Journal of Economic Entomology, XX(X), 2018, 1–5
doi: 10.1093/jee/toy047
Research article

Forest Entomology

Revisiting the Distribution of *Thaumetopoea pityocampa* (Lepidoptera: Notodontidae) and *T. pityocampa* ENA Clade in Greece

Dimitrios N. Avtzis,[1] Dimitrios Petsopoulos,[1] George I. Memtsas,[1] Nickolas G. Kavallieratos,[2,3,6] Christos G. Athanassiou,[4] and Maria C. Boukouvala[2,3,5]

[1]Laboratory of Forest Entomology, Forest Research Institute, Hellenic Agricultural Organization Demeter, Vassilika, 57006 Thessaloniki, Greece, [2]Laboratory of Agricultural Zoology and Entomology, Department of Crop Science, Agricultural University of Athens, 75 Iera Odos Street, 11855 Athens, Attica, Greece, [3]Laboratory of Agricultural Entomology, Department of Entomology and Agricultural Zoology, Benaki Phytopathological Institute, 8 Stefanou Delta Street, 14561 Kifissia, Attica, Greece, [4]Laboratory of Entomology and Agricultural Zoology, Department of Agriculture Crop Production and Rural Environment, University of Thessaly, Phytokou Street, Nea Ionia, 38446 Magnissia, Greece, [5]Laboratory of Organic Chemistry, Department of Chemistry, University of Ioannina, Panepistimioupolis, 45110 Ioannina, Greece, and [6]Corresponding author, e-mail: nick_kaval@hotmail.com

Subject Editor: Timothy Schowalter

Received 26 November 2017; Editorial decision 30 January 2018

Abstract

In the present work, we sampled individuals of the processionary pine moth, *Thaumetopoea pityocampa* (Denis and Schiffermüller; Lepidoptera: Notodontidae) from different areas of Greece between 2014 and 2016. These samples were sequenced for a 760-bp long mtDNA COI locus and the haplotypes retrieved clearly showed that the occurrence of *T. pityocampa* in Greece is being considerably restricted, with only 8 individuals out of the 221 exhibiting *T. pityocampa* haplotypes and the rest being identified as *T. pityocampa* ENA clade haplotypes. To that, one haplotype in particular exhibited the highest abundance and broadest geographic distribution, occurring both in mainland and on islands. Our data suggest a rather recent and rapid population expansion of the ENA clade in Greece and a concomitant recent displacement of *T. pityocampa*. It thus seems that the relation between *T. pityocampa* and *T. pityocampa* ENA clade needs to be further and thorough analyzed before the taxonomic status of *T. pityocampa* ENA clade can be concluded with confidence.

Key words: *Thaumetopoea pityocampa*, *T. pityocampa* ENA clade, mtDNA, expansion, displacement

Thaumetopoea (Lepidoptera: Notodontidae) species include some of the most important pests of conifer and broadleaved trees (Battisti et al. 2015), occurring mostly in the Mediterranean and Iranoturanic regions (Kiriakoff 1970, Basso et al. 2017). Apart from their profound ecological impact, most of these species pose also a serious health threat for humans and animals due to the urticating setae they possess (Battisti et al. 2011), which may cause serious respiration disorders, skin and eye irritations (Vega et al. 2011). One of the most notorious species of this genus, combining both ecological and health issues, is the processionary pine moth, *Thaumetopoea pityocampa* (Denis and Schiffermüller; Lepidoptera: Notodontidae), and for that its distribution, ecology and behavior have been extensively studied in the western parts of the Mediterranean basin (Athanassiou et al. 2007, Kerdelhué et al. 2015, Korsch et al. 2015, Athanassiou et al. 2017, Colacci et al. 2018). Even though its sister species, the Cyprus processionary caterpillar, *Thaumetopoea wilkinsoni* Tams predominates in the Eastern Mediterranean basin, it has been recently found to coexist in some areas with *T. pityocampa* (Kerdelhué et al. 2015). Despite the extent of these investigations, only recently it has become known that in addition to these two already known pine infesting species, that broadly occur in the Mediterranean basin, there is a distinctly diverged mtDNA clade located in north Algeria, Tunisia and Libya (East-North Africa), which has therefore been defined in a preliminary way as ENA clade (Kerdelhué et al. 2009). Until recently, Greece was considered to occur on the borderline between *T. pityocampa* and *T. wilkinsoni*, with the former inhabiting Greek mainland and islands of the north Aegean and Ionian Sea, and *T. wilkinsoni* being restricted on islands of the south Aegean Sea (e.g., Crete, Cyprus, Samos, Rhodes) (Kerdelhué et al. 2009). The occurrence of *T. pityocampa* has been verified by Korsch et al. (2015), who analyzed 15 populations in an effort to conclude about its phylogeography. Nevertheless, not much later, haplotypes belonging to ENA clade have been detected for the first time in a region away from East-North Africa, namely Attica (Greece) (Avtzis et al. 2016). Out

