey were very similar in cases of wings and genitalia alike. The 'spring' and the 'summer arion' differentiated significantly (fore-wing: Wilks' $\lambda=0.253$, $p < 0.001$; hind-wing: Wilks' $\lambda=0.358$, $p < 0.001$; genitalia: Wilks' $\lambda=0.399$, $p < 0.001$). More than 85% of the individuals were correctly classified by a cross-validated method in both wings and genitalia (Appendix 3A–C). The best classification was obtained in the case of fore-wing (the lowest Wilks' λ with the highest classification value –93.0%).

The samples of the 'spring' and the 'summer arion' clustered separately both on the phenogram of wings (Fig. 5a,b) and on that of genitalia (Fig. 5c). The samples from the same locality (AZab and LZab) clustered separately according to the phenology. In cases of wings, a geographical pattern can be recognized. The Slovenian sample differentiated from the other ones at the highest extent in wings, but it is clustered together with one of the Transylvanian samples on the basis of genital traits. The group centroids of the two different types of *M. arion* samples also differentiated from each other at the first axis (Fig. 5d–f). Moreover, we experienced significant differences ($p < 0.001$) in size between the 'spring' and 'summer arion'. The Transylvanian and Slovenian samples were clearly grouped into the 'summer arion' on the basis of size (Fig. 5g–i).

Discussion

The genetic and morphometric patterns revealed by our studies are discordant. While we experienced significant differences in morphology between the 'spring' and 'summer type' of

