

MORPHOMETRIC STUDY ON THE GENITALIA
OF SIBLING SPECIES *MELITAEA PHOEBE* AND *M. TELONA*
(LEPIDOPTERA: NYMPHALIDAE)

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Taxonomy and phylogeny of the diverse nymphalid genus *Melitaea* was often considered during the last decade. *Melitaea phoebe* (Knapweed Fritillary) and *M. telona* has been considered as conspecific until the last few years. Morphometric characters of genital structures were analysed by traditional morphometric method. Significant but slightly overlapping differences were found in both sexes. In male genitalia we observed that *M. telona* can be characterised by a more notched saccus, and more symmetric processus posteriore than *M. phoebe*. In females, *Melitaea phoebe* has more circle shaped, while *M. telona* has more elliptic shaped posterior lamella. The furca is usually smaller in *M. phoebe*. *M. phoebe ornata* specimens from the South Ural, Russia, were clustered together with *M. telona* in the analyses.

Keywords: morphometrics, *Melitaea phoebe*, *Melitaea telona*, taxonomic separation, male and female genitalia

INTRODUCTION

Molecular taxonomy and phylogeny of the nymphalid genus *Melitaea* was thoroughly studied during the last decade (WAHLBERG & ZIMMERMANN 2000, WAHLBERG *et al.* 2003, WAHLBERG *et al.* 2005, LENEVEU *et al.* 2009). The taxonomy of the “*phoebe* group” (= subg. *Cinclidia* HÜBNER, [1819]) was also discussed in several recent papers (RUSSELL *et al.* 2005, VARGA *et al.* 2005, RUSSELL *et al.* 2007, VARGA 2007).

Melitaea phoebe ([DENIS & SCHIFFERMÜLLER], 1775) is a polytypic species with several described subspecies and infra-subspecific forms (HIGGINS 1941). Its range extends from North Africa over Eurasia to the Far East. *M. phoebe* and its subspecies are generally polyphagous, their host-plants are different species of Asteraceae (*Cirsium*, *Centaurea*, *Serratula*, *Saussurea*, *Stemmacantha*) and Plantaginaceae (KORSHUNOV & GORBUNOV 1995, TUZOV *et al.* 2000, RUSSELL *et al.* 2007, VARGA 2007, TOLMAN & LEWINGTON 2008). They are mostly bivoltine (except some high mountain populations), and its L4 larvae have black head capsule. The western Mediterranean populations also share these characters.

Table 1. Ecological traits of *Melitaea telona* and *M. phoebe*

	<i>M. phoebe</i>	<i>M. telona</i>
Distribution	Euro-Siberian nearly continuous	Ponto-Mediterranean disjunct (insular)
Habitats	Euryoecious; wide range in altitude (lowland to sub-alpine)	Stenoecious; nature-like xeric or mesic grasslands; narrow range of altitude (lowland to montane)
Larval food plants	Polyphagous on Asteraceae (<i>Cirsium</i> , <i>Centaurea</i> , <i>Serratula</i> , <i>Saussurea</i> , <i>Stemmacantha</i>) and <i>Plantago</i>	Oligophagous on local (endemic) Asteraceae
Voltinism	Generally bivoltine, in high altitudes monovoltine	Strictly monovoltine (even in Mediterranean habitats)
Morphology of larvae	Variable but usually with black head capsule of hibernated larvae	Generally black with brick-red head capsule of hibernated larvae

Several taxa from the Eastern Mediterranean region were also described as subspecies of *M. phoebe*: *Melitaea phoebe telona* FRUHSTORFER, 1908 from Jerusalem (Israel), *Melitaea phoebe ogygia* FRUHSTORFER, 1908 from Poros (Greece), *Melitaea phoebe totila* STAUDER, 1914 from southern Italy, *Melitaea phoebe amanica* REBEL, 1917 from Amanus Mt. (Turkey, Asia Minor), *Melitaea phoebe emipunica* VERITY, 1919 from Palermo (Sicily), *Melitaea phoebe nigrogygia* VERITY, 1938 from Opatija (Croatia). All of these taxa have, however, some important common, distinctive characters: they are usually univoltine, the L4 larvae have a red head capsule, and they are feeding on some, regionally different specific, often endemic *Cirsium* or *Centaurea* host-plants (RUSSELL *et al.* 2007). Also the Hungarian subspecies *Melitaea phoebe kovacsi* VARGA, 1967 described from Central Hungary (Budakeszi) shows the same characters. RUSSELL *et al.* (2005, 2007) and VARGA *et al.* (2005) suggested, based on these similarities, the separation of these taxa as *Melitaea telona* FRUHSTORFER, 1908 (syn: *M. ogygia* FRUHSTORFER, 1908). *M. phoebe ornata* Christoph, 1893 described from Guberlya, South Ural was mentioned as a subspecies (KORSHUNOV & GORBUNOV 1995) but recently it was used as a synonym of *M. phoebe phoebe* (TUZOV *et al.* 2000). However, this taxon shows the typical wing pattern on the undersides of the wings and the shape of antennae like *M. telona* figured already by VARGA (1967).

