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Abstract

The pine processionary moth, *Thaumetopoea pityocampa*, is an iconic insect in the Mediterranean culture because of its economic and medical importance and the unique traits of the life history, namely the winter feeding and the construction of conspicuous silk tents by the larvae. Its taxonomic status, however, is unclear because the type material is not available and there is confusion among the several species and subspecies described in the last centuries. In the present study, a metadata analysis of morphological and molecular data of the species in the *T. pityocampa* complex were considered for species delimitation, using more than 400 individuals from more than 120 geographic locations. A reconstruction of the origin of the material used for the first formal description has allowed to identify the type locality and to design a neotype. In addition, as Denis and Schiffermüller were referring to the work of Réaumur for details about the species, the description provided by Réaumur was reviewed. The results indicate that the barcode region of mitochondrial DNA is a reliable trait to separate species in most cases whereas morphological traits are not. Hybridization among taxa makes it difficult to delimit species in contact zones when mating barriers are not present. In other cases, such as the populations of Crete Island, the lack of gene flow with the mainland population may support species delimitation even when morphological traits are not conclusive. Thus, the new species *Thaumetopoea cretensis* is described here based on the evidence obtained from a previous study. Species delimitation based on both mitochondrial and nuclear markers allowed maintenance of three species of the complex (*T. cretensis*, *T. pityocampa*, and *T. wilkinsoni*) while more data are needed to determine the status of two recently described species: *T. hellenica* and *T. mediterranea*.

Keywords

1.   Introduction

The pine processionary moth, *Thaumetopoea pityocampa* (Denis and Schiffermüller, 1775), is an iconic insect in the Mediterranean culture. Known as *pityocampe* (πιτυοκάμπη) in ancient Greek and as *pityocampa* in Latin, which means the pine (*pityo*) caterpillar (*campa*), has fascinated naturalists for its gregarious behaviour (e.g., Fabre 1899). This is probably one of the reasons why the lepidopterologist Jacob Hübner created the genus *Thaumetopoea* (Hübner, 1820) (literally, to make wonderful things) to allocate *pityocampa* and other related species (Hübner 1820). Besides the unique behaviour, the species attracted medical doctors since the times of Dioscorides (40–90 AD) because of the urtication caused by the larvae on human skin and because larval extracts have been used to poison people since the ancient times (Theophrastus of Eresos, 371–287 BC) (Roques and Battisti 2015).

A total evidence phylogeny of the genus *Thaumetopoea* suggested that the species associated with conifer hosts stemmed from an ancestor associated with broad-leaved trees in the eastern Mediterranean area (Basso et al. 2017a). *Thaumetopoea pityocampa* is the best studied species in the clade of conifer-feeding *Thaumetopoea*. According to Salvato et al. (2002), two species were identified based on molecular markers, i.e., *T. pityocampa* in the west and *T. wilkinsoni* Tams, 1925 in the east Mediterranean. The latter was originally described from Cyprus Island, and the divergence of the two species was estimated to be at least 5 million years ago (Simonato et al. 2007). Kerdelhué et al. (2009) explored the role of the Mediterranean Sea desiccation in the Miocene-Pliocene transition and of the Quaternary periodic glaciations on the genetic structure of the species, confirming the role of the Messinian Salinity Crisis in shaping the origin of the species and identifying the role of glacial refugia. In addition, the same authors identified a new clade called ENA (populations of Eastern-North Africa) being genetically different from *T. pityocampa* and *T. wilkinsoni* based on mitochondrial data. The occurrence of the ENA clade was further corroborated by El Mokhefi et al. (2016), based on a clear separation from the *pityocampa* populations of NW Africa found in mitochondrial but not in nuclear markers. Recently, the potential hybridization between *T. pityocampa* and *T. wilkinsoni* was documented both through laboratory crossing (Petrucco-Toffolo et al. 2018) and in the field, as a natural hybrid zone was identified in western Turkey (İpekdal et al. 2020). Interestingly, the viable F1 and F2 hybrids *pityocampa* x *wilkinsoni* obtained under controlled conditions were intermediate in all the morphological and genetic traits considered (Petrucco-Toffolo et al. 2018).

A large variation in the morphological traits of *T. pityocampa* was already pointed out in the first revision of the genus done by Agenjo (1941), where several synonymous varieties were identified based mainly on wing colour (Table 1). The same author casted doubts about the possibility to morphologically distinguish *pityocampa* from *wilkinsoni*, although the second was retained because of its distribution and life history traits described by Tams (1925) and Wilkinson (1926). In the revision by Kiriakoff (1970), the two species were considered valid. Moreover, two of the *pityocampa* varieties considered by Agenjo (1941) were retained, namely *T. p. orana* (Staudinger, 1901) and *T. p. ceballosi* (Agenjo, 1941), likely because of their restricted geographic distribution, in North Africa (Algeria and Morocco) and in Turkey (Anatolia), respectively. Later, de Freina and Witt (1987) and Schintlmeister (2013) considered *pityocampa* and *wilkinsoni* as separate species and synonymized most varieties described by Agenjo as well as *T. galaica* Palanca Soler, Castan Lanaspa and Calle Pascual, 1982, a further described species in NW Spain (Palanca Soler et al. 1982), with *pityocampa*. Only *T. pityocampa orana* was retained at subspecies level. In addition, de Freina and Witt (1987) proposed to assign the species to the genus *Traumatocampa* Wallengren, 1871. However, the phylogenies by Simonato et al. (2013) and Basso et al. (2017a) suggested that all the species in the group should be included in the genus *Thaumetopoea*. In the same work, the individuals included in the ENA clade were potentially identified as different species based on molecular data, confirming the previous results of Kerdelhué et al. (2009). A species named *T. mediterranea* Trematerra and Scalercio, 2017 was later described by Trematerra et al. (2017) based on the material from Pantelleria Island, which falls within the range of the ENA clade. In the same paper, another species, *T. hellenica* Trematerra and Scalercio, 2017, was described from continental Greece. Both species were described using morphological and molecular traits.

The aim of this paper is to reconsider all available morphological and molecular traits of the species in the *T. pityocampa* complex, including populations for which both types of data are available, even if they are not from the same individuals. The original material on which *T. pityocampa* was described is not available and a thorough analysis of the old literature has allowed us to clearly identify the type locality of the species and to propose a neotype for the nominal taxon. The expectation is to delimit the species in the group and to provide traits for their identification. A clear definition of taxa within *T. pityocampa* species complex is important because of the consequences it could have on the study of evolution of the genus and on the management of these important pests that threaten trees, human and animal health.

2.   Materials and Methods

2.1.   Taxa description

The taxa included in the *T. pityocampa* complex are presented in Table 1. Four are considered as valid species (in chronological order of their description, *T. pityocampa*, *T. wilkinsoni*, *T. hellenica*, and *T. mediterranea*), one as a subspecies (*T. pityocampa orana*), and 15 are synonyms of *T. pityocampa*. Types of all species, subspecies and

Table 1. List of taxa described in the *Thaumetopoea pityocampa* complex and their current status based on Schintlmeister (2013) and further descriptions.

| Species	Subspecies	Synonyms	Author	Year	Terra
pityocampa			Denis & Schiffermüller	1775	Italy: South Tyrol
pityocampa		*maritima*	Herrich-Schäffer	1843	Portugal
pityocampa		*nigra*	Bang-Haas	1910	Switzerland: Tessin
pityocampa		*insignipennis*	Strand	1913	France: Cannes
pityocampa		*obscura*	Vorbrodt & Muller-Rutz	1914	Switzerland: Tessin
pityocampa		*renegata*	Dannehl	1925	Italy: South Tyrol
pityocampa		*convergens*	Dannehl	1925	Italy: South Tyrol
pityocampa		*bicolor*	Reisser	1928	Spain: Granada
pityocampa		*illineata*	Schawerda	1932	France: Corsica
pityocampa		*nigrofasciata*	Nitsche	1933	Croatia: Sabbioncello Orevic
pityocampa		*pujoli*	Agenjo	1941	Spain: Madrid, Barcelona
pityocampa		*cancioi*	Agenjo	1941	Spain: Segovia
pityocampa		*clara*	Agenjo	1941	Spain: Murcia, Madrid
pityocampa		*vareai*	Agenjo	1941	Spain: Malaga
pityocampa		*ceballosi*	Agenjo	1941	Turkey: Aydın
pityocampa		*galaica*	Palanca Soler et al.	1982	Spain: Vigo Pontevedra
pityocampa	orana		Staudinger	1901	Algeria: Oran
wilkinsoni			Tams	1925	Cyprus
hellenica			Trematerra & Scalercio	2017	Greece: Thessaly, Athens
mediterranea			Trematerra & Scalercio	2017	Italy: Pantelleria

synonyms were examined, either from museum collections (listed in Table S1a) or from publications, spanning over 120 locations in Europe and the Mediterranean Basin (Fig. 1). Whenever possible, populations close to the type locality of taxa were used for both morphological and molecular analyses.