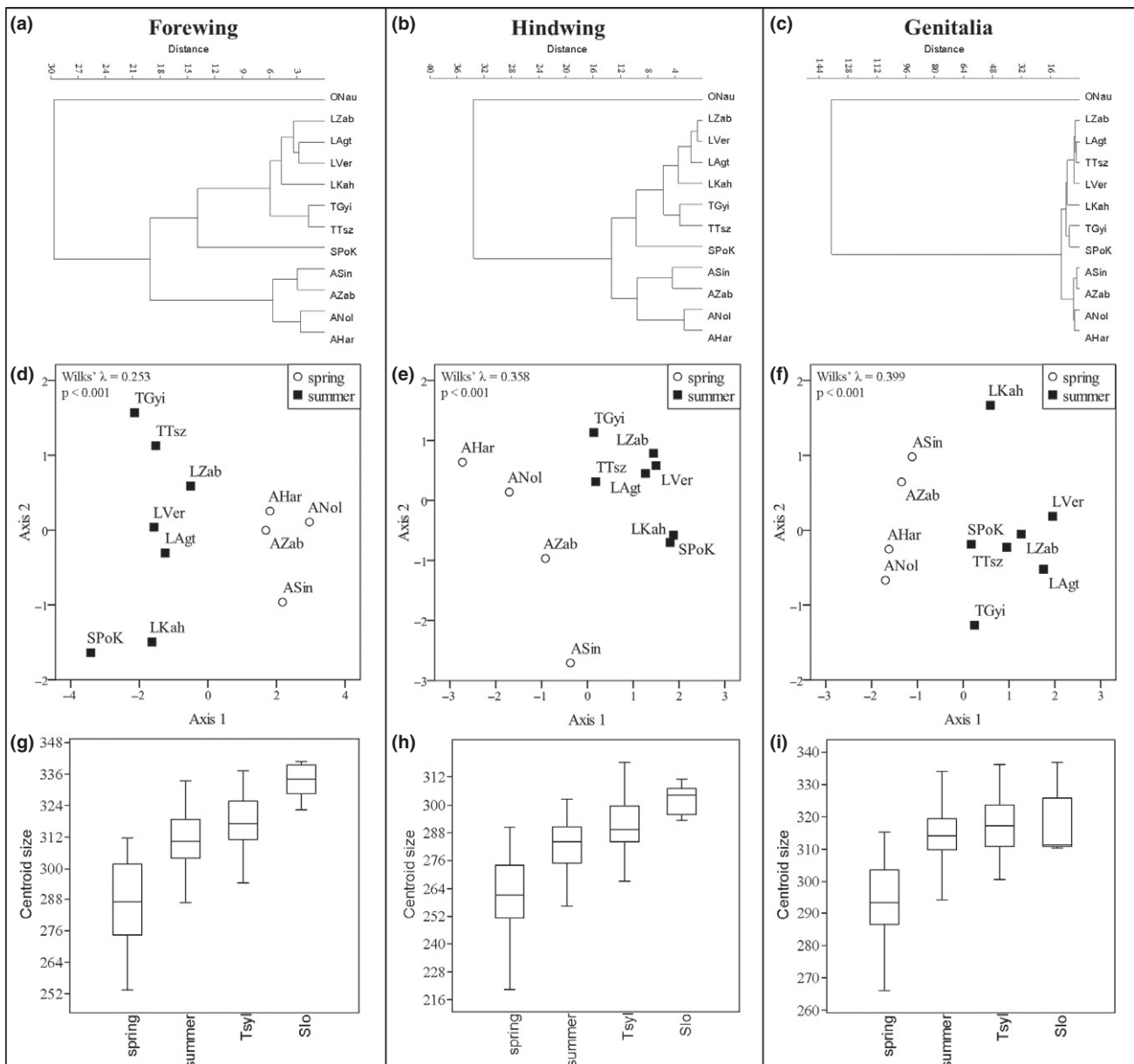


Fig. 5. The results of morphometric analyses. UPGMA phenograms based on Mahalanobis distances: (a) fore-wing, (b) hind-wing, (c) genitalia. On the CVA scatterplots, the group centroids of samples are represented. See the abbreviations in Appendix 2. (d) fore-wing, (e) hind-wing, (f) genitalia. Box plots indicating the distribution of the centroid size. Spring – the ‘spring arion’; summer – the ‘summer arion’; Tsy – Transylvanian samples; Slo – the Slovenian sample. (g) fore-wing, (h) hind-wing, (i) genitalia

M. arion, the two phenological forms show genetic differentiation neither on the mitochondrial sequence nor at the allozyme level.

The variability of the studied mitochondrial regions was highly reduced. Only the Slovenian specimens from Kamnik formed a highly supported clade nested within the otherwise unresolved clade of *M. arion*. At the same time, all *M. arion* specimens proved to be infected by *Wolbachia* A. *M. alcon* individuals, used here as outgroups, were also infected but with *Wolbachia* B. Similar to our results, Sielezniew (2012) and Sielezniew et al. (2012) reported the infection of the same types of *Wolbachia* and a reduced mtDNA diversity compared to nuclear variation in the populations of *M. arion* and *M. alcon* in Poland and Lithuania. Therefore, the lack of resolution within *M. arion* found here is presumably the consequence of indirect selection mediated by *Wolbachia* (Ballard and William 2000a; Jiggins 2003; Hurst and

Jiggins 2005) rather than that of population dynamic events. Thus, the usage of additional nuclear markers and regular assays for *Wolbachia* presence as well as multilocus sequence typing (MLST) should be applied in phylogenetic and phylogeographical studies despite the fact that *Wolbachia* COI was only present in 0.16% of >2 million insect COI sequences (Smith et al. 2012). Although detailed population dynamic studies (mark-release-recapture) were carried out only in one population from Vértesszomsza (‘LVer’) in Hungary (Körösi et al. 2005) and sex ratio distortion was not recorded (Körösi, pers. comm.), further studies are needed to elucidate the possible effect and phenotype of *Wolbachia* in *M. arion* populations in the Carpathian Basin. Besides, it would be worth to use nuclear markers mostly in the couples of samples originated from the same locality.

On the basis of our allozymes studies, genetic differentiation among *M. arion* populations is low. The isolation of the

1 Slovenian sample was the highest. Our results coincide with
2 those of Polish authors based on microsatellites (Rutkowski et al.
3 2009; Sielezniew and Rutkowski 2012) who also experienced
4 small and moderate differentiation among *M. arion* samples.

