Relationships within the *Melitaea phoebe* species group (Lepidoptera: Nymphalidae): new insights from molecular and morphometric information

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Abstract
The genus *Melitaea* consists of about 80 species, divided into 10 species groups, which are all restricted to the Palaearctic region. The *Melitaea phoebe* group was defined by Higgins based on morphological characters such as wing pattern and genital structures. According to his interpretation, the *M. phoebe* group included seven species: *M. phoebe*, *M. sibina*, *M. scotosia*, *M. aetherie*, *M. collina*, *M. consulis* and *M. turkmanica*. The taxonomy of the *phoebe* species group has been poorly resolved and recent results on the species composition within the group suggest the need for a re-evaluation. In this study molecular sequences (5985 bp) including one mitochondrial (COI) and up to six nuclear (CAD, EF-1α , GAPDH, MDH, RpS5 and wingless) gene regions from 38 specimens of the *Melitaea phoebe* species group *sensu* Higgins and some closely related taxa from the Palaearctic region were analysed. The possible evolution of the processus posterior of the male genitalia was also reconstructed based on a shape mapping technique. The analysis of the combined data shows a very clear pattern and almost all relationships are highly supported. Based on the combined Bayesian tree and the shape of the processus posterior of the male genitalia four main groups are recognised: (1) *collina* group, (2) *arduinna* group, (3) *aetherie* group and (4) *phoebe* group. The status of *M. ornata*, *M. zagrosi* and *M. scotosia* as species is confirmed, and the results also indicate that *M. telona* (*s. str.*) from Israel is a separate species.

Introduction
Recent phylogenetic work on Lepidoptera has deepened our understanding of their evolutionary history (Kristensen *et al*., 2007; Mutanen *et al*., 2010; Wahlberg *et al*., 2013). Several publications have investigated the position of Papilionoidea within the huge Ditrysian radiation and clarified the phylogenetic relationships of the constituent families (e.g. Regier *et al*., 2009; Heikkilä *et al*., 2012). A number of studies have given considerable attention to the phylogenetic relationships within the highly diverse family Nymphalidae (Wahlberg *et al*.,

2003; Freitas & Brown, 2004; Wahlberg *et al.*, 2009), including its subfamilies (e.g. Peña *et al.*, 2006; Simonsen, 2006; Peña & Wahlberg, 2008). Among them, several papers were devoted to different aspects of phylogeny and population biology of butterflies in the tribe Melitaeini. These have long been model organisms in population biology (Ehrlich & Hanski, 2004), giving insights into e.g. how populations survive in fragmented landscapes (Hanski, 1999), how host plant specialization evolves (Singer *et al.*, 1993; Wahlberg, 2001) and how host-parasitoid interactions affect insect populations (van Nouhuys & Hanski, 2002). Within the tribe, the genus *Melitaea* has been shown to be a large monophyletic unit (Wahlberg & Zimmermann, 2000; Leneveu *et al.*, 2009), consisting of about 80 species. The genus is restricted to the Palaearctic region, and thus this rather diverse group of species is one of the typical butterfly genera of this biogeographic region.

The first significant review of this relatively species-rich genus was published by Higgins (1941) with several comments on its taxonomy. He defined the *Melitaea phoebe* group based on morphological characters such as wing pattern as well as male and female genitalia. According to his interpretation, the *M. phoebe* group consists of seven species. The best-known species is *Melitaea phoebe* ([Denis & Schiffermüller]) (TL: Vienna, Austria) with a wide trans-Palaearctic distribution from North Africa to the Far East with several described subspecies. *Melitaea sibina* Alphéraky (TL: Kuldja, China), from the high mountain regions of Central Asia, shows a very characteristic vivid reddish colouration and reduced dark pattern on the upper side of the wings. The largest species, *Melitaea scotosia* Butler (TL: Japan), is known from the Far East: Amur and Ussuri region, Manchuria, Korea, north-eastern China and Japan. The strongly sexually dimorphic *Melitaea aetherie* (Hübner) (TL: Spain) is a western Mediterranean species distributed only in North Africa, the southern part of the Iberian Peninsula and Sicily. Finally, three smaller species are confined to a relatively restricted area in the Middle East: *M. turkmanica* Higgins (TL: Ashkhabad, Turkmenia), *M. consulis* Wiltshire (TL: Shiraz, Iran) and *M. collina* Lederer (TL: Antioch, Syria). At the time of Higgins' (1941) review, two species, *Melitaea sarvistana* Wiltshire and *M. tangigharuensis* Eckweiler, also with restricted ranges, were unknown.