The type material was available for all taxa except the nominal one, which was probably lost in the fire of the Vienna Hofburg on 31 October 1848 during the Vienna Uprising or October Revolution (personal communication of Sabine Gaal-Haszler, curator of the Naturhistorisches Museum, Vienna, Austria). In this regard, Denis and Schiffermüller (1776) merely reported the species from Dioscorides (40–90 AD) as *pityocampa* and from Réaumur (1734) as *la chenille du pin* (translation of *pityocampa*) in France, considering the latter as reliable based on the careful description of the larval, pupal, and adult stages on material received from the Bordeaux region (see Fig. S1). Denis and Schiffermüller (1776) mentioned that they did not see any adults and were hoping to get them from the rearing of larvae provided by the naturalist Dr. Popowitsch, who received them from the baron (Freiherr) von Sperges from Tyrol, without precise indication of the locality. At that time there was a lawyer and historian named Joseph Freiherr von Sperges (1725-1791), who was active in South Tyrol (http://worldcat.org/identities/lccn-n80040576/) so the larvae were most likely collected in that area. About two hundred years earlier, Matthioli (1562) described the *bruchi de pini* (translation in Italian of *pityocampa*) as abundant in today's Trento district (Fiemme and Non valleys), formerly part of South Tyrol. Denis and Schiffermüller (1776) formally described the larvae as *Bombyx pityocampa* and added the German name *Fichtenspinner* (translated as the

spruce caterpillar, with spruce being *Picea abies*), based on the observation that the captive larvae were feeding on both Norway spruce and Scots pine (*Pinus sylvestris*). However, while it is known that under captive conditions the mature larvae may feed on spruce (Hellrigl 1995), this is not observed in the field (Battisti et al. 2015). In the world revision of Notodontidae types, Schintlmeister (2013) attributed the taxon to Denis and Schiffermüller (1776) and indicated both France and Tyrol (Austria) as *terra typica* of *T. pityocampa*, reporting that reference material was not available for any of these locations. After a thorough discussion with Alexander Schintlmeister in April 2022, it was concluded that the best solution was to define a neotype from the area where von Sperges collected the larvae, identified as today's South Tyrol in Italy. This is supported by the evidence that the moth was well documented in this area by Kitschelt (1925) and Hellrigl (1995).

2.2. Outgroup definition

To investigate the evolutionary relationships among taxa, it is crucial to select the appropriate outgroup set unambiguously outside the studied clade (Grimaldi and Engel 2005). Therefore, when considering different datasets belonging to the same specimens, it is essential to verify the phylogenetic signal associated with plesiomorphic and apomorphic characters (Sereno 2007). When molecular data are analysed, the richness of the dataset is often adequate to discern separate clades, even when they are closely related. Conversely, the use of morphological traits is challenging because they result from the expression of mutations accumulated over evolutionary
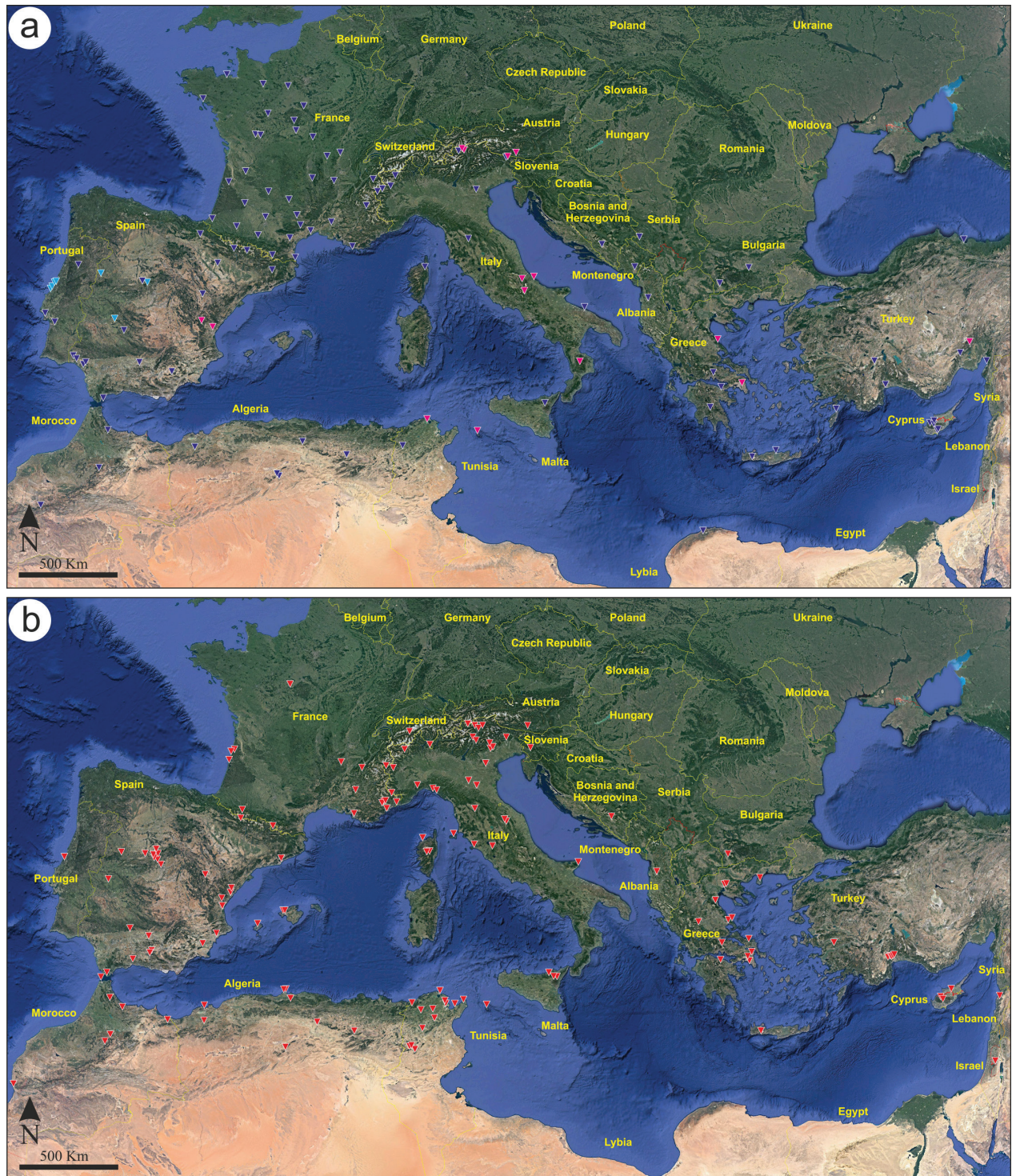
Figure 1. Sites where individuals of the *Thaumetopoea pityocampa* species complex were collected for the molecular (**a** above) and morphological (**b** below) analyses. In map «a», different pin colours indicate BOLD-records in purple, data from Burban et al. (2016) in light blue and new sequences in dark blue.

time in multiple genes (Sereno 2007). Based on previous results (Simonato et al. 2013, Basso et al. 2017a), ten species were selected as outgroups to provide a representative sample of taxonomic diversity within the genus *Thaumetopoea*. Basso et al. (2017a) characterized this genus with 165 morphological traits, although most of them were fixed within the *T. pityocampa* complex. Therefore, to avoid computational noise from excessive plesiomorphic traits widespread in the ingroup spec-

imens, the morphological matrix was reduced to the variable traits only, and some of the traits were re-coded (see File S1) in a straightforward perspective (Basso et al. 2017a). On the contrary, the reduction of the dataset, considering only the hyper-variable traits, may directly affect the conveyed phylogenetic signal and mislead the results. Preliminary analyses of the moth morphological traits revealed that the inclusion of the broadleaved-feeding species produced unstable tree topologies due to the

divergent apomorphic traits of these moths (Basso et al. 2017a). Thus, these species were removed and the trees rooted on conifer-feeding *Thaumetopoea*, such as *T. pinivora* (Treitschke, 1834) and the closely related *T. bonjeani* Powell, 1922, *T. cheela* Moore, 1883, *T. ispartaensis* Doganlar and Avci, 2001, *T. libanotica* Kiriakoff and Talhouk, 1975, *T. sedirica* (Doganlar, 2005), and *T. torosica* (Doganlar, 2005) (Simonato et al. 2013; Basso et al. 2017a). During molecular analyses, we considered all the available variability using sequences retrieved from public databases (BOLD and GenBank) and rooting the trees on broadleaved-feeding species such as *T. herculeana* (Rambur, 1837), *T. processionea* (L., 1758), and *T. solitaria* (Freyer, 1838) (Basso et al. 2017a).

2.3. Morphological data

Finally, for male and female specimens, 44 and 31 traits, respectively, showed polymorphism and were used to explore morphological differentiation (Table S1). The moth samples used in the study were collected with different methods (e.g., rearing from larvae, light/pheromone trapping) following all applicable international and local permitting requirements. There was some variation in the specimen quality and suitability for a comparative analysis of several traits. For this reason, only individuals in a reasonably good preservation condition were used. Male genitalia preparation and female scale extraction from anal tuft followed the method described in Basso et al. (2017a). Geographic coordinates and name of the collection site were recorded for each specimen, to which a unique alphanumeric code was assigned indicating country, locality, and number of specimens. Countries were indicated with NATO digram codes (https://en.wikipedia.org/wiki/List_of_NATO_country_codes), localities were scored using capital letters (A to Z), and the number of specimens was marked with two-digit progressive numbers (starting from 01) (see Table S1a).