5 This pattern is probably in relation to the biological character-
6 istics of this species. Only one or two larvae of *M. arion* may
7 survive in an ant-nest, because one predatory caterpillar requires
8 on average 350 ant workers to be raised compared with 50 for a
9 'cuckoo' larva (Elmes et al. 1991; Thomas and Wardlaw 1992).
10 In addition, the majority of *Myrmica* species live in small colonies
11 of 200–500 workers (Elmes et al. 1998) such as *M. sabuleti*,
12 the primary host ant of *M. arion* in Western Europe. Therefore,
13 only few *Myrmica* colonies are large enough to support just a
14 single *M. arion* larva, and small colonies (or multiple infected
15 large ones) are probably to be completely exploited by caterpillar
16 (s) in the spring (Thomas and Wardlaw 1992), because they
17 experience dramatic reductions in colony fitness by infection
18 (Thomas et al. 1989). Consequently, the depleted colonies may
19 be less suitable as a host for *M. arion* in the following year
20 although the ant colony may be replaced by another one (Tolman
21 and Lewington 2009). This intimate butterfly–ant relationship
22 may lead to strong oscillations in population census size such as
23 reported in the northernmost Finnish population (Kolev 1998)
24 and in Vértes population in Hungary (LVer). In the latter popula-
25 tion, approximately 300 individuals were marked in a two-ha
26 area in 2002, but mark–release–recapture studies failed due to
27 the absence of *M. arion* in 2004–2005 (Körösi et al. 2005). Con-
28 sequently, the genetic diversity may primarily be maintained by
29 gene flow among local low-density populations.

30 Additionally, *M. arion* has high dispersal ability, which has
31 been revealed by capture–recapture studies (Pajari 1992). At the
32 same time, molecular studies indicated that gene flow occurs
33 over distances 15 times longer than the maximum distance
34 recorded from mark–recapture studies, and *M. arion* can main-
35 tain fully functional metapopulations where the suitable habitat
36 patches are not further apart than approximately 10 km (Ugelvig
37 et al. 2011a, 2012). Therefore, the high potential of gene flow
38 may also contribute to the low genetic differentiation found
39 among *M. arion* populations.

40 Our morphometric research showed significant differences
41 between the 'spring and summer arion' both in wings and in
42 genitalia. In the case of wings, a geographical pattern was
43 observed. Numerous studies revealed that certain wing traits –
44 for example size, melanization level – may be determined envi-
45 ronmentally along climatic (latitudinal or altitudinal) gradients
46 (Dennis 1977; Dennis and Shreeve 1989; Smyllie 1992) and
47 greatly exposed to environmental stress (Talloen et al. 2009).
48 Sielezniew and Dziekańska (2011) found that the melanization
49 level was higher in the north-eastern part of Poland than in the
50 South, and the mid-eastern region showed intermediate character-
51 istics. At the same time, other wing traits are probably not
52 exposed to environmental effects considerably, but determined
53 rather by genetic factors (Talloen et al. 2009), for example the
54 position of spots on the underside, which is used for the identi-
55 fication in *Maculinea* taxonomy (Sibatani et al. 1994). Since we
56 experienced clear geographical pattern hardly explained by
57 climatic factors, we suppose that the wing traits involved in our
58 study are probably determined genetically rather than environ-
59 mentally. Nonetheless, this pattern may also be in relation to the
60 different host-ant use (Gadeberg and Boomsma 1997; Sielezniew
61 and Dziekańska 2011; Sielezniew and Rutkowski 2012).

62 At the same time, good agreement has been revealed between
63 the outcome of the molecular studies and that of male genitalia
morphometrics in numerous analyses (Cesaroni et al. 1989,
1994; Garnier et al. 2005). This suggests that selective pressures

controlling genital structures are relatively homogeneous across
taxa and the patterns of divergence in genital morphology may
reflect overall genetic divergence rather than differential adaptive
responses. Consequently, the quantitative traits of male genitalia
may be good estimators of the overall divergence among popula-
tions and closely related species and generally considered reliable
taxonomic characteristics for traditional systematic work at the
species level.