Recently several new observations have brought into question the taxonomy of the *phoebe* group, especially concerning the close relatives of *M. phoebe*. Two research groups independently realized that there was an unrecognised species in Europe under the name of *M. phoebe* (Russell *et al.*, 2005; Varga *et al.*, 2005). The separation of this cryptic species was based on larval morphology from the fourth instar onwards. *M. phoebe* larvae have a black head capsule while the larvae of this recently recognised Ponto-Mediterranean species have a brick-red head capsule (Russell *et al.*, 2007). Based on these observations, the name *M. telona* Fruhstorfer (TL: Jerusalem, Israel) was taken into use for this species.

In a recent molecular study, *Melitaea* was shown to comprise two main clades that correspond to *Melitaea s. str.* and *Didymaeformia*, and that the *M. phoebe* group forms a monophyletic clade within the subgenus *Didymaeformia* (Leneveu *et al.*, 2009). Although that study provided important results regarding the systematics of the genus, the members of the *phoebe* species group were poorly represented, and the need for a detailed examination of this group remained. One of the important results was the corroboration of the species rank of *M. telona* and the suggestion that the taxon *punica* (TL: Lambessa, Algeria) may represent a separate species from both *M. telona* and *M. phoebe*. In contrast to other studies, Leneveu *et al.* (2009) reported that *M. sibina* and *M. scotosia* did not differ genetically from *M. phoebe*.

Another recent study on the morphometry of genitalia in males and females of the *phoebe* species group provided additional information (Tóth & Varga, 2011). An analysis of a large number of specimens (568) from the Palaearctic showed that *Melitaea telona* is not restricted to the Ponto-Mediterranean region since several new localities were found, including the Orenburg region (Russia), northern Iran and the easternmost border of Kazakhstan. Since the

name *ornata* described by Christoph in 1893 (TL: South-Urals, Russia) is older than the name *telona*, the authors began to use *M. ornata* as the valid name for this species following the rule of priority. Most of the results of Tóth and Varga (2011) are in concordance with the results of Leneveu *et al*. (2009), except that *Melitaea scotosia* proved to be a distinct species based not only on the morphology of genitalia but also the wing pattern. Also, based on the significant difference in male and female genitalia and the distinct wing pattern, a new species was described from Iran as *Melitaea zagrosi* Tóth & Varga, 2011.

Landmark based genitalia morphometry proved to be a useful tool for species delimitation in the case of *Melitaea phoebe* group, but the phylogenetic relationships among the species remained unclear. Therefore, we attempted to clarify the phylogenetic relationships of the *M. phoebe* group by using DNA sequence data from seven gene regions from multiple individuals of almost all species belonging to the group. Through synthesis of results from previous research and by combining molecular and morphometric methods, we provide the most comprehensive picture of the *Melitaea phoebe* group to date.

Material and Methods

Samples
We sampled 38 specimens of the *Melitaea phoebe* species group *sensu* Higgins (except *M. turkmanica*, samples of which were unavailable) and some closely related taxa from the Palaearctic region (Table 1, Fig. 1). The identification was based on wing characters as well as on genitalia in certain cases (Tóth & Varga, 2011).

DNA studies
For samples processed in Debrecen, Hungary, DNA was extracted by homogenising the head 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 3 h. The DNA was pelleted by centrifugation at 14,000 rpm for 10 minutes 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 re-dissolved in 50 µl elution buffer (10 mM Tris HCl, pH 8.0 and 0.5 mM EDTA, pH 9.0). For samples processed in Turku, Finland, DNA was extracted following protocols in e.g. Matos-Maraví *et al*. (2013). DNA aliquots were stored at 4˚C.