LENEVEU *et al.* (2009) analysed a mitochondrial and two nuclear genes from many taxa belonging to the *Melitaea* genus. Their results suggest that *Melitaea phoebe*, *M. telona* and *M. punica* are three well-differentiated species. Enzyme electrophoretic study of Hungarian populations has also shown obvious differences between *M. phoebe* and *M. telona* without any mark of hybridisation (PECSENYE *et al.* 2007).

Genitalia are often species-specific, and their forms are often more divergent among closely related species than are the forms of other traits. This relatively rapid divergence of genitalia is extremely widespread taxonomically, and has made them especially useful in distinguishing closely related species (EBERHARD 1985, SHAPIRO & PORTER 1989). A combination of morphometric studies and multivariate analysis has been used in the last few years and is growing in importance as an approach (WAKEHAM-DAWSON *et al.* 2004, SIMONSEN 2005, MUTANEN *et al.* 2006, SIMONSEN 2006, SIMONSEN *et al.* 2006, HERNÁNDEZ-ROLDÁN & MUNGUIRA 2008, PRIETO *et al.* 2008).

M. phoebe and *M. telona* have so far, only been analysed by molecular methods. In this paper we would like to focus on the morphological differences of genitalia using traditional morphometrics.

MATERIAL AND METHODS

In our study we surveyed 115 specimens to identify the potential morphological differences allowing the separation of these two species. The genital characters are much more conservative than external characters, and they are insignificantly influenced by environmental factors (CESARONI *et al.* 1994, DAPPORIO *et al.* 2009).

M. phoebe (23 ♂, 26 ♀) and *M. telona* (32 ♂, 34 ♀) specimens originated mostly from Hungary (72) but the sample also includes some museum specimens from Greece (5), Macedonia (12), Albania (2), Romania (1), Bulgaria (6), Turkey (3), Serbia (1) and Russia (6). The specimens belong to the Hungarian Natural History Museum, Zoological State Collection in Munich and to the collection of the University of Debrecen.

We made genitalia slides and digitalised those by the combination of an Olympus camera and a Canon stereo microscope. Set of distance measurements from the structures of male and female genitalia were taken using the ImageJ 1.34 Java image processing program.

In permanent genital slides some parts of the genitalia can be deformed in different way, so they are unsuitable for the measures even if they possibly bear important traits. However, the preparation is necessary because the sclerotised parts must be fixed in a standard way in order to measure at least some characters. Moreover, this process can be repeated in any time. For females structures we could find only a few characters what we could measure.

We grouped the specimens according to the characters of the wing pattern and colouration, morphology of antennae, forelegs (VARGA 1967), the occurrence and collection time.

Bilaterally symmetrical features were measured on the left side of the genitalia. Seven characters were measured on males genitalia (Fig. 1a) and three on females (Fig. 1b) Two ratios were calculated from these measurements. We used ratio G to describe the curvature of the harpe, it was calculated as follows: $G = HI / ((HH \times \pi) / 2)$ and ratio P = PLH / PLW to demonstrate how close to oval the posterior lamella is in females.

Measurement error was calculated by the following formula: $ME = s^2_{within} / (s^2_{within} + s^2_{among}) \times 100$ (LESSELLS & BOAG 1987) using nested ANOVA (BAILEY & BYRNES 1990, YEZERINAC *et al.* 1992). The repeatability of measurements was then calculated as 100%-ME.

Most of the characters were highly repeatable ME < 1% in both sex, but some traits have slightly higher measurement error: PW (ME = 7.13%), PI (ME = 4.02%) and HE (ME = 3.25%).

Univariate and multivariate analysis were used in statistics. Statistical analysis was carried out using the statistical software package SPSS 16.0. The measurements were tested independently by ANOVA (Analysis of Variance). Multiple Discriminant Analysis (MDA) was used to unravel the relationship between the previously sorted groups (expected species) and find the most discriminative traits. All variables were entered simultaneously. Wilks' lambda (λ) measures the discriminatory power of the model. Its value ranges from 0 (perfect discriminatory power) to 1 (no discriminatory power). Some specimens (3 ♂, 3 ♀) were grouped to *M. telona* from new localities: Magnitogorsk and Guberlya in S Urals Russia. MDA was also used to classify these specimens. These were set as ungrouped cases. PCA (Principal Component Analysis) was also used to demonstrate the relationship between the specimens.