Our molecular and morphometric analyses lead to discordant
results, but we have to consider some basic facts. First, the diver-
sity of the barcoding gene is proved to be reduced presumably
due to *Wolbachia* infection. Second, the allozymes of *Maculinea*
species are generally less polymorphic than those of other Euro-
pean lycaenid species (Schmitt and Seitz 2001, 2002a,b; Aagaard
et al. 2002; Schmitt et al. 2002, 2003, 2005; Schmitt and Hewitt
2004). Therefore, it is possible that allozyme and mitochondrial
DNA studies are not suitable for the detection of the divergence
between the 'spring and summer arion'. Nevertheless, our mor-
phometric studies have revealed significant differences between
M. a. arion and *M. a. ligurica*. Although certain wing traits may
not represent reliable tracers of phylogeny because of their par-
ticular adaptive significance, the wing characteristics involved in
our research are probably determined genetically. Additionally,
the significant differentiation of male genitalia also indicates
incipient prezygotic isolation arising from phenological differen-
tiation between the two types of *M. arion*.

In summary, our study clearly indicates that the combined use
of different approaches and data sets is highly necessary to clar-
ify systematic and evolutionary relationships among taxa despite
the fact that molecular data often tend to receive more emphasis
than morphological ones. Although we did not find differences
between the two forms of *M. arion* on the basis of molecular
data, it is not at all unlikely that our markers are not suitable for
the detection of the divergence between them. It is possible that
all extant differences of the two forms are attributable to (1) dif-
ferent host-ant use, (2) incipient speciation, (3) CI by *Wolbachia*
or a combination of these factors. Further molecular and ecologi-
cal studies are needed to elucidate the pattern of variation.

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Zusammenfassung

Der gefährdete sozialparasitische Bläuling *Maculinea arion* stand im Vor-
dergrund dieser naturschutzbiologischen Untersuchungen. In der vorlie-
genden Arbeit werden die Unterschiede zwischen den phänologischen
Formen von *Maculinea arion* aufgrund von vier verschiedenen Merk-
malsgruppen (mitochondriale Sequenzen, Allozyme, Morphometrie der
männlichen Genitalien und Flügelmuster) besprochen. Distanzmatrizes,
phylogenetische Bäume und Ordinationsmuster werden verglichen. Wir
haben gegensätzliche Ergebnisse in der genetischen bzw. morphometris-
chen Differenzierungen erhalten. Während in den morphometrischen
Merkmalen der Flügel und Genitalien erhebliche Unterschiede zwischen
den phänologischen 'Frühjahrs-' und 'Sommer'-Formen gefunden wur-
den, zeigten die beiden mitochondrialen Loci und auch das Allozymmus-
ter keine Differenzierung. Alle untersuchten Individuen waren jedoch mit
Wolbachia infiziert. Obwohl die meisten Flügelmerkmale wegen ihrer
adaptiven Plastizität phylogenetisch als irrelevant gelten, sollten die von
uns gewählten Merkmale einen genetischen Hintergrund haben. Außer-
dem deuten die nachgewiesenen Unterschiede in den männlichen Geni-
talien auf eine anfängliche präzygotische Isolation zwischen den beiden

phänologischen Formen hin. Als mögliche Gründe für eine solche Differenzierung könnten (1) die verschiedenen Wirtsameisen, (2) beginnende Speziation bzw. (3) zytoplasmatische Inkompatibilität durch *Wolbachia* oder die Kombination diese Faktoren gelten. Die gegensätzlichen Ergebnisse sollten die Notwendigkeit der kombinierten Anwendung verschiedener Methoden und Datensätze in der Untersuchung der systematischen und evolutionären Verhältnisse unterstreichen.