2.4. Molecular data

So far, a few DNA fragments have been used to explore the genetic diversity in the *T. pityocampa* complex at both mitochondrial and nuclear levels (Kerdelhué et al. 2015). The available information from different laboratories and periods of time could be merged to obtain a unique picture, although this required the identification of a sequence shared among the datasets. A preliminary exploration of the sequences available for most taxa described so far identified the mitochondrial region 5p of the gene *cox1* (primers LCO1490 – HCO2198, Folmer et al. 1994) as suitable for the study. Even though the choice involved discarding several studies and samples, it allowed a satisfactory and sufficient coverage of the distribution range and a good matching of both morphological and molecular datasets.

For those locations lacking the target sequence, specimens were retrieved from collections starting in the 1990s that were preserved either frozen or in ethanol. Individuals consisted of adult moths obtained from light/pheromone trapping, larvae from either rearing, colonies on trees, or pupation processions, from a total of 92 locations in Europe and the Mediterranean Basin (Fig. 1). Total DNA extraction was performed from each specimen individually using Invisorb® Spin Tissue Mini Kit, following manufacturer's instructions to extract from muscle. The extractions from larvae were performed from the head (except for the first instar larvae, where extractions were performed on a bulk of 20-25 siblings), while the extractions from adults were carried out from the thorax muscles or legs to keep the head and abdomen for morphological analyses (Basso et al. 2017a). Quality and quantity of extracted DNA were evaluated using both NanoDrop ND-1000 (Thermo Fisher Scientific, USA) and Qubit 4 (Invitrogen, USA) while DNA integrity was assessed on agarose gel (0.8%). The barcode sequences (Ratnasingham et al. 2007) were amplified using universal primers (LCO 1490 and HCO 2198) and following the PCR conditions used in Salvato et al. (2002). Obtained amplicons were purified with Exo-Sap enzymes protocol and sequenced using both the forward and reverse primers previously used in the amplification step (Basso et al. 2017b). Sequencing was performed at BMR Genomics (https://www.bmr-genomics.it/) and INRAE France. The quality of the chromatograms was assessed using Chromas Lite software (http://technelysium.com.au/wp/chromas). Consensus sequences (385 new sequences with lengths spanning from 530 to 710 nucleotides) were assembled using the DNASTAR software (Lasergene, Madison, WI). In addition, 59 sequences belonging to the genus *Thaumetopoea* were retrieved in February 2021 (see Table S1b) from NCBI and BOLD databases (Ratnasingham et al. 2007; Mutanen et al. 2010; Huemer et al. 2012; Simonato et al. 2013; Mutanen et al. 2016; Basso et al. 2017; Trematerra et al. 2017a). Among these, a total of 45 sequences (voucher specimens) were used as references to assign species identification in the phylogenetic analyses (see Table S1b).

2.5. Data analyses

A full set morphological matrix was built in Mesquite software (Maddison et al. 2018) and traits were encoded as described in File S1 following the encoding used in Basso et al. (2017a). The traits were divided by sex, creating two matrices (File S2a, File S2b) of 187 × 44 and 137 × 31 (specimens x traits), respectively. The outgroup specimens were scored together based on Basso et al. (2017a). Furthermore, the "inapplicable characters (–)" for these specimens were coded as "absent" into the matrices. Principal Component Analysis (PCA) was carried out for both matrices to explore the variance among the multidimensional datasets and to investigate the traits that mainly contribute to outlining the groups (Legendre and Legendre 1998; Loko 2015; Zimisuhara 2015). The analyses were performed with PAST 4.11 (Hammer et al. 2001) under multivariate ordination setting, using a

correlation matrix and scaling principal component (PC) by eigenvalue. The relevant PCs were detected by scree-plot, cutting off the point equal or under the broken-stick distributions. The contribution of single variables (traits) in each PC was deemed significant when ≥ |0.4|, while the signs of the values were interpreted for positive (+) or negative (-) associations. Concurrently, the matrices were analysed separately using a phylogenetic approach under both Maximum Parsimony (MP) and Maximum Likelihood (ML) criteria (see File S2a, File S2b) rooting trees in the *T. pinivora* clade. The MP approach was carried out using TNT v1.5 software (Goloboff et al. 2016). The software was set according to Basso et al. (2017a), and the analyses were performed both on equal (EW) and implied weighting (IW). IW parameters (k) were calculated using setk.run script (Santos et al. 2015; Badano et al. 2018). Each MP analysis produced more than 100 trees. The consensus trees obtained for both datasets resulted in polytomies, assuming the absence of enough discriminating signals among the datasets, these results were discarded and not further discussed. The ML approach was carried out using Iqtree v1.6.12 software (Nguyen et al. 2015) using the nucleotide substitution model determined by ModelFinder (Kalyaanamoorthy et al. 2017).

A total of 430 sequences belonging to *T. pityocampa* group and 14 sequences belonging to another ten *Thaumetopoea* species were first translated into putative proteins to identify the proper reading frame. Then multiple alignments were carried out "in-frame" using MAFFT tools implemented in the TranslatorX (http://www.translatorx.co.uk/) pipeline (Abascal et al. 2010) with default settings. To study the variability conveyed by the barcode marker and due to the concordance in the sequences between siblings and specimens from the same geographic zone, we decided to use data-supported even by single chromatogram. Hence, the longer sequences were trimmed, while the shorter sequences were expanded in both 5' and 3' ends, manually curating each chromatogram and reporting the nucleotide called in small letters. Sequences retrieved from databases were used as found. The unique haplotypes and the presence of identical sequences in the dataset were assessed with DNAsp v.6.12.03 x64 (Rozas et al. 2017). Empty positions were treated as missing data and considered in the analysis; ambiguous data were not detected. In the case of identical sequences, the one with the longest length was chosen to represent the haplotype. The final multiple alignments set spanned 657 positions for 94 unique (65 presented in this work) sequences representing the haplotypes' variability of the *T. pityocampa* complex around the Mediterranean Basin. Initially, a double approach for the phylogenetic investigation was carried out, performing the analyses with NJ and ML methods.

According to the BOLD method (Ratnasingham et al. 2007), the NJ analysis was carried out in MEGA X and used as a reference for the specimens' identification. The ML analysis was carried out using Iqtree v1.6.12 software (Nguyen et al. 2015), applying K2P+G4 evolutionary model. The NJ tree was discarded during the preliminary evaluation due to inconsistencies between BOLD assignment and NJ topology obtained from MEGA X software. Indeed, the sequence belonging to the *T. mediterranea* clade stands apart from the main clade encompassing the other *T. mediterranea* and *T. hellenica* sequences. This choice was corroborated by genetic distances evaluation as well. Thus, we performed the following analyses with the ML approach, which was consistent with the clade designation of BOLD. Fifty independent runs were carried out with the settings described above, and each Log-likelihood score was assessed to identify the most likely tree describing the relationship between the clades. This tree was used as a starting point in the calculation of supports. Node supports were evaluated by standard Bootstrap (BT) (Felsenstein 1985) and Ultrafast bootstrap (UFB) (Minh et al. 2013; Hoang et al. 2018), while supports to the branches were estimated by SH-like approximate likelihood ratio test (SH-aLRT) (Guindon et al. 2010). Each test was performed for 10,000 iterations, and values only > 90 in both nodes and branches were reported. Tree was rooted on the clade of broadleaved-feeding species *T. herculeana*, *T. processionea*, and *T. solitaria*. Genetic distances on unique haplotypes between specimens of the detected genetic clades were estimated in MEGA X using the K2P substitution model (Kimura 1980) for the BIN evaluation (Ratnasingham et al. 2007). Finally, the outcomes of distance analyses were compared with results obtained via two typical web applications to detect the species gap. The dataset comprising only T. pityocampa complex was submitted to the Automatic Barcode Gap Discovery (ABGD) (Puillandre et al. 2012) (https://bio-info.mnhn.fr/abi/public/abgd) using 1000 iteration and running the analyses with default settings for JC69, K2P, and SD models. The relative gap width was set to X = 1.5. The ML tree was evaluated with bPTP server (https://species.h-its.org), using 100,000 MCMC generation and with a thinning of 1000 and a burn-in of 0.3 (Zhang et al. 2013). Haplotype networks were carried out separately by species, using TCS setting under popART 1.7 software (Leigh et al. 2015).