The cytochrome c oxidase subunit I gene (COI), which is commonly used in barcoding animal life (Hebert et al. 2003; Wiemers and Fiedler 2007), offers an adequate tool to obtain insight into the phylogeny of taxa at species level. We therefore sequenced this section of the mitochondrial genome together with the nuclear elongation factor 1α (EF-1α), CAD, GAPDH, MDH, RpS5 and wingless. These genes were amplified by specific primers modified at their 5'-end to include the universal sequencing primer T7promoter (Wahlberg and Wheat 2008). Amplification from 1 µl of DNA extracts was carried out in 25 µl final reaction volumes containing 10x PCR buffer, 2 mM $MgCl_2$, 0.2 mM dNTPs, 0.02 units/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, 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, Seoul). Sequences were edited and revised manually by Chromas Lite v. 2.01, aligned by MEGA v. 5.2 (Tamura *et al.*, 2011). DNA sequences and voucher data were stored in and dataset for phylogenetic analyses, as well as FASTA files for submission to GenBank were produced by the software VoSeq (Peña & Malm, 2012) (see Table 1 for accession numbers).

Bayesian analyses were conducted on single-gene, nuclear genes only and all-gene datasets by using MrBayes 3.2.1 (Ronquist *et al.*, 2012). The all-genes dataset was partitioned by gene. Various possible models of molecular evolution were sampled for each gene (both single and combined data) during the analysis by taking advantage of the model-jumping feature of MrBayes v3.2 through the command "lset applyto = (all) nucmodel=4by4 nst=mixed rates=gamma covarion=no;". Two independent MCMC runs each with four simultaneous chains (one cold and three heated) for each analysis were run for 10 million generations and the sampling of trees and parameters was set to every 1000 generations. Convergence of the two runs was determined by the stationary distribution plot of the log likelihood values against number of generations and confirmed by the average standard deviation of split frequencies, which were lower than 0.05 in all cases. We discarded the first 2,500,000 generations as burn-in and trees were summarized under the 50 percent majority rule method.

Morphometry

To reconstruct the possible evolution of the processus posterior (male genitalia) we applied a shape mapping technique on the phylogenetic tree resulting from the all-gene analysis (Klingenberg & Gidaszewski, 2010). Fixed and sliding landmarks were used to define the shape of the processus posterior (Fig. 2) for all the surveyed species. Since the applied method uses only one shape per taxon, we chose the most typical if it was possible. Unfortunately, we had only female specimens from certain taxa *(M. collina*, *M. consulis*, *M. avinovi*, *M. sarvistana)*. In these cases we used figures from previous publications (Higgins, 1941; Eckweiler, 2008) for obtaining landmark data. These figures were made by using camera lucida which allows great precision. For fitting landmark data we used a generalized Procrustes analysis. The degree of phylogenetic signal present in shape data was estimated based on a Brownian motion model of evolution using the 'geomorph' (Adams & Otarola-Castillo, 2013) package in the R computing environment (R Core Team, 2013) where we used the consensus topology from the all-genes Bayesian analysis. The phylogenetic signal is estimated as the sum of squared changes in shape along all branches of the phylogenetic tree (Klingenberg & Gidaszewski, 2010). A permutation test for phylogenetic signal was applied using 10,000 iterations where ancestral states were estimated in each iteration.

Results

The analysis of the all-genes dataset shows a very clear pattern, with almost all relationships highly supported (Fig. 4). The clade including *Melitaea consulis* and *M. collina* is sister to the rest of the *M. phoebe* species group, followed by *M. arduinna* and *M. avinovi*. Next up is a clade without statistical support (posterior probability: 0.74) comprising *M. sarvistana+M. tangigharuensis* and *M. aetherie*. It is not entirely clear whether these are sisters to each other, or whether one or the other is sister to the *M. phoebe* clade. Within the large *M. phoebe* clade, most species are genetically distinct from each other, and thus *a priori* species delimitation is corroborated. The only taxon that is not well differentiated is *M. sibina*, which is genetically identical to *M. phoebe*. Surprisingly, the *Melitaea telona* (*s. str.*) specimens from Israel are well separated from *M. ornata*. The recently described *M. zagrosi* also shows clear separation from *M. ornata* and a close relation to *M. scotosia*, which is well separated from *M. phoebe*.

The single gene analyses are not in conflict with the combined analysis (supplementary: Fig. 1). All six nuclear genes, however, show very little variation among the sampled individuals

and thus there is very low resolution of the relationships of the species in the single gene analyses. Nevertheless, combining the nuclear genes does give a phylogenetic hypothesis which is very similar to the COI topology as well as the all-genes analysis (supplementary: Fig. 1). This suggests that all of the sequenced gene regions share a common evolutionary history and the revealed relationships are for the most part robust.

The shape of processus posterior showed significant phylogenetic signal (0.62, p<0.001) based on the all-genes Bayesian tree. Thus the shape of the processus posterior of the male genitalia as well as the phylogenetic relationships of the sampled species (Fig. 5) suggest that the *phoebe* group has four well-defined groups within it, of which the first three consist of species with rather restricted ranges, while the much more diverse fourth one, the *phoebe* group *s. str.* is formed from both geographically restricted and widespread species.

The main groups are:

(1) *collina* group: *M. collina* and *M. consulis*, with the inner process missing on the processus posterior;

(2) *arduinna* group: *M. arduinna* and *M. avinovi*, with the middle and inner process missing while the outer process is well developed;

(3) *aetherie* group: *M. aetherie, M. tangigharuensis* and *M. sarvistana* with the middle process missing;

and (4) *phoebe* group: *M. phoebe*, *M. punica*, *M. telona*, *M. zagrosi*, *M. scotosia* and *M. ornata*, with all the processi well developed.

Discussion

In this study the phylogenetic relationships among the members of *M. phoebe* species group have been determined based on seven gene regions. According to our results, the monophyly of Higgins' (1941) *phoebe* group is supported. Within the clade there are four well-defined species groups based on both molecular data and the morphology of the male genitalia (processi posteriores).

The *collina* group (*M. collina*: Asia Minor, Syria; *M. consulis*: Iran, Shiraz region) was already recognised by Leneveu *et al*. (2009). Species in this group show very uniform morphology of the processus posterior (see: Fig 5). Although *M. turkmanica* (Armenia, Iran and Turkmenistan) was not included in this analysis, it is possible to place this species in the *collina* group based on the morphology of the male genitalia. The biology of these species is poorly known. They are found in a relatively small area in the Middle East (Tshikolovets, 2011) in hot and dry climatic conditions.

M. arduinna and *M. avinovi* belong to the *arduinna* group based on the very uniform wing pattern, the peculiar shape of the processus posterior and molecular data. *M. arduinna* is relatively widely distributed in the south-eastern part of western Palaearctic region (south-eastern Europe, north-western Kazakhstan, Caucasus, Transcaucasia, Asia Minor, north-eastern Iraq and Iran, mountains of Central Asia) under hot and dry climate conditions, while *M. avinovi* has a restricted distribution as it is only known from the Pamir Mountains (Gorbunov & Kosterin, 2007; Higgins, 1950).

Although *M. aetherie* has a very different wing pattern from the other two members of the *aetherie* group, some similarities are present in the shape of the processus posterior, namely the reduction of the middle process. All the species of this group have a restricted distribution. *M. sarvistana* and *M. tangigharuensis* (Eckweiler, 2008) are only known from Iran, while *M. aetherie* is distributed in some parts of North Africa, southern part of the Iberian Peninsula and in Sicily under hot and dry climate conditions (Tolman & Lewington, 2008; Tshikolovets, 2011).

In this analysis, the largest clade is comprised by the closest relatives of *M. phoebe* that could be defined as the *phoebe* group *s. str.* The shape of the processus posterior is uniform in that

all the processi are developed; however, the length, the symmetry and the directions of these processi show species level differences (Fig. 3) (Tóth & Varga, 2011).