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Appendix 1. Information on the specimens used in the DNA studies

Taxa	Locality	Abbr.	Sampling time	DNA extraction	<i>Wolbachia</i> supergroup
Spring arion (Hungary)	Almás-tető	AAlm	2011-05-26	Thorax	A
	Boszorkány-völgy	ABos	2011-05-25	Thorax	A
	Haragistya	AHar	2011-05-31	Thorax	A
	Kánó	AKan	2011-05-24	Thorax	A
	Korlát-hegy	AKor	2011-05-25	Thorax	A
	Perkupa	APer	2011-05-28	Thorax	A
	Sóshartyán	ASos	2002-05-19	Head	A
	Szin	ASin	2011-05-25	Thorax	A
	Zabanyik	AZab	2011-05-24	Thorax	A
	Summer arion (Hungary)	Aggtelek	LAgt	2002-08-06	Head
Kaszonyi hegy		LKah	2002-07-23	Head	A
Korlát-hegy		LKor	2011-08-05	Thorax	A
Perkupa		LPer	2011-08-04	Thorax	A
Szin		LSin	2011-08-02	Thorax	A
Szólóhegy		LSol	2011-07-14	Thorax	A
Vértesszomszék		LVer	2002-07-10	Head	A
Zabanyik		LZab	2011-08-03	Thorax	A
Summer arion (Transylvania)	Gyilkos tó	TGyi	2002-07-02	Head	A
	Gyimesbükk	TGim	2011-07-16	Thorax	A
Summer arion (Slovenia)	Kamnik	SKam1	2003-07-04	Head	A
		SKam2	2003-07-04	Head	A
	Polovnik	SPol1	2002-07-12	Head	A
		SPol2	2002-07-12	Head	A
		SPol3	2003-07-04	Head	A
<i>M. alcon</i>	Tohonya-hát	OAlc1	2011-06-23	Thorax	B
	Hochschwab	OAlc2	2003-07-11	Head	B
<i>M. nausithous</i>	Kétvölgy	ONau	2003-08-01	Head	–
<i>M. teleius</i>	Aggtelek	OTel	2005-07-29	Thorax	?

Abbr.: the abbreviations of localities (Fig. 1). See in boldface the couples of 'spring and summer arion' from the same locality. *Wolbachia* supergroup: the type of *Wolbachia* infection.

Appendix 2. Information on the samples used in the allozyme and morphometric analyses

Taxa	Locality	Abbr.	Sampling time	N
Spring arion (Hungary)	Haragistya	AHar	2005-06-06/07	16
	Nagyoldal	ANol	2003-05-28; 2004-06-09	19
	Szin	ASin	2011-05-25	9
	Zabanyik	AZab	2005-05-30; 2005-06-05/06; 2011-05-24	16
Summer arion (Hungary)	Zabanyik	LZab	2005-07-28, 29; 2011-07-14	9
	Aggtelek	LAgt	2005-07-28/29	18
	Kaszonyi hill	LKah	2003-07-21; 2003-08-05	15
	Vértesszomszka	LVer	2002-07-01/03; 2003-07-02/07	12
Summer arion (Transylvania)	Lake Gyilkos	TGyi	2004-07-24	13
	Torokószentgyörgy	TTsz	2004-07-22/23	11
Summer arion (Slovenia)	Polovnik, Kamnik	SPoK	2002-07-12; 2003-07-04	5
<i>M. nausithous</i> (outgroup)	Kétvölgy	ONau	2004-08-05	16
Total				159

Abbr.: the abbreviations of localities (Fig. 1). See in boldface the pair of samples from the same syntopic population. N: number of individuals.

Appendix 3. Classification results of the canonical variates analysis (CVA)

Type	Classification results ^{1,2}			Total
	Predicted group membership			
	1	2		
(A) Fore-wing				
Original				
Count	Spring	56	4	60
	Summer	4	79	83
%	Spring	93.3	6.7	100.0
	Summer	4.8	95.2	100.0
Cross-validated ³				
Count	Spring	55	5	60
	Summer	5	78	83
%	Spring	91.7	8.3	100.0
	Summer	6.0	94.0	100.0
(B) Hind-wing				
Original				
Count	Spring	51	9	60
	Summer	5	78	83
%	Spring	85.0	15.0	100.0
	Summer	6.0	94.0	100.0
Cross-validated ⁶				
Count	Spring	48	12	60
	Summer	8	75	83
%	Spring	80.0	20.0	100.0
	Summer	9.6	90.4	100.0
(C) Genitalia				
Original				
Count	Spring	53	7	60
	Summer	13	70	83
%	Spring	88.3	11.7	100.0
	Summer	15.7	84.3	100.0
Cross-validated ⁹				
Count	Spring	53	7	60
	Summer	14	69	83
%	Spring	88.3	11.7	100.0
	Summer	16.9	83.1	100.0

¹94.4% of original grouped cases correctly classified.