3.   Results

3.1.   Morphological analysis

PCAs were carried out separately on male and female matrices to summarize the information carried by morphological data. The three most relevant PCs explained 34.59% of the total variation when analysing the male dataset and this value increased to 39.59% when analysing the female dataset. The relative variance of the first three PCs in both datasets spanned from 17.25% to 9.78%, with single variables positively or negatively correlated to each PC, as summarized in Table S2. The score plots of PC1-3, conveyed by the male matrix, showed a clear separation between the outgroup and the *T. pityocampa* complex. On the contrary, in the female matrix, the separation between species groups was less

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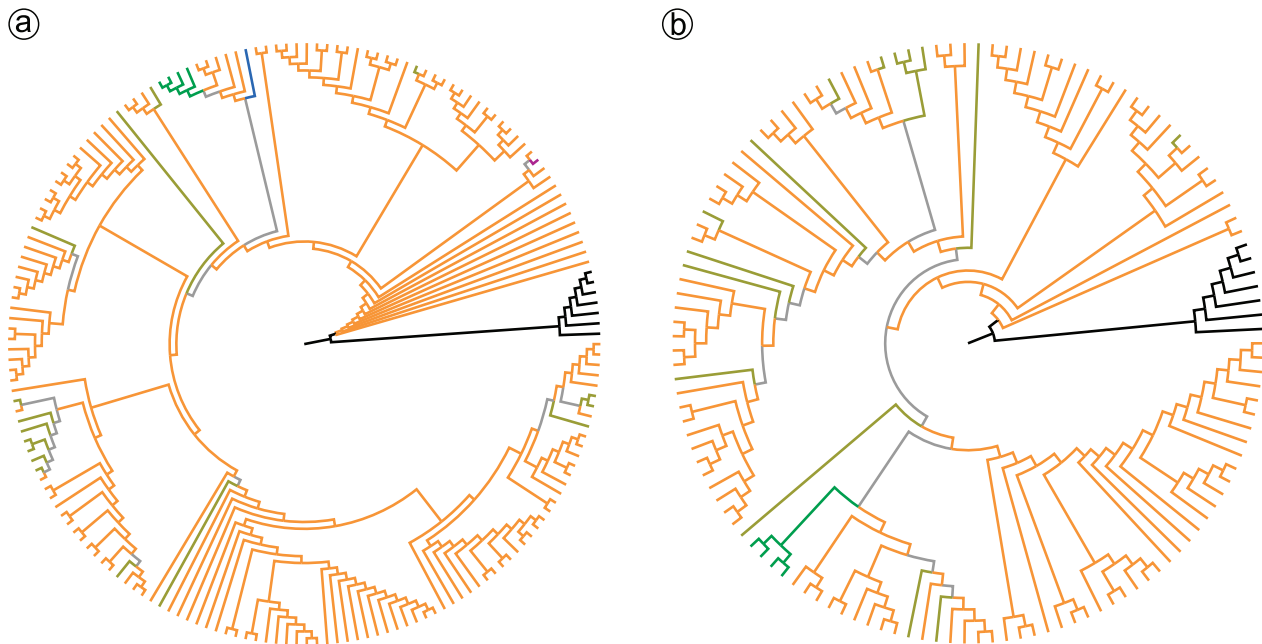
Figure 2. Maximum likelihood trees inferred from morphological matrices; **a** dendrogram from male specimens (182) using MK+FQ+I+G4 substitution model; **b** dendrogram from female specimens (132) using MK+FQ+ASC+R3 substitution model. Branch-colour indicates the clades: black, *T. pinivora*; green, *T. cretensis*; blue, *T. hellenica*; purple, *T. mediterranea*; orange, *T. pityocampa*; dark yellow, *T. wilkinsoni*. Grey branches highlight taxonomical inconsistencies.

defined. Conversely, in both cases, a complete overlap of the specimens was noticed within the *T. pityocampa* complex without chances to determine a defined pattern to distinguish taxa (Fig. S2). The results of ML analyses performed on morphological traits were shown in Figure 2 separately for female and male moths. In both cases, the *T. pinivora* clade (*T. pinivora* and related species) stood out clearly, forming a separate clade at the base of the tree. The other species that are associated with angiosperms cluster in consistent groups (e.g., *apologetica* Strand, 1909 – *dhofarensis* Wiltshire, 1980 - *jordana* Staudinger, 1887 and *processionea* – *solitaria*) but remained in the *T. pityocampa* complex. All the individuals of the *T. pityocampa* complex were mixed up and no patterns were detected with the exception that individuals from the same population group together, irrespective of the putative species (*T. hellenica*, *T. mediterranea*, and *T. wilkinsoni*), or subspecies (*T. pityocampa orana*) they belong to (Fig. S3). Even when single traits were considered in a comparative way across species and subspecies, a large overlap was observed irrespective of the type of trait (e.g., colour, female anal scale, male genitalia, shape of the canthus, wing venation) or taxonomic allocation (data not shown).

3.2. Molecular analysis

The outcome of the molecular study based on the barcode region of the mitochondrial DNA is presented in Figure 3 and in Fig. S4. The ML tree exhibited eight major clades. The two first clades corresponded to species associated with angiosperms in Europe and Northern Africa (*T. herculeana, T. processionea, T. solitaria*) and in Central Af-

rica and the Arabian Peninsula (*T. apologetica abyssinica* Strand, 1911*, T. apologetica, T. dhofarensis*). The next clade included the species feeding on conifers in summer (*T. bonjeani, T. ispartaensis*, *T. libanotica, T. pinivora*). The ingroup was organized into three larger clades corresponding to species feeding on conifers in winter and differentiated according to geographic distribution. Following the phylogenetic order, *T. hellenica*, from Greece and Libya (clade 1: BIBSA1724-16, MW756044-45), was retrieved to stand apart from *T. mediterranea* from Algeria, Pantelleria Island, Tunisia (clade 2: BCLEP001-16, GBGL12179-13, MW756046-52). The second lineage included *T. cretensis* sp. n., collected in Crete Island (clade 3: MW756053-54, MZ425543-49), and *T. wilkinsoni* from islands of Cyprus and Rhodes, and Turkey (clade 4: GBGL12176-13, MW756055-64). The *T. pityocampa* clade spanning from Greece to NW Africa was split into four statistically supported subclades structured according to different geographic locations. Specimens from Corsica Island, Algeria and Morocco were clearly identified, while the others were not so distinct and included individuals from the Balkans to Spain and Portugal. Sequences from S Algeria and S Morocco (MW756065-66) were included in a subclade distinct from that including individuals from Corsica Island, NW Algeria and N Morocco (MW756067-69), which were sister to European specimens..

The haplotype network in the complete dataset revealed the presence of 94 haplotypes, which were divided by species, as shown in the ML phylogenetic tree (Fig. 3). The interspecific genetic distances of the *cox*1 marker between ingroup clades ranged from 3.8% (*T. hellenica – T. mediterranea*) to 9.4% (*T. hellenica – T. cretensis* or *T. wilkinsoni*) (Table 2). Seven sequences for *T. hellenica*
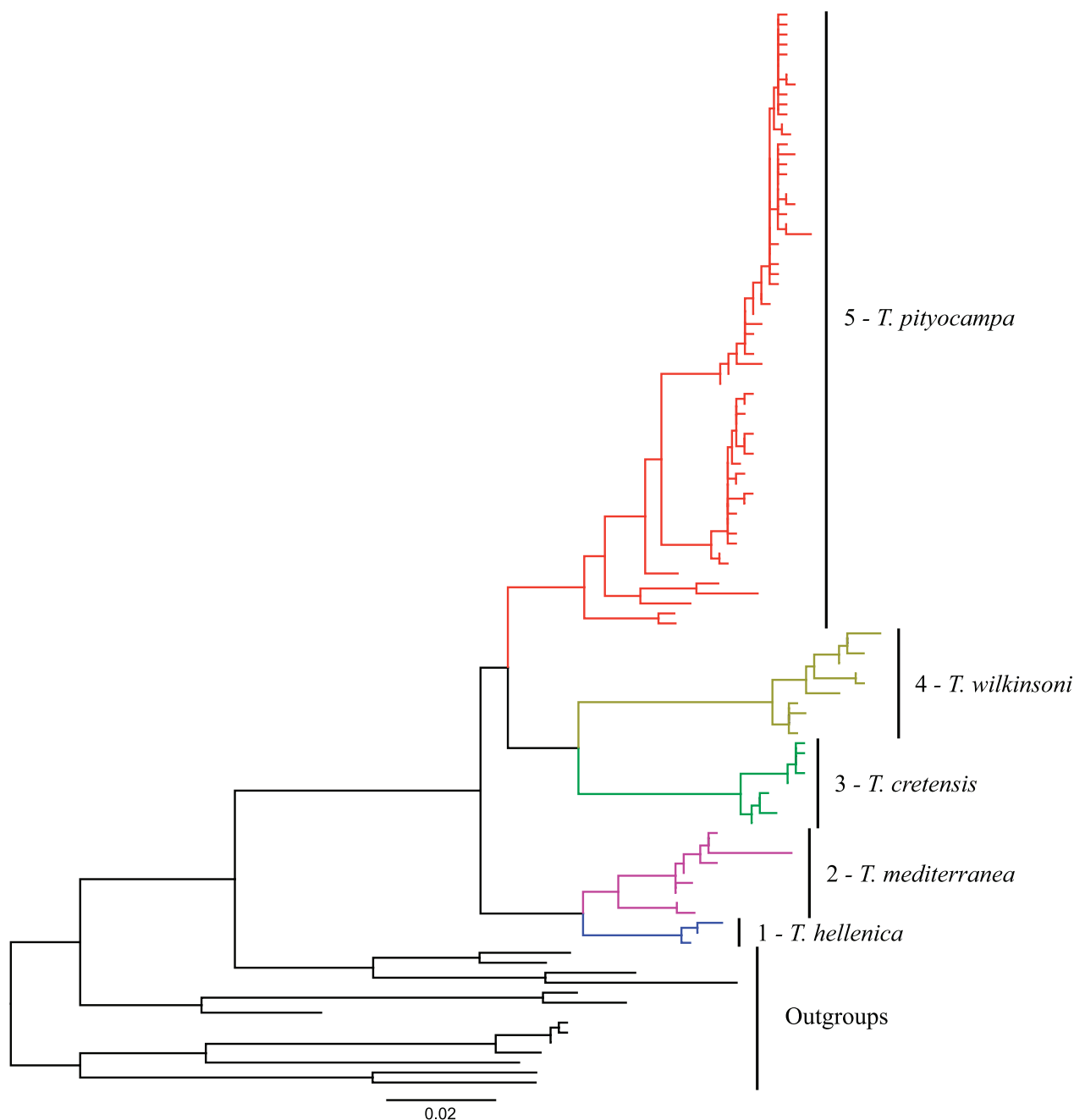
Figure 3. Simplified cladogram of ML phylogenetic analysis (-ln= 4358.6671) of the sequences considered in this work. A complete version is provided in Fig. S4.