In the light of the phylogenetic analyses, we discuss three important taxonomic consequences. First, the species status of the recently described *Melitaea zagrosi* is corroborated. Hopefully our knowledge of this species will increase in the coming years. The species was known only from Iran, but recently it has also been found in Azerbaijan (G. Kuznetsov pers. com.).

Second, contrary to the previous phylogenetic study of *Melitaea* (Leneveu *et al.*, 2009), *Melitaea scotosia* represents a separate species. This finding is in agreement with the wing pattern, the structure of male and female genitalia (Tóth & Varga 2011) and is also supported by the very distinct morphology of caterpillars and pupae (Yasuhiro Nakamura pers. com.). The contrasting result in Leneveu *et al*. (2009) was actually caused by the misidentification of the sampled specimen (sample NW27-11), which on re-examination is in fact *M. phoebe*. The correct taxonomic status of *M. scotosia* is an important issue since this species has a high conservation importance, especially in Japan, where its rate of decline is extremely high (98%) (Nakamura, 2011).

Finally, surprisingly, *M. telona* (*s. str.*) from Israel and *M. ornata* proved to be different taxa. Our previous surveys have already revealed differences in the genital structures of both sexes (Tóth *et al.,* 2013; Tóth & Varga, 2011); however, *M. telona* seems to be morphometrically very close to *Melitaea ornata*. Moreover, previous studies did not find any differences between these two species in larval morphology and wing pattern. Molecular data suggest that the two taxa are genetically distinct from each other and that they are sisters although this clade has no statistical support at this point (posterior probability: 0.6), meaning that one or the other might be more closely related to the *M. scotosia+M. zagrosi* clade. Based on these results we can conclude that *M. telona* is not a subspecies of *M. ornata* but a species in its own right. Previous studies showed that *M. sibina* is not a distinct species from *M. phoebe*. The results of DNA analysis (Leneveu *et al.*, 2009) and the genitalia morphometry (Tóth & Varga, 2011) agree that the two cannot be separated. The present analysis also supports the previous conclusions. Originally, the separation of *M. sibina* from *M. phoebe* was based on the characteristic wing pattern on the upper side of the wing, which is probably highly influenced by environmental factors as it has been shown in other butterflies (Kingsolver & Wiernasz, 1991; Cesaroni *et al.*, 1994). It is noteworthy that the underside of the hind wing, which has proved to be a reliable character in most cases, is not different from *M. phoebe*.

Most of the species in the *phoebe* group are adapted to hot and dry climate conditions like the members of the other three closely related species groups, with two exceptions. One is *Melitaea scotosia* with a restricted distribution in the most north-eastern part of China, south-eastern part of Russia, the Korean Peninsula and Japan under humid climate conditions. However, *M. phoebe* can be found in many different kinds of habitats, but it appears to show a preference for a humid climate. This species is widely distributed at medium altitudes of West Palaearctic high mountains (e.g. Alps, Carpathians, Balkans, Caucasus) but is usually rare under hot and dry climate conditions where other members of this group (*M. ornata, M. telona* or *M. zagrosi*) are often more abundant than *M. phoebe*. However, we have to note, this pattern does not hold in the case of the Iberian Peninsula and Iran, where dry adapted, probably well-differentiated forms of *M. phoebe* occur.

In this study, the DNA based phylogenetic hypothesis for species delimitation in the *M. phoebe*-group agrees generally quite well with the previous morphometric studies (Tóth & Varga, 2011), although some morphometrically close species seem to be more distant relatives of each other based on the phylogenetic reconstruction than was expected. It has also become clear that the head colouration of the larvae in the close relatives of *M. phoebe* cannot be considered as a species-specific character since *M. ornata*, *M. telona* and *M. zagrosi* (G. Kuznetsov pers. com.) all have red head capsules. On the other hand, the black head capsule

does not necessarily indicate a conspecific relationship, since *M. scotosia* larvae also have black head capsule. A black head capsule is known in *M. aetherie* and *M. arduinna*, suggesting that this may be the ancestral state. However, species in other groups of the *Didymaeformia* clade have orange coloured head capsules, which may or may not be homologous to the brick red colour found in the *M. phoebe*-group. Therefore, the specific colouration of the head capsule cannot be considered as either a plesiomorphic or apomorphic trait, but as a possible parallelism, having evolved on multiple occasions from an originally polymorphic state with repeated loss of "black" or "red" alleles.

We have shown here that a multigene approach can resolve the phylogenetic relationships of multiple previously cryptic species and in doing so lead to clarification of the taxonomic composition of such a species group. We made a large step forward in our understanding of the *Melitea phoebe* group, but a number of questions remains. In the future it would be interesting to add data from e.g. other populations of *M. phoebe* that appear well differentiated and asses them both using molecular data and morphometrics.

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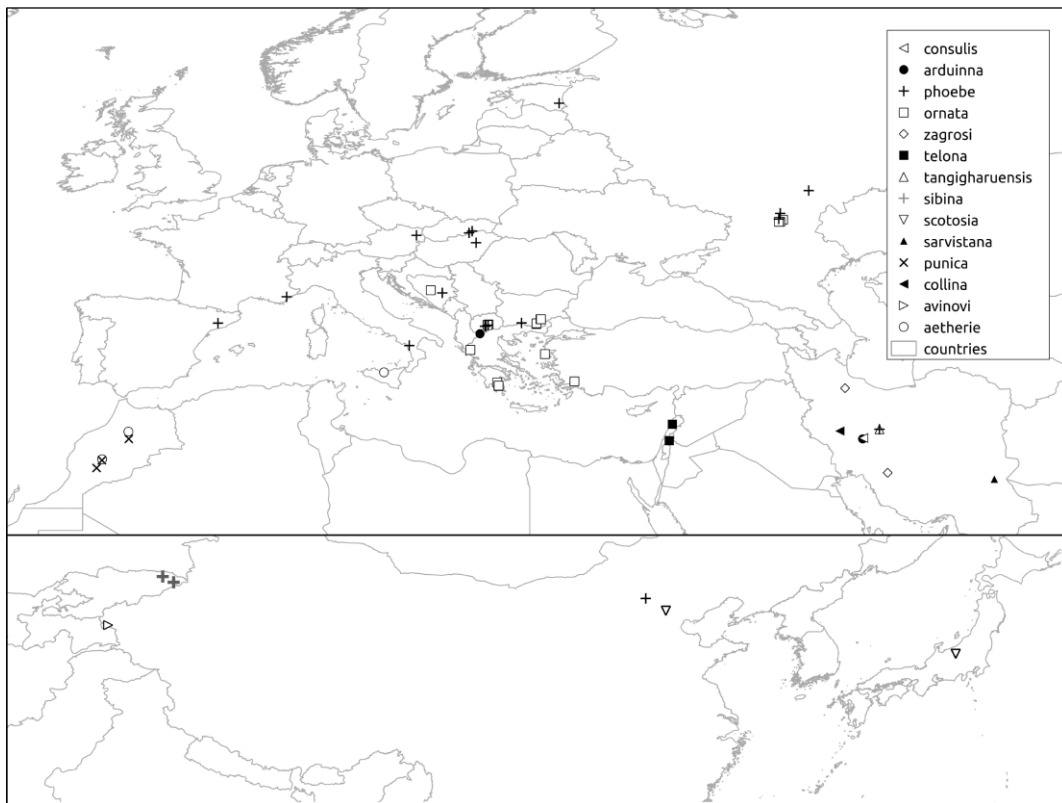
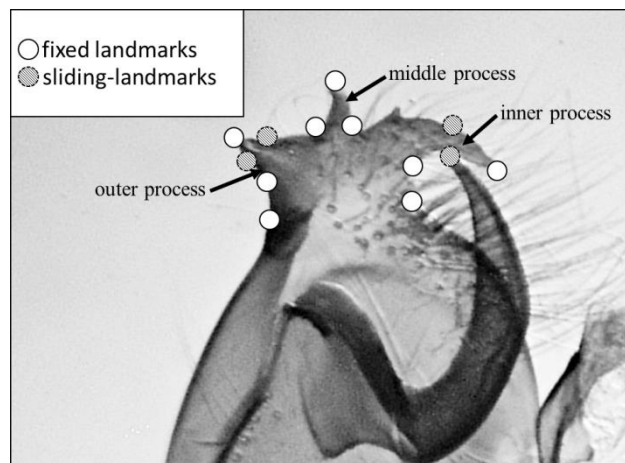Figures

Fig. 1. Sample locations

Fig. 2. Landmarks on the processus posterior (*Melitaea phoebe* male genitalia)
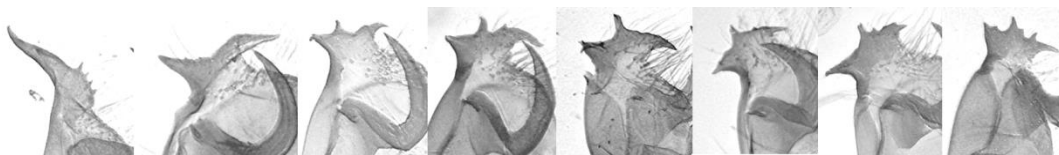

Fig. 3. Processus posterior of some *Melitaea* species. From left to the right: *M. arduinna*, *M. aetherie*, *M. punica*, *M. phoebe*, *M. telona*, *M. ornata*, *M. scotosia*, *M. zagrosi*.
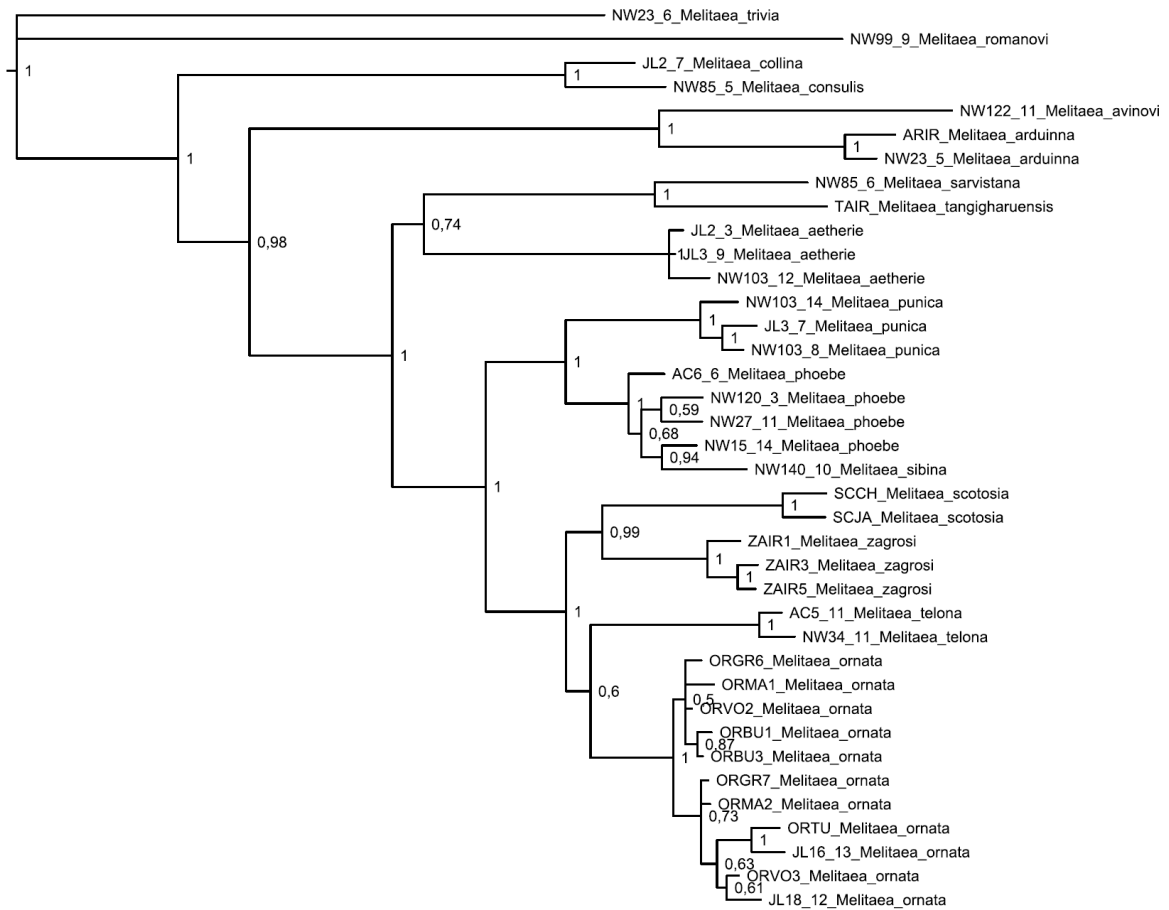
Fig. 4. Consensus phylogeny from the Bayesian inference analysis based on the combined dataset of seven region (COI, EF-1α, CAD, GAPDH, MDH, RpS5 and wingless).
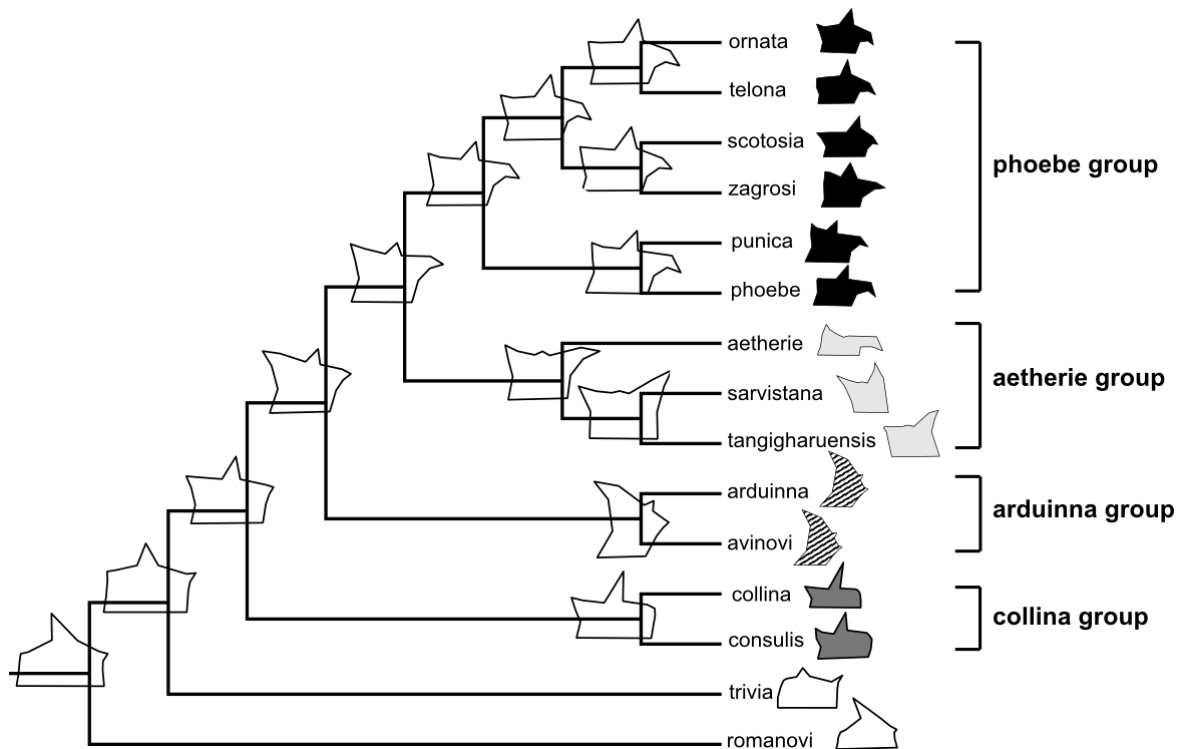

Fig. 5. The combined Bayesian tree and the shape of the processus posterior of the male genitalia.