²93.0% of cross-validated grouped cases correctly classified.

³Cross-validation is carried out only for those cases in the analysis. In cross-validation, each case is classified by the functions derived from all cases other than that case.

⁴90.2% of original grouped cases correctly classified.

⁵86.0% of cross-validated grouped cases correctly classified.

⁶Cross-validation is carried out only for those cases in the analysis. In cross-validation, each case is classified by the functions derived from all cases other than that case.

⁷86.0% of original grouped cases correctly classified.

⁸85.3% of cross-validated grouped cases correctly classified.

⁹Cross-validation is carried out only for those cases in the analysis. In cross-validation, each case is classified by the functions derived from all cases other than that case.

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During the copy-editing of your paper, the following queries arose. Please respond to these by marking up your proofs with the necessary changes/additions. Please write your answers on the query sheet if there is insufficient space on the page proofs. Please write clearly and follow the conventions shown on the attached corrections sheet. If returning the proof by fax do not write too close to the paper's edge. Please remember that illegible mark-ups may delay publication. Many thanks for your assistance.

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19	AUTHOR: Please check the usage of “?” here.	

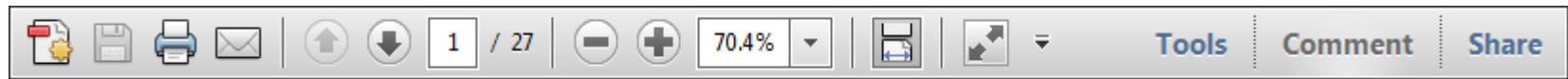
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USING e-ANNOTATION TOOLS FOR ELECTRONIC PROOF CORRECTION

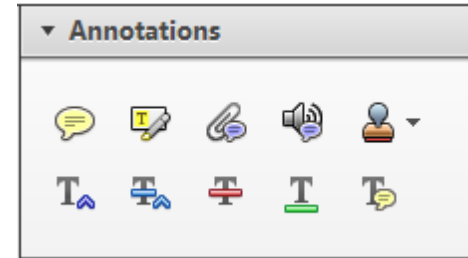
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Once you have Acrobat Reader open on your computer, click on the [Comment](#) tab at the right of the toolbar:



This will open up a panel down the right side of the document. The majority of tools you will use for annotating your proof will be in the [Annotations](#) section, pictured opposite. We've picked out some of these tools below:



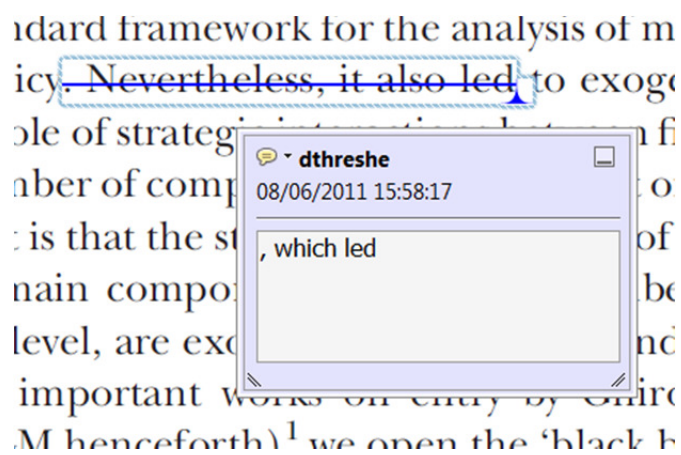
1. Replace (Ins) Tool – for replacing text.



Strikes a line through text and opens up a text box where replacement text can be entered.