of the 14 populations studied then, only one ('Gerakas') contained *T. pityocampa* haplotypes, with every other population exhibiting only ENA clade haplotypes. Furthermore, the fact that these haplotypes resembled those originating from Libya favored the scenario of human-mediated transport from that region (Avtzis et al. 2016), as this distance exceeds by far the active flight capacity of the species (Battisti et al. 2005, Battisti et al. 2015).

The occurrence of a contact zone between *T. pityocampa* and the mtDNA diverged ENA clade individuals has been implied by Kerdelhué et al. (2009) to occur in Algeria, and only recently it has been verified to be located in Maghreb (El Mokhefi et al. 2016). Nevertheless, the mechanism that lies behind this zone remains less clear due to the inconsistency between mtDNA and microsatellite data (El Mokhefi et al. 2016). As a consequence, the first detection of ENA clade mtDNA haplotypes in a place other than the place of origin (East-North Africa) cannot be easily interpreted as new species (Trematerra et al. 2017) but definitely justifies an even more thorough screening of populations with multiple markers as previous works were solely based on uniparental mtDNA markers (Korsch et al. 2015, Avtzis et al. 2016). Therefore, the present work aims to reassess the distribution of *T. pityocampa* and *T. pityocampa* ENA clade in an effort to better understand and delimit their taxonomic identity.

Materials and Methods

During 2014–2016, 221 larval individuals have been sampled from 13 different locations of Greece (Table 1). The selection of sampling sites was based on the findings of the previous study of ENA clade in Greece (Avtzis et al. 2016) and aimed at extending the investigation, not only in space (sampling new areas) but also in time (sampling populations that were previously analyzed). Particularly, the population 'Gerakas' that contained only *T. pityocampa* haplotypes (Avtzis et al. 2016) was sampled twice. Sampling was conducted during winter time, and only one larva per nest was sampled in order to avoid the induction of bias for the mtDNA analysis. Specimens were immediately put into Eppendorf filled with alcohol and were transported to the Laboratory of Forest Entomology (Forest Research Institute, Thessaloniki). DNA extraction was conducted with the Mammalian Genomic DNA Miniprep Kit (Sigma-Aldrich) following the manufacturer's instructions, with the only adaptation at the initial grinding phase, which was done with the help of small grinding balls. DNA amplification was run in 25 µl volumes, with Pat-Jerry primer pair (Simon et al. 1994) that aims at the Cytochrome Oxidase One mitochondrial gene. PCR protocol was identical with the one employed by Avtzis et al. (2016). Finally, purification was performed using the PureLink PCR Purification Kit (Invitrogen) following the manufacturer's protocol and the sequencing of purified products took place at CEMIA SA (Larissa, Greece) using an ABI 3730XL sequencer. Sequences were visually examined with Chromas Lite and then aligned using Clustal X (Thompson et al. 1997). In order to identify the taxonomic assignment of these sequences, they were aligned with haplotypes retrieved in the previous work by Avtzis et al. (2016) (Accession numbers: KT768149–KT768170). Population dynamics were estimated using Tajima's D (Tajima 1989) neutrality test and a mismatch distribution analysis performed by MEGA 6 (Tamura et al. 2013) and DNAsp (Librado and Rozas 2009). Finally, a network of ENA clade haplotypes retrieved was constructed using ANeCA (Panchal 2007), that employs TCS (Clement et al. 2000) and GeoDis (Posada et al. 2000) in order to demonstrate the geographical distribution of haplotypes and also conclude about the demographic processes that influenced the current distribution of genetic diversity.

Table 1. Populations sampled and haplotypes retrieved after the analysis of 221 individuals

	Population	Latitude	Longitude	Year	*N*	*T. pityocampa* ENA clade haplotypes	*T. pityocampa* haplotypes
Pop1	Halkida 1	38°26′48.18″N	23°44′22.14″E	2015	4	TPEna19[1]	TP01[2] ***TP04***[1]
Pop2	Halkida 2	38°27′54.73″N	23°35′5.42″E	2015	6	TPEna05[5]	TP01[1]
Pop3	Halkida 4	38°24′25.04″N	23°31′50.92″E	2015	9	TPEna04[1]–TPEna05[3] TPEna19[2]–***TPEna25***[1]	TP01[2]
Pop4	Gerakas	38°0′15.32″N	23°51′0.08″E	2015	10	TPEna19[2]–***TPEna24***[8]	
Pop5	Gerakas			2014	13	TPEna10[4]–TPEna19[4] ***TPEna20***[5]	
Pop6	Volos	39°21′41.82″N	22°56′32.07″E	2016	33	TPEna06[2]–TPEna19[7] ***TPEna21***[24]	
Pop7	Skiathos	39°9′47.20″N	23°29′24.58″E	2016	28	TPEna06[2]–TPEna10[7] TPEna19[19]	
Pop8	Skopelos	39°7′9.98″N	23°43′46.72″E	2016	15	TPEna19[15]	
Pop9	Varibobi	38°7′41.75″N	23°47′12.34″E	2016	6	TPEna05[6]	
Pop10	Amaroussion	38°3′38.95″N	23°48′58.95″E	2016	51	TPEna05[1]–TPEna08[1] TPEna19[22]–***TPEna23***[27]	
Pop11	Glyfada	37°52′49.95″N	23°46′25.58″E	2016	6	TPEna09[1]–TPEna19[5]	
Pop12	Kifissia	38°5′8.73″N	23°50′8.51″E	2016	16	TPEna03[16]	
Pop13	Egina	37°43′12.75″N	23°30′1.25″E	2016	6	TPEna10[2]–TPEna19[3] ***TPEna26***[1]	
Pop14	Poros	37°31′13.93″N	23°28′17.89″E	2016	18	TPEna09[2]–TPEna10[1] TPEna19[11]–***TPEna22***[2]	TP01[2]
Total					221	213	8

Haplotypes identified in this study (TPEna20-26 and TP04) are bold, whereas the other haplotypes (TPEna01-19 and TP01-03) have been identified before (Avtzis et al. 2016). In brackets is the number of individuals that share the same haplotype.

Results

Analysis of the 760 base pairs from the 221 individuals collected from the locations described in Table 1, revealed that *T. pityocampa* has a much more limited geographic distribution in Greece than previously thought. Only eight individuals contained *T. pityocampa* haplotypes, seven of which belonged to haplotype TP01 (Avtzis et al. 2016) and only one individual bearing a new haplotype (TP04). The remaining 213 individuals all belonged to ENA clade exhibiting in total 15 haplotypes. Out of the 15 haplotypes, six (TPEna20-25) were found for the first time (GenBank Accession Numbers: MG808366-MG808372), whereas the other nine have already been described by Avtzis et al. (2016). TPEna19 was the most abundant ENA clade haplotype among the 213 ENA clade individuals analyzed, being detected in 91 individuals, but also the most widespread, being found in areas remote to Attica. This is congruent with the previous study that already identified TPEna19 as the most abundant ENA clade haplotype in Greece (Avtzis et al. 2016). ENA clade haplotypes were retrieved even in two populations that contained only *T. pityocampa* haplotypes in previous studies, namely 'Gerakas' (Pops 4 + 5) (Avtzis et al. 2016) and 'Halkida 2' (Pop2) (Korsch et al. 2015). Co-occurrence of ENA clade and *T. pityocampa* haplotypes observed in all populations of 'Halkida' (Pops 1–3) and the population of 'Poros' (Pop 14). 'Skiathos' and 'Skopelos', the islands located near Volos were found to be inhabited only by ENA clade haplotypes (Fig. 1, Table 1). Finally, mismatch analysis (unimodal distribution; data not shown here) and Tajima's neutrality test ($D = -1.80847$, $P < 0.05$) both indicated a recent population expansion of ENA clade haplotypes in Greece. Finally, the congruence between the haplotypes retrieved in this study with the previous ENA clade haplotypes (Avtzis et al. 2016) is further demonstrated by NCA, with the new haplotypes blending harmonically into the network (Fig. 2). Demographic inference was drawn for the major sub clusters of haplotypes (namely 2-1 and 2-2), but not for the total cladogram; for both clusters it was suggested that restricted gene flow with isolation by distance were the processes that gave rise to the observed pattern of genetic diversity (Fig. 2).

Discussion

Even though the circum-Mediterranean distribution of *Thaumetopoea* species has been thoroughly studied and circumscribed (Salvato et al. 2002, Simonato et al. 2007, Kerdelhué et al. 2009), recent investigations suggest that the exact delimitation of each species distribution is more difficult to be concluded. To that, it has been shown that human-mediated transport can easily create infestation points beyond the natural distribution of a species (Robinet et al. 2012, Avtzis et al. 2016), whereas the frequent hybridization among species proves to be an almost impenetrable difficulty in accurately defining the geographic between species (İpekdal et al. 2015, El Mokhefi et al. 2016, Petrucco Toffolo et al. 2018) and human-mediated transport (Robinet et al. 2012, Avtzis et al. 2016). Apparently, hybridization between closely related species is not uncommon in Lepidoptera (Mallet et al. 2007, Gompert et al. 2010); nevertheless, it is often difficult to disentangle the interactions between the species involved (Bridle and Vines 2007). In the *T. pityocampa/ wilkinsoni* complex, the contact zone that occurs in Anatolia shows signs of asymmetrical introgression, as *T. wilkinsoni* individuals identified by nuclear DNA, were found to be carrying mtDNA haplotypes of *T. pityocampa*, but never *T. pityocampa* individuals carrying mtDNA haplotypes of *T. wilkinsoni* (İpekdal et al. 2015).

Kerdelhué et al. (2009) were the first to imply that a similar contact zone should be located in Algeria between ENA clade and *T. pityocampa*, something that was investigated and verified a few years later (El Mokhefi et al. 2016). Even though the location of this contact zone was accurately defined, it was not feasible to infer whether ENA clade is indeed a separate taxonomic unit, as it has been suggested by Simonato et al. (2013), because of the inconsistency between the two different markers (mtDNA and microsatellites) employed (El Mokhefi et al. 2016). The discovery of ENA

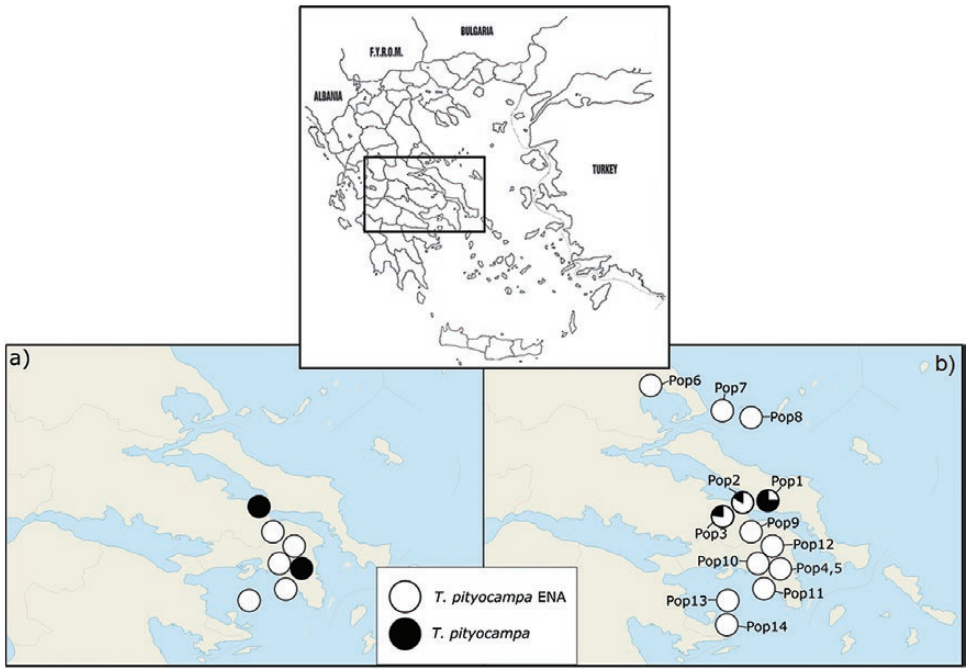

Fig. 1. Map showing the distribution of *T. pityocampa* ENA clade against *T. pityocampa* in previous studies (Korsch et al. 2015, Avtzis et al. 2016) (a) and the current study (b). On the top is map of Greece.

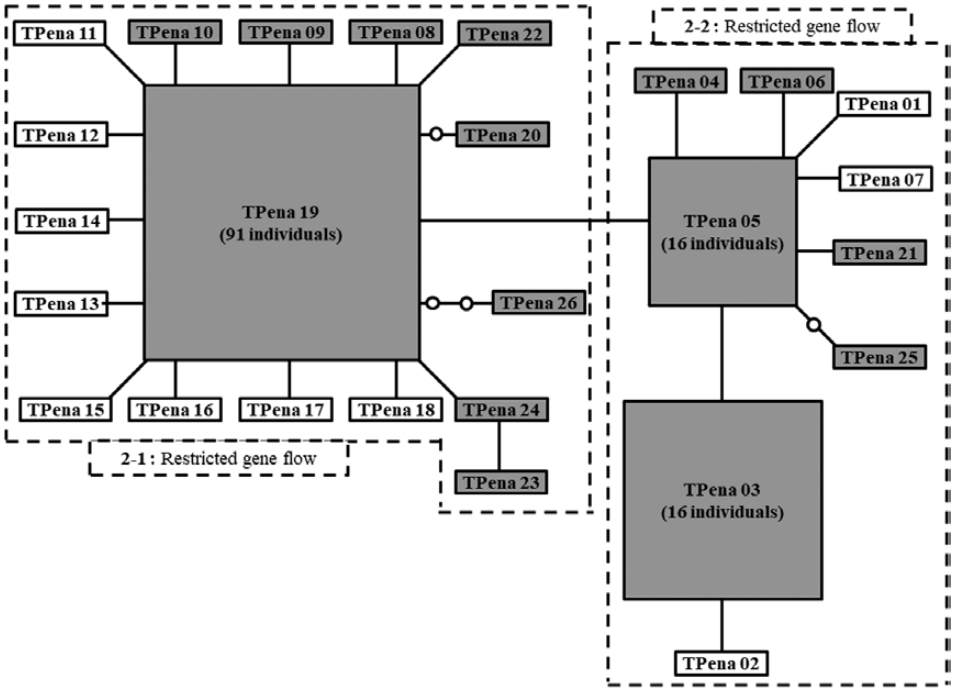

Fig. 2. ENA clade haplotype network. Haplotypes retrieved in the current study are gray shaded, while the haplotypes that were identified only in Avtzis et al. (2016) are white. Empty circles indicate missing haplotypes. The number of individuals that share the same haplotype are shown in parentheses for TPEna03, TPEna05 and TPEna19. Dotted lines separate the two major subclades (2-1 and 2-2) of the analysis complemented by the demographic inferences concluded.

clade haplotypes in Greece complicated further the clarification of its taxonomic status, as the only plausible scenario supporting this finding involved a human-mediated translocation from Africa (Avtzis et al. 2016). A thorough sampling repeated in the same areas of Attica, complemented with other near-by located sites, demonstrated that ENA clade haplotypes is not only abundant and established in Attica, but shows also indications of replacing *T. pityocampa* in 'Halkida' (Pops 1–3) and 'Gerakas' (Pops 4–5) that in previous and recent studies was absent (Korsch et al. 2015, Avtzis et al. 2016). Given the short period of time between the successive samplings that show the replacement of *T. pityocampa* in concert with the discrepancy demonstrated between different markers in previous studies (El Mokhefi et al. 2016), one cannot exclude the possibility of mtDNA introgression from ENA clade to *T. pityocampa*. MtDNA has been shown to be prone to introgress between initially allopatric, interbreeding species that came in sympatry afterwards (Lehman et al. 1991, Mallet 2005, Daras and Aron 2015). More to that, this introgressive hybridization is far more frequent for mitochondrial than nuclear DNA to such an extent that a complete mtDNA replacement is evident with little or no nuclear introgression (Zakharov et al. 2009, Pons et al. 2014, Zielinski et al. 2013). However, mtDNA introgression was often asymmetric, directing mostly from the local to the colonizing species (Toews and Brelsford 2012). However, in due course, this asymmetry has been found to weaken and gradually evolved into reciprocal introgression (Mastrantonio et al. 2016). In our case however, the alleged colonizing lineage (ENA clade) seems to be replacing what was supposed to be the local one (*T. pityocampa*). This observation supports the hypothesis that the initial colonization occurred a long time ago, providing the time necessary not only to overturn the asymmetry but actually become established in the region. The occurrence of the most abundant ENA clade haplotype (TPEna 19) in remote or even isolated areas indicates also the intervention of a given time since its initial introduction.

Summarizing the findings of the current investigation with previous studies on *T. pityocampa* ENA clade (Avtzis et al. 2016), it becomes evident that resolving the taxonomic status of this distinct mtDNA clade is more complicated than previously thought (Trematerra et al. 2017), requires the analysis of many more individuals and cannot be solely relied on a single, uniparentally inherited marker due to incongruence with nuclear DNA (El Mokhefi et al. 2016). For that, a deeper and thorough examination of morphological traits (Petrucco Toffolo et al. 2018) coupled with molecular data from several markers could possibly provide a valid explanation of its taxonomic assignment.

Acknowledgments

This study was partially funded by the EU grant 'Innovative Eco Friendly Traps for the Control of Pine Lepidoptera in Urban and Recreational Places' (LIFE PISA: LIFE13 ENV/ES/000504). We would like to thank Dr Antonios Michaelakis and Dr Dimitrios P. Papachristos (Benaki Phytopathological Institute) for providing samples from the region of Attica.

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