revealed three different haplotypes, each characterized by three nucleotide substitutions with the intraspecific genic distance of 0.6%. The haplotype distribution of Clade 1 showed that two sequences were located near the Libyan coast (MW756044-45). In contrast, a single haplotype from the reference sequences (BIBSA1724-16) belonged to males collected with pheromone traps in two sampling sites in Greece (Trematerra et al. 2017b) (Fig. S5). *Thaumetopoea mediterranea* was represented by 11 sequences split into nine unique haplotypes, with a 1.6% genetic distance (Table 2). The network analyses showed a subdivision into two groups supported by 13 nucleotide changes (Fig. S6). This set showed the two reference sequences reported, respectively, from Pantelleria Island (BCLEP001-16) (Trematerra et al. 2017b) and in Bizerte

(GBGL12179-13) (Kerdelhue et al. 2009), diverged from those obtained by inland specimens (MW756046-52). The same distribution was obtained in the ML phylogenetic tree, with reference sequences (BCLEP001-16, GBGL12179-13) clustered together and were sisters to the others (MW756046-52). The haplotype distribution located the Clade 2 between Algeria and Tunisia, along the Mediterranean side of the Atlas Mountains, where more genetic variability was detected (MW756046-52).

The 18 sequences of *T. cretensis* were split into nine exclusive haplotypes with an overall genetic distance of 0.9%. The network grouped the haplotypes in two main clades (MZ425547-49 + MW756054 and MZ425543-46 + MW756053), separated by seven changes equally distributed in Crete Island (Fig. S7). This partition was sup-

Table 2. Genetic distances (base substitutions/site) calculated with MEGA X. Analyses were performed using the Kimura 2-parameter model involving 94 nucleotide sequences. Distances calculated among clades were reported on the diagonal and in bold. Distances calculated between species were reported in the lower-left matrix.

	T. hellenica	*T. mediterranea*	*T. pityocampa*	*T. cretensis*	*T. wilkinsoni*
T. hellenica	**0.006**				
T. mediterranea	0.038	**0.017**			
T. pityocampa	0.081	0.075	**0.020**		
T. cretensis	0.094	0.092	0.084	**0.009**	
T. wilkinsoni	0.094	0.094	0.083	0.086	**0.015**

ported, also by nucleotide substitutions (A>T) involving position 130 of the alignment and it was shown in the phylogenetic tree where it was sustained by a fully supported node (Figs 3 and S4). In addition, this substitution reflected the isolation of this species, corroborated by two unique amino acid substitutions (I>L and L>M) occurring in positions 41 and 93 of the amino acid alignment.

The haplotype network of 19 sequences belonging to *T. wilkinsoni* detected 11 specific polymorphisms with an overall interspecific distance >8% and an intraspecific genetic distance of 1.4%. The analysis showed three main clusters separated by a hypothetical ancestor. The first clade included sequences from NE and SW Turkey (MW756061-64), which resulted sister group of the other clades comprising sequences from Cyprus (MW756055-56), and samples collected near Adana region in the SE Turkey (GBGL12176-13, MW756057-59). Sequence MW756060, retrieved from SW Turkey, was linked to this latter, with six substitutions from the reference sequence (Fig. S8).

Thaumetopoea pityocampa included 62 exclusive haplotypes from 375 analysed sequences arranged into four groups. The overall genetic difference calculated within the group was 2%, while the interspecific distances were >7.5% (Table 2). In addition, the TCS network analyses showed several substitution steps separating the subclades from S Algeria – S Morocco (MW756065-66), NW Algeria – N Morocco, and Corsica Island (MW756067-69) by European clades. The ancestors to link these sequences with the other from Europe were not detected. The group from Spain included sequences from the summer form of *T. pityocampa* and other sequences from the Iberian Peninsula. The TCS network identified haplotype KT768188 as a common ancestor of sequences retrieved from Spain and Portugal. The same mutation was distributed also in France, collected from specimens near Pyrenean and Brittany regions. The other sequences were combined in a large group from Pyreneans to Greece showed three clades of differentiation, linked to each other and with a few substitutions (BIBSA1730-16, BIBSA1727-16, ABOLD143-16) (Figs S9 and S10). During the analyses, MW756107 from southern France was discarded by the software after detecting length differences among sequences >5%. Therefore, it was added manually at the end of the analysis associating according to the MP principle (Fig. S10).

The barcode-gap evaluation carried out with ABGD resembled the findings above with slight differences. Through the recursive partition analysis, the software retrieved 11 groups (p-values = 0.001–0.004), estimating the barcode gap boundary at 2%. Furthermore, the clear species delimitation was evaluated at around 5–5.5%, where a second split between distance values was detected. In detail, single clades were identified within Clade 1, 3, and 4, while Clade 2 was separated into two sub-groups based on geographic distribution. Finally, the sequences of *T. pityocampa* (Clade 5) were arranged into six other subgroups. Each of them agreed with the layout shown in the phylogenetic tree (Figs 3 and S4). The gap delimitation obtained with bPTP was generally congruent with previous results. However, it tended to over-fragment the *T. pityocampa* clade, retrieving up to seven sub-groups.

3.3. Taxa description

3.3.1. *Thaumetopoea pityocampa*

https://zoobank.org/01298407-43D8-44F7-A107-688796AF2-7B3

B.[*ombyx*] *Pityocampa* [Denis & Schiffermüller], 1775: 58

Neotype. Adult ♀. Label: Italy, Val Venosta, Silandro, 20/06/2022.

Collection data. Collected by Andrea Battisti in the field in a forest of *Pinus nigra* and *P. sylvestris*. Coordinates: 46°37′30″N, 10°48′12″E, altitude 810 m. Neotype deposited at the Naturhistorisches Museum Wien, Vienna, Austria.

Diagnosis. The species can be distinguished from other *Thaumetopoea* species based on several morphological and biological traits (de Freina and Witt 1987). The separation from other taxa included in the same group (Table 1) is difficult because of substantial variation of morphological traits within taxa (e.g., individuals from the original site of the material studied and described by Réaumur, Fig. S1 and S11). However, the barcode sequence allows an easy discrimination of *T. pityocampa* from the three other similar species (*T. wilkinsoni*, *T. hellenica*, and *T. mediterranea*), but not from the synonymous *T. pityocampa orana*.

Description (Fig. 4). Forewing length 23 mm. Antenna bipectinate, rami more or less equal in length except in apical tenth, where rami become shorter; axis and rami pale yellow-brown. Head small, labial palpus short, directed ventrally, light yellow and covered completely in long, dense brownish hair-like setae; eye surrounded by long brownish hair-like setae; chitinous shiny frontal process (crest, canthus) with 6 teeth, each of them with lateral horns; first tooth pointing dorsally and the others frontally; angle of less than 90° between the first and the second tooth; height of the first tooth about one half of the second, height of other teeth progressively decreasing until sixth, which is about one tenth of second; spacing between first and second tooth larger than that between other teeth, maximum width at level of fourth tooth; frontal process surrounded medially and basally by long yellow hair-like setae with brown apex; vertex covered by long yellow hair-like setae with brown apex; compound eye large, globular. Thorax, tegula and collar covered in dense, very long light brown hair-like setae; abdomen orange brown dorsally and covered with short hair-like-setaes, yellowish with longer hair-like setae laterally and ventrally; eighth and ninth tergites covered with dense pack of scales, partially covered by long hair-like setae. Scales elongated, maximum width at apex, 2.1 times longer than wide, light brown in apical fifth. Forewing long, broad triangular, costal and ventral margin straight, outer margin evenly arcuate, tornal angle broad, apex narrowly rounded. Forewing ground colour pale light brown, costal margin yellowish for two-thirds from the base; basal line faint, ante-median line visible in the upper half and faint in the lower half, post-median line extended from costal to ventral margin, more distinct in the upper half, discal spot well defined, c-shaped, brown, terminal line distinct and brown. Hindwing off-white, with yellowish venation; discal spot faint; terminal line present with a clear anal spot. Underside of forewing as the upperside but generally less distinct; underside of hindwing homogenous whitish as upperside but with darker shade, discal spot present but faint.

Barcode sequence. GenBank MW756090, BOLD ID GBMNF23029-22, see Table S1b.

Distribution. Known from Northern Africa to southern Europe and western Asia (western Turkey)

Material examined. Type material: Adult ♀. Label: Italy, Val Venosta, Silandro, 20/06/2022. — **Other material**: Other individuals (3 ♀, 1 ♂) from the same locality present another haplotype MW756096 (label data: Italy, Val Venosta, Silandro, 30/07/2014, 46°37′45″N, 10°47′55″W, 903 m, ex larva *Pinus nigra*, leg. A. Battisti).