How to use it

- Highlight a word or sentence.
- Click on the [Replace \(Ins\)](#) icon in the Annotations section.
- Type the replacement text into the blue box that appears.



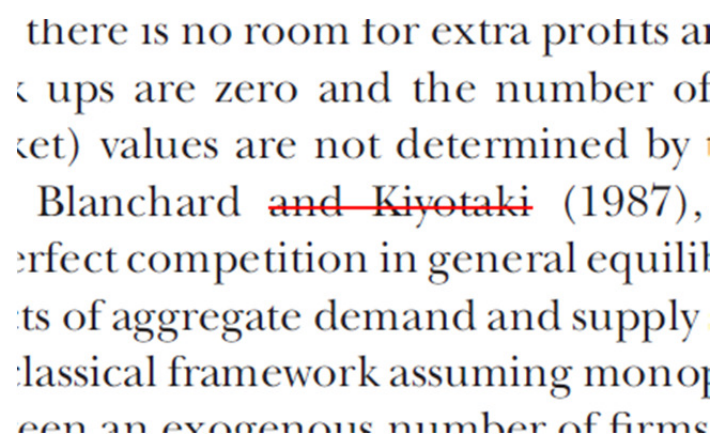
2. Strikethrough (Del) Tool – for deleting text.



Strikes a red line through text that is to be deleted.

How to use it

- Highlight a word or sentence.
- Click on the [Strikethrough \(Del\)](#) icon in the Annotations section.



3. Add note to text Tool – for highlighting a section to be changed to bold or italic.

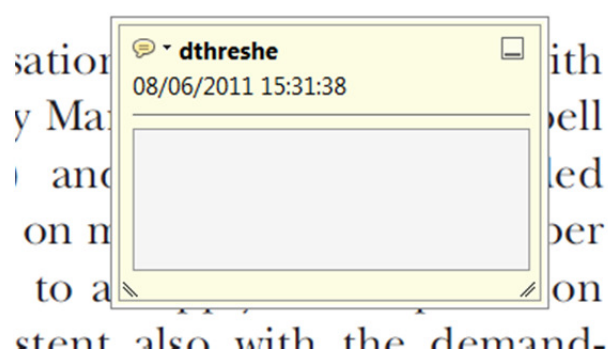


Highlights text in yellow and opens up a text box where comments can be entered.

How to use it

- Highlight the relevant section of text.
- Click on the [Add note to text](#) icon in the Annotations section.
- Type instruction on what should be changed regarding the text into the yellow box that appears.

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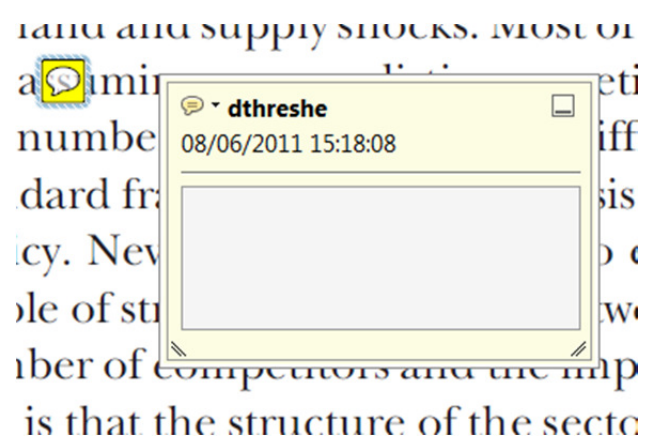
4. Add sticky note Tool – for making notes at specific points in the text.



Marks a point in the proof where a comment needs to be highlighted.

How to use it

- Click on the [Add sticky note](#) icon in the Annotations section.
- Click at the point in the proof where the comment should be inserted.
- Type the comment into the yellow box that appears.