RESULTS

Shapiro-Wilks test supported that all the measured variables showed normal distribution. MDA demonstrated significant differences between the two species in males ($p < 0.001$, Wilks' $\lambda = 0.19$) and in females ($p < 0.001$, Wilks' $\lambda = 0.29$).

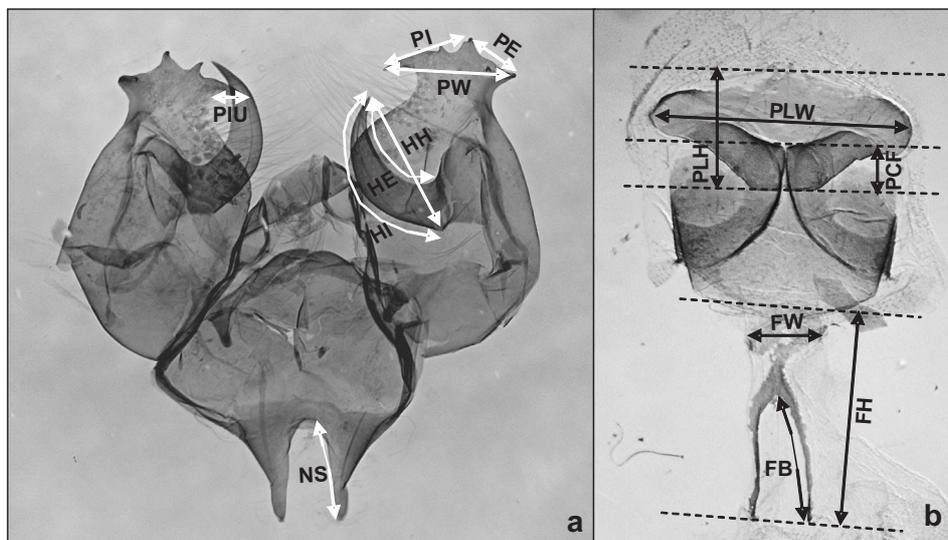
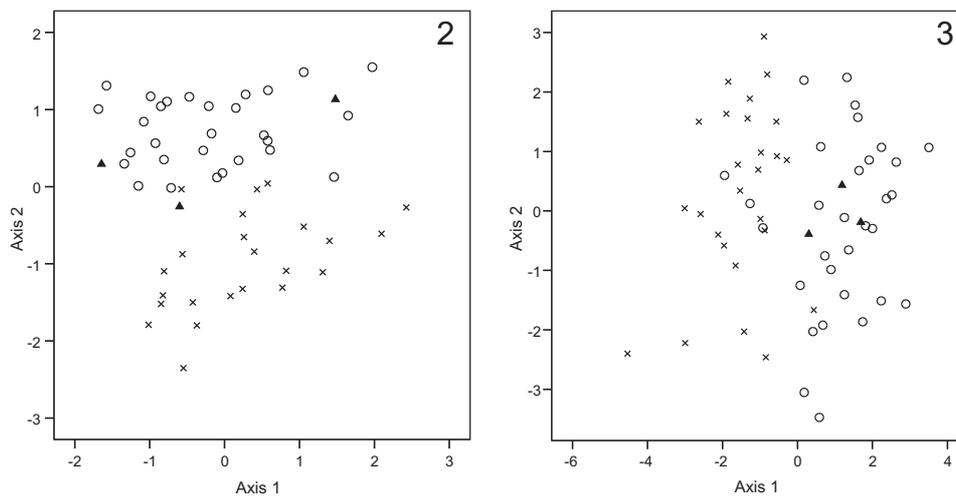


Fig. 1. Male (a) and female (b) measured characters. Abbreviation: NS = length of the central notch of saccus, PE = external process of the processus posterior PI = inner process of the processus posterior PW = width of the processus posterior, HE = external arc of the harpe, HI = internal arc of the harpe HE = external arc of the harpe, HH = height of the harpe, PIU = underside length of the inner process of the processus posterior, PLW = width of the posterior lamella, PLH = height of the posterior lamella, PCF = cover flap of the posterior lamella, FB = left branch of the furca, FH = height of the furca, FW = width of the base of furca

Table 2. Results of the univariate ANOVA of the male characters

	Tests of equality of group means				
	Wilks' λ	F	df1	df2	Sig.
NS	0.449	61.284	1	50	0.000
PE	0.884	6.550	1	50	0.014
PI	0.984	0.818	1	50	0.370
PW	0.960	2.086	1	50	0.155
PIU	0.816	11.298	1	50	0.001
HI	0.840	9.527	1	50	0.003
HE	0.929	3.836	1	50	0.056
HH	0.992	0.423	1	50	0.518
G	0.662	25.530	1	50	0.000

Males. The MDA correctly classified 96.2% of male specimens (we got the same results with the cross validation procedure), only two specimens were misclassified. *M. "phoebe" ornata* specimens were set as ungrouped cases and they all were classified as *M. telona*. The NS show the biggest correlation with the discriminative function. The univariate ANOVA tests showed significant differences except some trait: PI, PW and HH. Trait HE shows just marginal significance (Table 2).



Figs 2–3. PCA plot of the surveyed specimens: 2 = males, 3 = females. X = *Melitaea phoebe*, O = *M. telona*, ▲ = *M. "telona ornata"*

Table 3. Results of the univariate ANOVA of the female characters

	Tests of equality of group means				
	Wilks' λ	F	df1	df2	Sig.
PLW	0.980	1.121	1	55	0.294
FW	0.915	5.092	1	55	0.028
FL	0.761	17.299	1	55	0.000
FBL	0.619	33.885	1	55	0.000
PCL	0.624	33.209	1	55	0.000
PLH	0.664	27.772	1	55	0.000
P	0.541	46.684	1	55	0.000

PCA (Principal Component Analysis) plot revealed an overlap between the two species, but we also observed the occurrence of some well segregated objects. Two surveyed specimens from Guberlya, Russia fell into the *telona* “cloud” well separated from the *M. phoebe* and one in the intermediate zone (Fig. 2). The first two axes accounted for 58.36 % of the variance. In Axis 1, traits of the harpe (HI, HE, HH) have the largest importance. In Axis 2 what is more important in species separation NS G showed the highest loadings.

Females. In females, we obtained very similar results. MDA correctly classified 93.0% (86.2% with the cross validation procedure) of original grouped cases. All specimens from Southern Urals were classified as *M. telona*. The P, PCL, PLH and FL showed the largest correlation with the discriminant function. Except PLW all character showed statistically significant differences ($p < 0.05$) between the two species when tested separately (Table 3, Fig. 4).

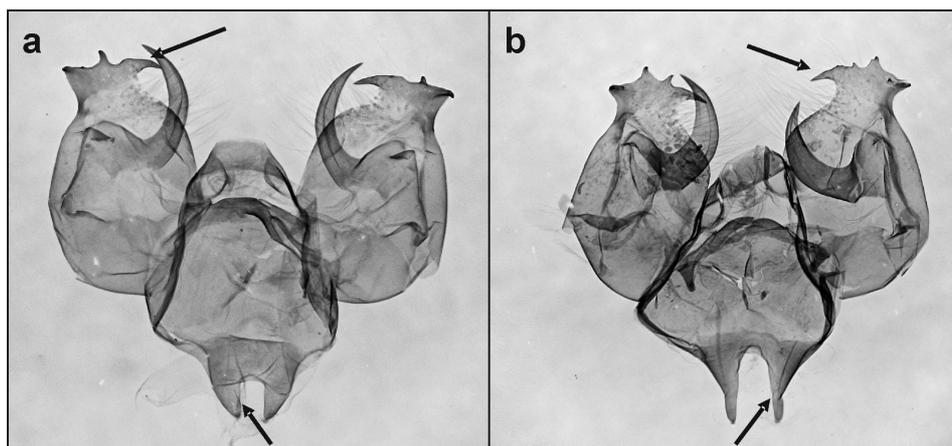


Fig. 4. Typical *Melitaea phoebe* (a) and *M. telona* (b) male genitalia. The arrows show the most discriminative characters

Using PCA method, we obtained quite similar results to males (Fig. 3) also revealing an overlapping between the two species. The animals from Magnitogorsk and Guberlya (Russia) are in the *telona* “cloud” (Fig. 3). The first two axes accumulated 73.7% of the variance. In species discrimination axis 1 had the largest importance. Traits P, PCL, PLH, FL, and FBL revealed the largest loadings in this axis.

DISCUSSION

In complete agreement with the distinctive characters between *M. phoebe* and *M. telona* reviewed in the Introduction, we found significant differences in univariate and multivariate level in the genital structures between the two species in both sexes. Using MDA method we could classify correctly most of the specimens and the low Wilks' λ also demonstrate that these two species are mostly identifiable based on some genitalia traits. The PCA plots are also demonstrated separation in the case of most of the specimens. However, PCA does not always give satisfactory results when it is applied in cases when two species show overlap (MUTANEN *et al.* 2006).

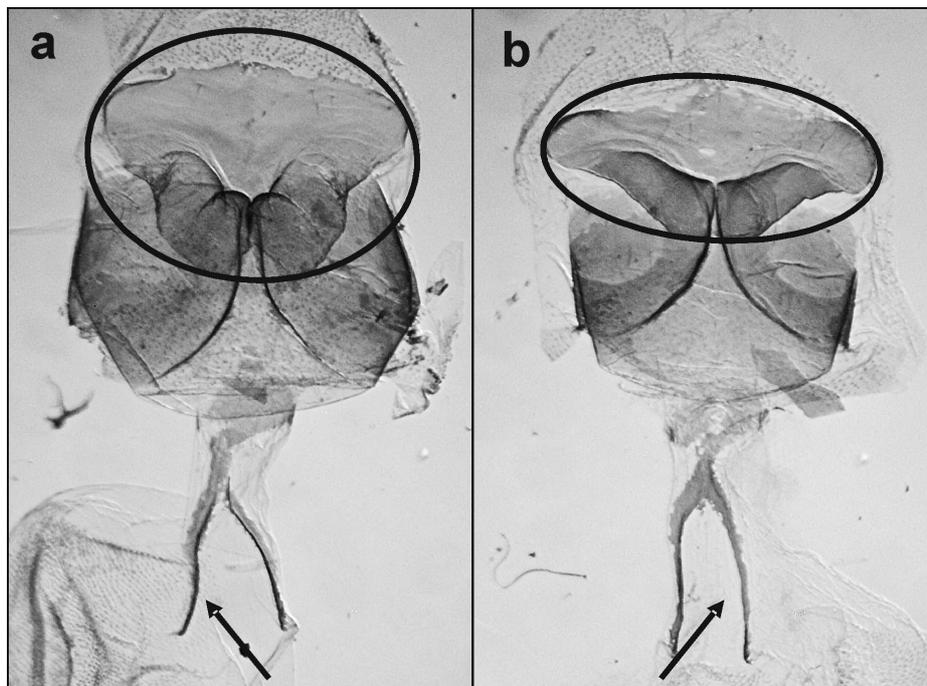


Fig. 5. Typical *Melitaea phoebe* (a) and *M. telona* (b) female genitalia. The arrows and ovals indicate the differences between the species

In males the depth of the central notch of the saccus proved to be the most important difference. Except for two characters all show significant differences, but if we separately consider these differences we found a major overlap. In general we observed that in *M. telona* we can see a more notched saccus, and more symmetric shape of processus posterior (in same side) because the inner process of the processus posterior is shorter than *M. phoebe* (Fig. 4). These two characters are essentially the same as the distinctive characters in the original description of *M. "phoebe" kovacsi* (VARGA 1967).

In case of the females we could measure only few characters. Although the width of the posterior lamella did not show a significant difference only in its height, its shape proved to be different significantly. *Melitaea phoebe* has a more rounded shape of lamella, thus its shape is closer to a circle. Oppositely, *M. telona* shows a more extended elliptic shape of the lamella. These are the most important differences between the females. The furca is usually shorter in *M. phoebe* (Fig. 5).

Surprisingly, the specimens from Magnitogorsk and Guberlya, South Ural (Russia) seem to belong to *M. telona* based on external and genital traits, too. Possibly they can be classified as an own subspecies (*M. telona ornata* CHRISTOPH, 1893, comb. n.), because of the conspicuous external colouration (dichroism) and also due to its widely separated occurrence from the nominotypical and other eastern Mediterranean forms of *M. telona*. More material has to be studied in order to clarify this enigmatic question, but these new findings draw our attention to the fact that the species is distributed much more to the East.

Despite of our results which demonstrate significant differences between the surveyed species, there is some overlap in both sexes. This figure might be the consequence of a very recent wave of diversification in this species group (LENEVEU *et al.* 2009). We assume that the slightly different genital structures cannot exclude the mating between these sibling species. Since the allozyme surveys have not shown any mark of hybridisation between *M. phoebe* and *M. telona kovacsi*, these results can be interpreted by two different ways (or a combination of both).

Either there are some prae-mating barriers e.g. the different habitat and food plant preferences combined with different behavioural or chemical (pheromone) signals. The different shape of the tip of antennae may be a signal of such differences. The other possibility is the lower fertility or survival chance of hybrids. The solution of this question might be of considerable significance in nature conservation because *M. telona kovacsi* is protected in Hungary.

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