Remarks. The type material was searched in vain at the Naturhistorisches Museum, Vienna, Austria where the Schiffermüller collection was preserved. It was probably lost in the fire of the Vienna Hofburg on 31 October 1848 during the Vienna Uprising or October Revolution (personal communication of Sabine Gaal-Haszler, curator of the Naturhistorisches Museum, Vienna, Austria).

a

10 mm

b

Italy, Val Venosta, Silandro
46°37'30''N, 10°48'12''W, 810 m
20/06/2022, leg. A. Battisti
Forest *Pinus nigra* and *P. sylvestris*

Thaumetopoea pityocampa
(Denis & Schiffermüller, 1775)
Neotype, female
Barcode sequence: MW756090

c

1 mm

d

1 mm

Figure 4. *Thaumetopoea pityocampa* neotype (**a** female dorsal view; **b** label; **c** canthus; **d** anal scale).

3.3.2. *Thaumetopoea cretensis* İpekdal & Avtzis, sp. nov.

https://zoobank.org/08D479CA-872C-44E5-91DD-80F0CE04-64D9

Holotype. Adult ♂. Label: Greece, Crete, Anopoli, Chania, 796 m, larva collected on 02 April 2019 on *Pinus brutia*, moth emerged on mid-August 2019, legit Kahraman İpekdal. Coordinates: 35°13′29″N, 24°02′10″E, elevation 796 m.

Paratypes. Three adult ♂ and four adult ♀, same label. Type material deposited at the Naturhistorisches Museum Wien, Austria.

Diagnosis. Smaller than other species of the *T. pityocampa* complex, from which it can be hardly separated based on morphological traits of both male and female moths. The anal scale of the female is more similar to *T. wilkinsoni* than to *T. pityocampa*. It can be separated from other species of the genus based on the gene sequence data of the barcode region *cox*1 (primers HCO – LCO) and from a clear separation at nuclear DNA level identified by Petsopoulos et al. (2018).

Description. (Figs 5, S12, S13). Adult ♂. Forewing length 15 mm. Antenna bipectinate, rami well developed and more or less equal in length except in the apex, where they become progressively shorter; axis and rami pale orange-brown. Head small, labial palpus extremely short, directed ventrally, covered completely in long, dense dark grey hair-like scales; eye surrounded by long dark brown hair-like setae; chitinous and shiny frontal process (crest, canthus) with 6 teeth; first tooth pointing dorsally and others frontally; angle of about 90° between first and second tooth; teeth from second to sixth with lateral horns; height of first tooth about one third of second, height of other teeth progressively decreasing until the sixth, which is about one tenth of second; spacing between first and second tooth larger than that between other teeth, maximum width at level of fourth tooth; frontal process surrounded medially and basally by long yellow hair-like setae with dark brown apex; vertex covered by long yellow hair-like setae with dark brown apex; compound eye large, globular. Thorax, tegula and collar covered in dense, very long dark brown hair-like setae. Abdomen orange-brown dorsally, dark grey laterally. Forewing ground colour pale light brown, costal margin yellowish for two-thirds from the base; basal line and ante-median lines complete, post-median line extended from costal to ventral margin, more distinct in the upper half, discal spot well defined, c-shaped, brown, terminal line distinct and brown. Hindwing off-white, with yellowish venation; discal spot faint; terminal line present with clear anal spot. Underside of forewing as upperside but generally less distinct; underside of hindwing homogenous whitish as upperside but with darker shade, discal spot present but faint. — ***Genitalia.*** Uncus short, relatively broad at base, apically with two hooks. Socii short, longer than uncus, broad at base, lateral and inner margins straight, apex rounded. Tegumen robust, as long as valvae; juxta wider than long, concave at superior margin, lateral and inferior margins convex, lateral angles rounded. Valvae broad at base, rounded quadrangular, cucullus rounded with sparse, short setae. Ventral margin of valvae convex basally and concave distally; dorsal margin of valvae straight basally and concave distally; upper ribbing of valvae faint in the basal part. Phallus as long as valvae, straight, apically rounded, constricted in the distal half.

Barcode sequence. GenBank MZ425548, BOLD ID GBMNF23047-22, see Table S1b.

Adult ♀. Forewing length 19 mm. Antenna bipectinate, rami more or less equal in length except in the apex, where they become shorter; axis and rami pale yellow brown. Head small, labial palpus short, directed ventrally, light yellow and covered completely by long, dense brownish hair-like setae; eye surrounded by long brownish hair-like setae; chitinous and shining frontal process (crest, canthus) with 6 teeth; first tooth pointing dorsally and the others frontally; angle of about 90° between the first and the second tooth; teeth from the second to the sixth with lateral horns; height of the first tooth about one fourth of the second, the height of other teeth progressively decreasing until the sixth, which is about one eighth of the second; teeth 2-6 with lateral horns; spacing between the first and second tooth larger than that between the other teeth, maximum width at level of fourth tooth; spacing between tooth 2 and 3 larger than between 4 and 5; frontal process surrounded medially and basally by long yellow hair-like setae with brown apex; vertex covered by long yellow hair-like setae with brown apex; compound eye large, globular. Thorax, tegula and collar covered by dense, very long light brown hair-like setae; abdomen orange brown dorsally and covered with short hair-like setae, yellowish with longer hair-like setae laterally and ventrally; 8th and 9th tergites covered with dense pack of scales, partially covered by long hair-like setae. Scales elongated, maximum width at apex, 1.75 times longer than wide, light brown except at the base. Forewing long, broad triangular, costal and ventral margin straight, outer margin evenly arcuate, tornal angle broad, apex narrowly rounded. Forewing ground colour pale light brown, costal margin yellowish for two-thirds from the base; basal line faint, ante-median line visible in the upper half and faint in the lower half, post-median line extended from costal to ventral margin but distinct only in the upper half, discal spot well defined, c-shaped, brown, terminal line distinct and brown. Hindwing off-white, with yellowish venation; discal spot faint; terminal line present with a clear anal spot. Underside of forewing as the upperside but generally less distinct; underside of hindwing homogenous whitish as upperside but with darker shade, discal spot present but faint.
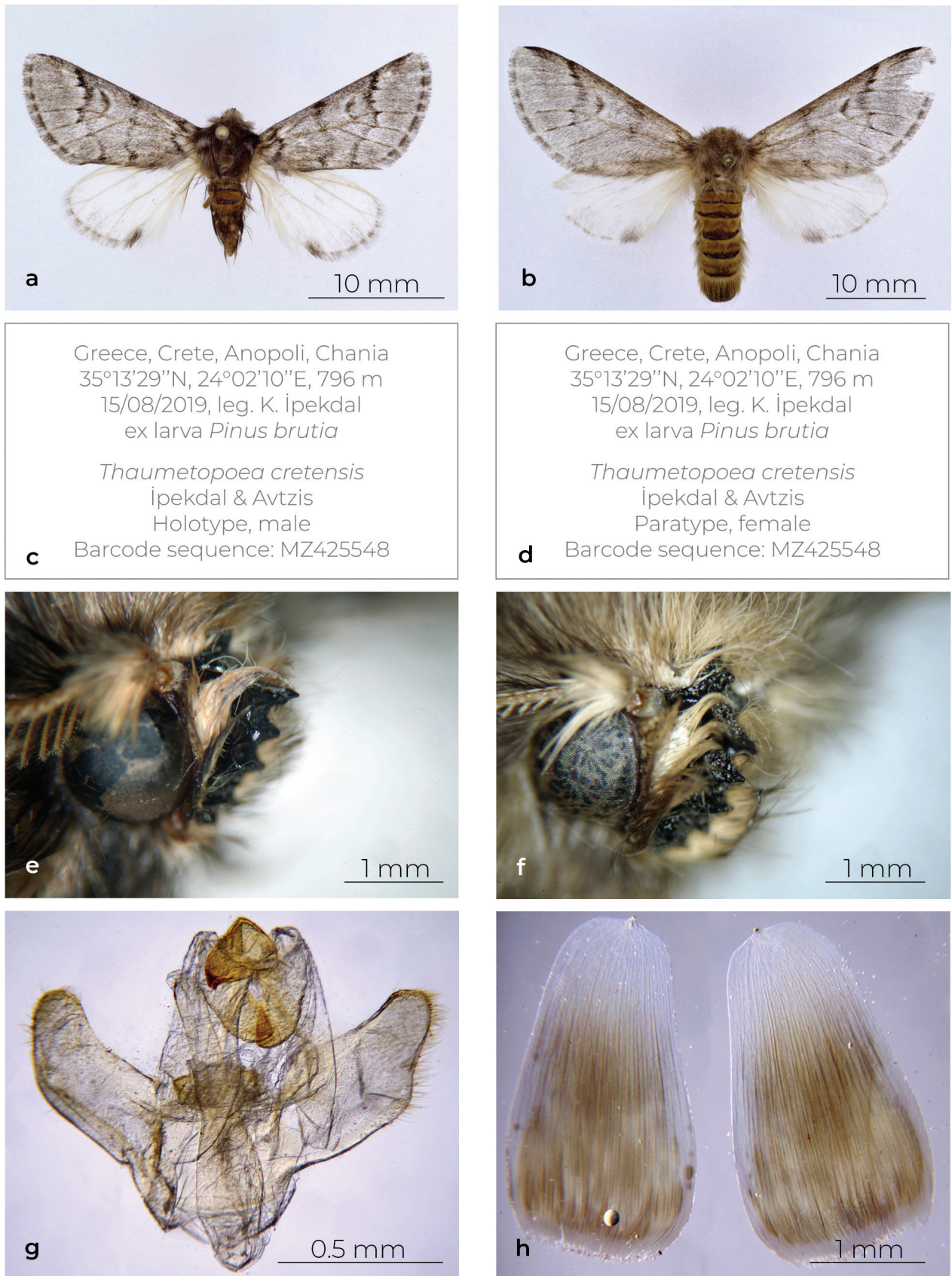
Figure 5. *Thaumetopoea cretensis* holotype (**a** male dorsal view; **c** male label; **e** male canthus; **g** genitalia) and paratype (**b** female dorsal view; **d** label; **f** canthus; **h** anal scale).

Barcode sequence. GenBank MZ425548, BOLD ID GBMNF23047-22, see Table S1b.

Distribution. Known from Crete Island.

Material examined. Type material: Adult ♂. Label: Greece, Crete, Anopoli, Chania, 796 m, larva collected on 02 April 2019 on *Pinus brutia*, moth emerged on mid-August 2019, legit Kahraman İpekdal. — **Other material**: Paratypes. Three adult ♂ and four adult ♀, same label.

Remarks. Etymology: from the island of Crete.

4. Discussion

The main results of the morphological and molecular analyses of the taxa included in the *T. pityocampa* complex indicate that morphology alone does not allow to delimit the species while the barcode region of mitochondrial DNA can be helpful. The lack of reliable morphological traits for the separation of the taxa was already mentioned in the first revision of Agenjo (1941), who considered the eastern pine processionary moth *T. wilkinsoni* as a self-standing species because of ecological and biogeographical traits, while morphological traits overlapped with those of *T. pityocampa*. Further efforts to find discriminant traits in male genitalia and female anal scales conducted by Démolin (1988) and Démolin et al. (1994), on individuals originating from populations associated with pines and cedars around the Mediterranean basin, failed to have a conclusive outcome on species identity. Some tendencies in the shape of the canthus, the middle rib of male genitalia valvae, or the shape of the female anal scales were evidenced, although they were never clear enough to discriminate among taxa even when these were geographically well separated.

Hybridization between the two main species in the group, *T. pityocampa* and *T. wilkinsoni*, was achieved under laboratory conditions first by Démolin (Battisti et al. 2015) and then by Petrucco-Toffolo et al. (2018). In both cases, the obstacle represented by different emergence times was overcome by manipulating the temperature regimes under which the diapausing pupae were maintained, making them emerge simultaneously in mating boxes. This suggests that phenology of adult emergence could act as a barrier to gene flow in the field. Interestingly, the viable F1 and F2 generations did not only show hybrid genomes but also intermediate morphological features (Petrucco-Toffolo et al. 2018). An exploration of the geographical contact zone between *T. pityocampa* and *T. wilkinsoni* in NW Turkey was carried out by İpekdal et al. (2020) through the analysis of mitochondrial genes and nuclear polymorphic SSR markers, evidencing recurrent introgression by *T. wilkinsoni* males in several *T. pityocampa* populations. The phenomenon, however, was not frequent and the occurrence of prezygotic isolation mechanisms, such as differences in timing of the adult emergences, was considered the most likely as no geographical or environmental barriers occur in that region. In addition, both species respond well to the same sexual pheromone ((Z)-13-hexadecen-11-yn-1-ol acetate), isolated first from *T. pityocampa* (Guerrero et al. 1981) but shown to be equally effective in *T. wilkinsoni* (Halperin

and Golan 1982). A thorough reassessment of the sexual pheromones confirmed the identity of the molecules in both taxa (Frérot and Démolin 1993).

Molecular markers used in the last decades have revealed a complex situation in the group of *T. pityocampa*. Here, the information differs depending greatly on the origin of genes considered, either mitochondrial or nuclear. Maternally inherited mitochondrial genes mirror the history of female lineages, that is generally restricted to small geographic regions because of females' short life (usually one night) and limited average flight range of few hundred metres (Battisti et al. 2015). Nuclear genes are strongly influenced by the gene flow caused by males, which live longer and spread over several kilometres, as suggested by trapping experiments (e.g., males were caught on mountain tops in the Alps, far from established populations) (Kitschelt 1925; Salvato et al. 2005). At both elevational and latitudinal expansion areas, nuclear markers revealed a larger admixture area than that identified by mtDNA markers, highlighting the role of male-driven gene flow (Salvato et al. 2005). The history of the pine processionary moth in the Mediterranean was reconstructed based on phylogeographic studies and it is essentially based on repeated isolation events in refugia since at least the Miocene, i.e., 10 million years ago (Kerdelhué et al. 2009, 2015). Those events may have given rise to the different taxa presently known in the group. However, some taxa could have come in secondary contact, in the past because of climatic oscillations or even in recent time because of extensive pine plantations in the Mediterranean. Consequently, gene flow in the nuclear genome could have occurred in the absence of barriers, as shown for example between Cyprus and the mainland (Kerdelhué et al. 2009) or between clades occurring in Northern Africa (El Mokhefi et al. 2016). Given that females are less mobile than males, the mitochondrial signature of former isolation may remain despite gene flow assured by males.

This situation has led to high haplotype diversity, as shown for example in the site where the neotype was collected, and to the definition of several clades mainly based on mitochondrial markers (Simonato et al. 2007; Kerdelhué et al. 2009), prompting the description of new taxa such as *T. hellenica* and *T. mediterranea* (Trematerra et al. 2017; Trematerra and Colacci 2018). Although these taxa are indistinguishable based on morphological traits, they are supported based on their genetic identity at the mitochondrial level. Basically, they represent two mitochondrial subclades described by Kerdelhué et al. (2009) included in the Eastern-Northern African (ENA) clade, the first subclade from East Algeria, Tunisia, and Pantelleria Island, and the second from the Cyrenaica peninsula in Libya. Interestingly, the haplotype found in Libya was later found in samples collected in the Attika region (Greece) (Avtzis et al. 2016, 2018), from which the name *T. hellenica* was chosen by Trematerra et al. (2017). Data suggest that the occurrence of Libyan-mtDNA bearing individuals in Greece is recent, as previous dense phylogeographical studies did not retrieve this corresponding divergent haplotype (Kerdelhué et al. 2009; Korsch et al. 2015). However, a thorough analysis of gene flow at

nuclear level would be necessary to conclude about the species status of the new taxa recently described, and to determine if *T. hellenica* occurs in Greece or if this situation mostly corresponds to a mitochondrial introgression. Such an analysis has been achieved for the populations of Algeria, where two clades (*pityocampa*, including the subspecies *orana*, and ENA) are coming into contact, and it showed that nuclear differentiation is limited, and gene flow commonly occurs among them despite the strong mitochondrial genetic structure (El Mokhefi et al. 2016). Based on these results, there is no substantial reason to consider *orana* as a separate taxon. A different situation was described for the populations of the Crete Island, which are a subclade of *T. wilkinsoni* (Kerdelhué et al. 2009). Here, a thorough analysis of all markers showed that Crete populations are isolated from all the other populations of the Asian mainland and the Aegean archipelago (Petsopoulos et al. 2018), leading to the description of the new species in this paper.

The economic and medical importance of the pine processionary moths is acknowledged in all its range. The species complex is well characterised based on the unique traits of the life history, namely the winter feeding and the construction of conspicuous silk tents by the larvae. There is, however, evidence that the group is subdivided into various taxa, characterized either by a typical geographic distribution or a particular phenology that may represent barriers to gene flow. In this concern, the recent finding of an allochronic population with shifted phenology, i.e., summer feeding in Portugal (Santos et al. 2007, 2011a), partially co-occurring with populations still exhibiting the classical winter-feeding phenology, has confirmed the large adaptation potential of the species to local conditions (Santos et al. 2011b, 2013), creating barriers to gene flow that may end up with new species formation (Burban et al. 2016, 2020). The exploration of the demographic history (Leblois et al. 2018) and of the underlying genetic mechanisms has just started (Gschloessl et al. 2014, 2018, 2022), and it could shed light on the speciation mechanism and species delimitation in the group.

The study of some traits of the life history as well as evidence from molecular data, mainly using the mitochondrial DNA-barcoding region, support the concept that the taxa included in the *T. pityocampa* complex are separately evolving lineages. Females of different species are very sedentary (Battisti et al. 2015) and possess distinct mitochondrial haplotypes that unambiguously set each taxon from others. The meta-populations of the five taxa are predominantly geographically separated, but when in contact they exhibit different phenological patterns, which minimizes but does not always prevent hybridization. Indeed, at least *T. pityocampa* and *T. wilkinsoni* were shown to produce hybrids both in laboratory and in the field (Petrucco-Toffolo et al. 2018; İpekdal et al. 2020). The production of fertile hybrids in the laboratory requires a strong manipulation of the phenology of the two taxa, thus enforcing very unnatural conditions. Furthermore, the occurrence in the field of hybrids is limited and restricted to a small overlapping portion of the distribution areas of *T. pityocampa* and *T. wilkinsoni* (İpekdal

et al. 2020). This phenomenon is typical of the early stages of differentiation of taxa that are separating according to a parapatric speciation mechanism. In *T. cretensis*, the speciation process seems to have progressed even further because this taxon has not been exchanging genetic material with other moths of the complex for a long time, aided by a complete geographic isolation within its distribution range (Petsopoulos et al. 2018). Information on *T. mediterranea* and *T. hellenica* is limited, but still some level of isolation seems to characterize both species as judged by DNA-barcoding outputs (present work; Trematerra et al. 2017; Trematerra and Colacci 2018).

5.   Conclusion

The five taxa included in the *T. pityocampa* complex seem to fulfil the fundamental property of the unified concept of species (De Queiroz 2007), i.e., they appear to evolve as separate lineages. However, it seems that the level of differentiation has not yet achieved the complete isolation in terms of reproduction for all taxa, and some of them still occupy grey zones within the cladogenetic branching pattern (De Queiroz 2007). Available knowledge favours the view that taxa within the *T. pityocampa* complex are experiencing a process of speciation. Thus, in this we suggest considering them as separate taxa, an approach that allows full appreciation to the biological diversity in this group of moths.

6.   Author contributions

Andrea Basso: conceptualization (equal); data curation (equal); formal analysis (equal); methodology (lead); writing (equal). Dimitrios Avtzis: data curation (supporting); methodology (supporting); writing (supporting). Christian Burban: data curation (equal); formal analysis (equal); writing (supporting). Carole Kerdelhué: conceptualization (equal); data curation (equal); formal analysis (supporting); writing (equal). Kahraman İpekdal: data curation (equal); formal analysis (supporting); writing (equal). Emmanuelle Magnoux: data curation (equal); formal analysis (equal); writing (supporting). Jérôme Rousselet: data curation (equal); formal analysis (equal); writing (supporting). Enrico Negrisolo: conceptualization (equal); data curation (equal); formal analysis (lead); methodology (equal); writing (equal). Andrea Battisti: conceptualization (lead); data curation (equal); formal analysis (equal); funding acquisition (lead); writing (lead).

7.   Statements

8.   Acknowledgements

9.   References

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Supplementary Material 1

File S1

Authors: Basso A, Avtzis D, Burban C, Kerdelhué C, İpekdal K, Magnoux E, Rousselet J, Negrisolo E, Battisti A
      (2023)
Data type: .docx
Explanation note: List of morphological traits used in the current work.
Link: https://doi.org/asp.81.e102928.suppl1

Supplementary Material 2

Files S2a, S2b

Authors: Basso A, Avtzis D, Burban C, Kerdelhué C, İpekdal K, Magnoux E, Rousselet J, Negrisolo E, Battisti A
      (2023)
Data type: .zip
Explanation notes: Files S2a and **S2b**. Morphological matrices in nexus format of the traits collected from specimens
      analysed (**a** male; **b** female). Outgroups were coded together.
Link: https://doi.org/asp.81.e102928.suppl2

Supplementary Material 3

Tables S1, S2

Authors: Basso A, Avtzis D, Burban C, Kerdelhué C, İpekdal K, Magnoux E, Rousselet J, Negrisolo E, Battisti A
      (2023)
Data type: .zip
Explanation notes: Table S1. (a) Individuals used for morphological analyses indicating the geographic zone and co-
      ordinates. Countries were expressed in digram, and localities were scored by capital letters (A–Z). Specimens were
      noted with progressive numbers. N/A means Not Available. **(b)** Individuals used for molecular analyses, with the
      indication of the geographic zone and coordinates. Countries were expressed in digram, and localities were scored
      by capital letters (A–Z). Specimens were noted with progressive numbers. Geographic Zone (GZ) links country and
      locality to Figs S7–S12. *, indicate the neotype of *T. pityocampa*. Records reported in grey were excluded because
      they reported identical sequences to those already considered. Outgroup sequences were reported in blue. — **Table
      S2.** Principal component (PC) correlation corrected by Eigenvalue on male and female morphological traits, which
      explain respectively 34.59% and 39.59% of the total variation.
Link: https://doi.org/asp.81.e102928.suppl3

Supplementary Material 4

Figures S1–S13

Authors: Basso A, Avtzis D, Burban C, Kerdelhué C, İpekdal K, Magnoux E, Rousselet J, Negrisolo E, Battisti A (2023)

Data type: .zip

Explanation notes: Figure S1. Description of *la chenille du pin* by Réaumur (1736). Permission to reproduce the material from the Réaumur collection was obtained from the Bibliothèque du Muséum des Sciences Naturelles, Paris France. — **Figure S2.** (**a**) Principal Component Analyses of morphological traits derived from male *Thaumetopoea.* PC1-3 express the 34.59% of variation through correlation analysis. (**b**) Principal Component Analyses of morphological traits derived from female *Thaumetopoea.* PC1-3 express the 39.59% of variation through correlation analysis. ○ indicate outgroups. Coloured circles indicate specimens of *Thaumetopoea pityocampa-wilkinsoni* complex: ● (green) *T. cretensis*; ● (blue) *T. hellenica*; ● (pink) *T. mediterranea*; ● (black) *T. pityocampa*; ● (brown) *T. wilkinsoni.* — **Figure S3.** (**a**) Maximum likelihood tree (-ln= 3829.3187) inferred from male morphological matrix including 182 specimens and using MK+FQ+I+G4 substitution model. (**b**) Maximum likelihood tree (-ln= 2261.2115) inferred from female morphological matrix including 132 specimens and using MK+FQ+ASC+R3 substitution model. Colours indicate the clades: ● (green) *T. cretensis*; ● (blue) *T. hellenica*; ● (pink), *T. mediterranea*; ● (black) *T. pinivora*; ● (orange) *T. pityocampa*; ● (brown) *T. wilkinsoni.* — **Figure S4.** (**a**) Phylogram from NJ phylogenetic analysis computed with Kimura 2-parameter evolutionary model and bootstrap test (10,000 replicates). Bold taxa indicate reference sequences from BOLD Systems; *, indicates sequence from neotype. Bootstrap values are reported at nodes. The scale bar scores the number of base substitutions per site. (**b**) Phylogram (-ln= 4358.6671) from ML phylogenetic analysis computed with Kimura 2-parameter evolutionary model. Bold taxa indicate reference sequences from BOLD Systems; *, indicates sequence from neotype. Numbers at nodes report the values of SH-aLRT, Ultrafast bootstrap and Boostratp tests. The scale bar scores the number of base substitutions per site. — **Figure S5.** On the left, unique haplotypes of *T. hellenica* are assigned to a colour and plotted on the map (pin coloured as in Fig. 1). The scale shows the number of samples analysed. The table shows the Geographic Zone (GZ), the colour assigned to the haplotype (HT); the databases accession number (Specimens code); species assigned post phylogenetic analysis (Species), and the number of the samples analysed (Frequencies). On the right, the TCS network of the haplotypes is reported according to the haplotype colour. ○ means undetected ancestors, while | indicates nucleotide-substitution occurred between linked haplotypes. — **Figure S6.** On the left, unique haplotypes of *T. mediterranea* are assigned to a colour and plotted on the map (pin coloured as in Fig. 1). The scale shows the number of samples analysed. The table shows the Geographic Zone (GZ), the colour assigned to the haplotype (HT); the databases accession number (Specimens code); species assigned post phylogenetic analysis (Species), and the number of the samples analysed (Frequencies). On the right, the TCS network of the haplotypes is reported according to the haplotype colour. ○ means undetected ancestors, while | indicates nucleotide-substitution occurred between linked haplotypes. — **Figure S7.** On the left, unique haplotypes of *T. cretensis* are assigned to a colour and plotted on the map (pin coloured as in Fig. 1). The scale shows the number of samples analysed. The table shows the Geographic Zone (GZ), the colour assigned to the haplotype (HT); the databases accession number (Specimens code); species assigned post phylogenetic analysis (Species), and the number of the samples analysed (Frequencies). On the right, the TCS network of the haplotypes is reported according to the haplotype colour. ○ means undetected ancestors, while | indicates nucleotide-substitution occurred between linked haplotypes. — **Figure S8.** On the left, unique haplotypes of *T. wilkinsoni* are assigned to a colour and plotted on the map (pin coloured as in Fig. 1). The scale shows the number of samples analysed. The table shows the Geographic Zone (GZ), the colour assigned to the haplotype (HT); the databases accession number (Specimens code); species assigned post phylogenetic analysis (Species), and the number of the samples analysed (Frequencies). On the right, the TCS network of the haplotypes is reported according to the haplotype colour. ○ means undetected ancestors, while | indicates nucleotide-substitution occurred between linked haplotypes. — **Figure S9a–c.** Unique haplotypes of *T. pityocampa* are assigned to a colour and plotted on the map (pin coloured as in Fig. 1). The scale shows the number of samples analysed. The table shows the Geographic Zone (GZ), the colour assigned to the haplotype (HT); the databases accession number (Specimens code); species assigned post phylogenetic analysis (Species), and the number of the samples analysed (Frequencies). — **Figure S10.** TCS network of *T. pityocampa* haplotypes reported according to the haplotype colour defined in Figure S9a–c. ○ means undetected ancestors, while | indicates nucleotide-substitution occurred between linked haplotypes. Dashed line boxes indicate the geographic clades discussed in the text. — **Figure S11.** *Thaumetopoea pityocampa* individuals from the collection of Guy Démolin (INRAE) originating from the collection site of the Réaumur individuals; **a** male dorsal view; **b** female dorsal view; **c** male canthus; **d** female canthus; **e** male genitalia; **f** female anal scale. — **Figure S12.** *Thaumetopoea cretensis* paratypes. Details of male genitalia, female anal scales, and canthus. — **Figure S13.** *Thaumetopoea cretensis* paratypes. Details of canthus in males (left) and females (right).

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