USING e-ANNOTATION TOOLS FOR ELECTRONIC PROOF CORRECTION

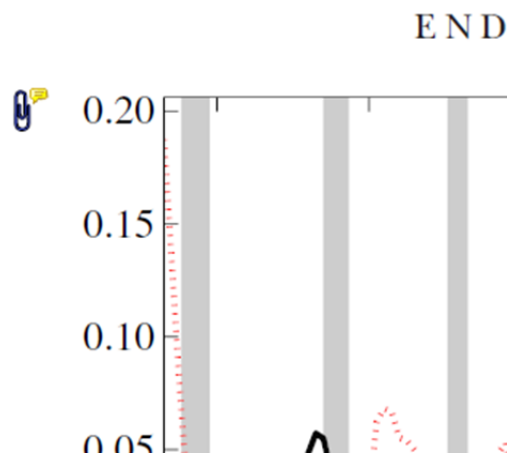
5. Attach File Tool – for inserting large amounts of text or replacement figures.



Inserts an icon linking to the attached file in the appropriate place in the text.

How to use it

- Click on the [Attach File](#) icon in the Annotations section.
- Click on the proof to where you'd like the attached file to be linked.
- Select the file to be attached from your computer or network.
- Select the colour and type of icon that will appear in the proof. Click OK.



6. Add stamp Tool – for approving a proof if no corrections are required.

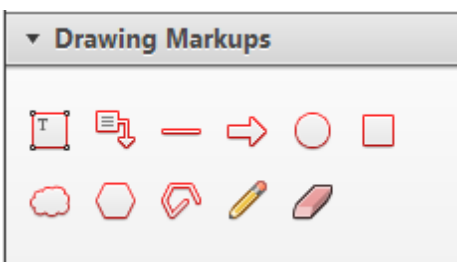


Inserts a selected stamp onto an appropriate place in the proof.

How to use it

- Click on the [Add stamp](#) icon in the Annotations section.
- Select the stamp you want to use. (The [Approved](#) stamp is usually available directly in the menu that appears).
- Click on the proof where you'd like the stamp to appear. (Where a proof is to be approved as it is, this would normally be on the first page).

of the business cycle, starting with the
 on perfect competition, constant return
 production. In this environment goods
 extra profits and the market
 he market. The New-Key
 otaki (1987), has introduced produc
 general equilibrium models with nomin
 ed and supply shocks. Most of this literat

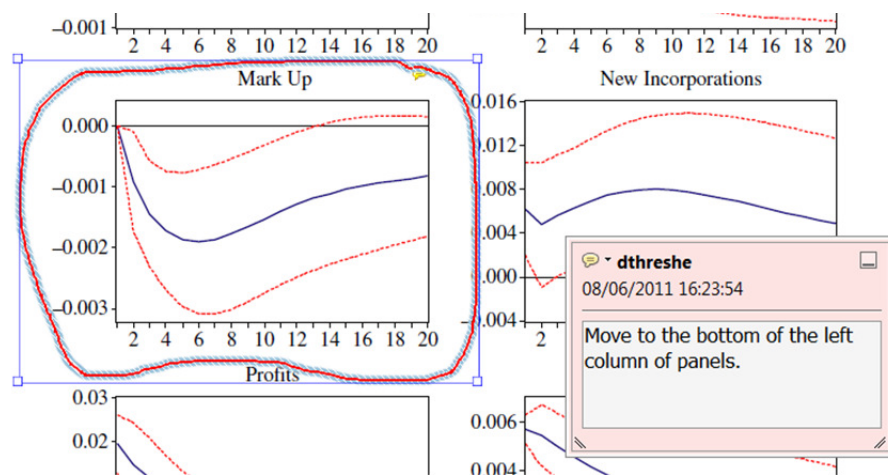


7. Drawing Markups Tools – for drawing shapes, lines and freeform annotations on proofs and commenting on these marks.

Allows shapes, lines and freeform annotations to be drawn on proofs and for comment to be made on these marks..

How to use it

- Click on one of the shapes in the [Drawing Markups](#) section.
- Click on the proof at the relevant point and draw the selected shape with the cursor.
- To add a comment to the drawn shape, move the cursor over the shape until an arrowhead appears.
- Double click on the shape and type any text in the red box that appears.



For further information on how to annotate proofs, click on the [Help](#) menu to reveal a list of further options:

