

New Data on the Range Expansion of the *Thaumetopoea pityocampa* (Lepidoptera: Notodontidae) ‘ENA clade’ in Greece: The Role of Bacterial Endosymbionts

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Abstract

The pine processionary moth, *Thaumetopoea pityocampa* (Denis and Schiffermüller), is an important insect in the Mediterranean region, as it defoliates pines and its urticating hairs can cause allergic reactions in humans and animals. Moreover, this species exhibits an interesting genetic structure as recently a distinct East-North African mtDNA lineage (‘ENA clade’) has been described. This clade has been recently detected in Greek populations where it has currently expanded its range by replacing the ‘endemic’ *T. pityocampa* lineages. Here, we report new data on the rapid spread of ‘ENA clade’ in the Greek island Evoia in only a few years. As the underlying mechanisms of the ‘ENA clade’ range expansion has not been studied so far, we screened *T. pityocampa* for an infection with the heritable bacterial endosymbionts *Wolbachia* (Bacteria: Anaplasmataceae), *Cardinium* (Bacteria: Bacteroidaceae), *Rickettsia* (Bacteria: Rickettsiaceae) and *Spiroplasma* (Bacteria: Spiroplasmataceae). These bacteria can manipulate the reproduction of infected hosts, something that could potentially explain the rapid spread of ‘ENA clade’ lineage. Therefore, we screened 28 individuals that exhibited *T. pityocampa* ‘ENA clade’ and ‘endemic’ *T. pityocampa* haplotypes from nine populations scattered all over Greece. None of them was infected with any of the four endosymbionts, suggesting that these bacteria do not cause reproductive manipulations in *T. pityocampa* lineages and, thus, other factors should be explored in future research efforts.

Key words: pine processionary moth, Greece, bacterial endosymbionts, *Wolbachia*

Among different pine defoliating insects that occur in the Mediterranean region, the pine processionary moth, *Thaumetopoea pityocampa* (Denis and Schiffermüller) is one of the most intriguing ones, since it defoliates pines (Battisti et al. 2015) and affects health of humans or animals through the urticating setae of the larvae (Battisti et al. 2011; Vega et al. 2011). Consequently, numerous studies have been focused on various aspects of this species, such as the evaluation of novel trap devices for the capture of male adults (Athanassiou et al. 2017) and larvae (Colacci et al. 2018),

the analysis of its phylogeny and phylogeography (Kerdelhué et al. 2009; Rousselet et al. 2010), or the unraveling of its genetic architecture on genomic and transcriptomic level (Gschloessl et al. 2018). One of the most recently emerged questions regards a mitochondrial lineage that was found for the first time in East-North Africa (called *T. pityocampa* ‘ENA clade’) (Kerdelhué et al. 2009) and was recently detected (2012 and 2013) near Athens, Greece (Avtzis et al. 2016). Since then, it is spreading rapidly by replacing the ‘endemic’ lineages of *T. pityocampa* (Avtzis et al. 2018).

The replacement of the ‘endemic’ *T. pityocampa* by the *T. pityocampa* ‘ENA clade’ has been documented through a series of consecutive studies, starting with the first attempt to investigate the phylogeography of *T. pityocampa* in Greece (Korsch et al. 2015). When individuals were collected from the largest city of Evoia (i.e., Halkida), which is the second largest island of Greece, and screened for the first time, they were solely assigned to the ‘endemic’ *T. pityocampa* haplotypes (Korsch et al. 2015). However, when individuals from Halkida were analyzed in a subsequent study (2014–2016), only 6 out of 19 individuals exhibited ‘endemic’ haplotypes and the majority belonged to *T. pityocampa* ‘ENA clade’ haplotypes (Avtzis et al. 2018). This finding showed the recent emergence of an active contact zone between the ‘endemic’ *T. pityocampa* and the *T. pityocampa* ‘ENA clade’, which has already been implied by Kerdelhué et al. (2009) but verified only recently in the Mahgreb region (El Mokhefi et al. 2016). However, as the latter study revealed contrasting patterns between mitochondrial and nuclear markers, the expansion dynamics of the *T. pityocampa* ‘ENA clade’ remains vague. Furthermore, underlying mechanisms that could drive the recent expansion of the *T. pityocampa* ‘ENA clade’ in Greece have been unknown as well. Changing range patterns of this clade could be a result of an infection with maternally heritable, bacterial endosymbionts. These bacteria, which are found in numerous arthropod species (Weinert et al. 2015), can manipulate the reproduction of their hosts by expressing reproductive phenotypes, such as cytoplasmic incompatibility (CI), feminization, male-killing, or parthenogenesis (Engelstadter and Hurst 2009). Common endosymbionts in terrestrial arthropods are *Wolbachia* (Bacteria: Anaplasmataceae), *Cardinium* (Bacteria: Bacteroidaceae), *Rickettsia* (Bacteria: Rickettsiaceae) and *Spiroplasma* (Bacteria: Spiroplasmataceae) (Weinert et al. 2015). By altering the reproductive outcome of endosymbiont-harboring hosts, these bacteria provide fitness benefits to infected individuals compared to uninfected conspecifics (Hurst and Jiggins 2005; Werren et al. 2008; Engelstadter and Hurst 2009). Therefore, we hypothesize that the current spread of the *T. pityocampa* ‘ENA clade’ is due to endosymbiont-related reproductive advantages of this lineage compared with ‘endemic’ mitochondrial haplotypes, yielding in replacement of the latter ones.

In order to understand the expansion dynamics of the *T. pityocampa* ‘ENA clade’ in Greece, we studied its spread in the inner parts of Evoia but also estimated the sampling effort needed to capture most of the haplotype diversity (Phillips et al. 2015) of both mitochondrial lineages. The latter would pinpoint the crucial number of individuals necessary to sufficiently describe their genetic structure in Greece, an important basis for future studies. Finally, we assessed an endosymbiont-driven influence on the expansion-replacement

dynamics of *T. pityocampa* lineages by screening individuals for *Cardinium*, *Rickettsia*, *Spiroplasma*, and *Wolbachia* in order to shed light on underlying mechanisms.

Materials and Methods

Sampling and Expansion of the *T. pityocampa* ‘ENA clade’ in Evoia, Greece

In 2017, 69 individuals (larvae) were collected from eight locations in Evoia (Table 1), which roughly cover the occurrence of pines on the island. In order to avoid any bias due to the analysis of siblings, only one individual per nest was collected. Specimens were stored in absolute ethanol and transported to the Laboratory of Forest Entomology (Forest Research Institute, Thessaloniki, Greece). DNA extraction from head capsules was conducted using the Mammalian Genomic DNA Miniprep Kit (Sigma–Aldrich, St. Louis, MO) following the manufacturer’s protocol with a slight modification in the grinding stage (Avtzis et al. 2018). To study the genetic structure on the mitochondrial level, polymerase chain reaction (PCR) was run in 25-μl volumes, with Pat/Jerry primers (Simon et al. 1994) that amplify a fragment of the mitochondrial COI gene (Avtzis et al. 2018). DNA was purified using the PureLink PCR Purification Kit (Invitrogen) following the manufacturer’s protocol and sequencing was performed at CEMIA SA (Larissa, Greece). After sequencing, reads were manually checked using Chromas Lite version 2.01 to exclude any case of base misincorporation as a result of a PCR error (Kobayashi et al. 1999) and subsequently aligned with the software package Clustal X (Thompson et al. 1997).

To estimate the sampling effort to describe the species’ genetic diversity, a modified version of Species Accumulation Curves known as Haplotype Accumulation Curves (Phillips et al. 2015) was constructed using the R software package (R CoreTeam 2015) with the function *specaccum* of the vegan software package (Oksanen et al. 2016) and, then, a power model was fit to the curves (Arrhenius 1921).

Screening for Endosymbionts

To assess an influence of heritable bacterial endosymbionts on range changes of *T. pityocampa* mitochondrial lineages, we performed a PCR screening with taxon-specific primers. In total, 28 individuals from nine populations all over Greece (Table 2) were analyzed for an infection with the bacteria *Cardinium*, *Rickettsia*, *Spiroplasma*, and *Wolbachia*.

PCR reactions for all four endosymbionts were performed as described in Schebeck et al. (2018). To study an infection of

Table 1. Sampled locations and haplotype assignment of *T. pityocampa* individuals in the current study

Population	Latitude	Longitude	Year	N	<i>T. pityocampa</i> ‘ENA clade’ haplotypes	‘endemic’ <i>T. pityocampa</i> haplotypes
1 Aliveri	38°24'33.41"N	24°2'24.23"E	2017	10	TPena5 [1]	Haplotype1 [1]; Haplotype10 [8];
2 Amarynthos	38°23'36.00"N	23°53'11.54"E	2017	6		Haplotype1 [6]
3 Eretria	38°25'9.68"N	23°52'55.33"E	2017	11		Haplotype1 [1]; Haplotype10 [9]; Haplotype16 [1]
4 Karystos	38°0'35.98"N	24°25'22.37"E	2017	10	TPena19 [10]	
5 Kymi	38°38'7.77"N	24°6'7.64"E	2017	11	TPena5 [2]	Haplotype1 [9]
6 Pefki	39°0'33.76"N	23°12'46.80"E	2017	5		Haplotype1 [5]
7 Psahna	38°34'38.95"N	23°38'30.25"E	2017	6		Haplotype1 [5]; Haplotype17 [1]
8 Styra	38°10'40.51"N	24°12'38.39"E	2017	10	TPena19 [10]	

Haplotype designation follows previous studies (Korsch et al. 2015; Avtzis et al. 2016), while the two new *T. pityocampa* haplotypes identified in this study (Haplotype16 and Haplotype17) follow the numbering of Korsch et al. (2015) and are highlighted in bold. The number of individuals exhibiting the same haplotype is given in brackets.

Table 2. Locations and individuals with their respective mitochondrial haplotypes that were screened for *Wolbachia*, *Cardinium*, *Spiroplasma*, and *Rickettsia*

	Population	Latitude	Longitude	N	Haplotype assignment		Reference
					<i>T. pityocampa</i> 'ENA clade'	'endemic' <i>T. pityocampa</i>	
1	Glyka Nera	37°59'24.63"N	23°50'49.68"E	2	TPena5 [1]; TPena10 [1]		Avtzis et al. (2016)
2	Kifissia	38°5'8.73"N	23°50'8.51"E	3	TPena3 [3]		Avtzis et al. (2016)
3	Gerakas	38°0'15.32"N	23°51'0.08"E	5	TPena10 [3]; TPena20 [2]		Avtzis et al. (2018)
4	Amaroussion	38°3'38.95"N	23°48'58.95"E	2	TPena19 [2]		Avtzis et al. (2018)
5	Skiathos	39°9'47.20"N	23°29'24.58"E	2	TPena10 [2]		Avtzis et al. (2018)
6	Skopelos	39°7'9.98"N	23°43'46.72"E	2	TPena19 [2]		Avtzis et al. (2018)
7	Thessaloniki	40°38'9.16"N	22°58'9.45"E	3		Haplotype1 [3]	Korsch et al. (2015)
8	Shinias	38°4'6.21"N	22°38'0.67"E	5		Haplotype1 [3]; Haplotype09 [2]	Korsch et al. (2015)
9	Edessa	40°53'7.24"N	21°48'6.05"E	4		Haplotype3 [3]; Haplotype14 [1]	Korsch et al. (2015)

References indicate where these individuals were screened for first time. The number of individuals that exhibit the same haplotype is given in brackets.

T. pityocampa with *Cardinium*, we used the primer pair CLO-F1/CLO-R1 (Weeks et al. 2003), with *Cardinium*-infected silverleaf whitefly, *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae) as positive control. To detect *Rickettsia*, PCR runs were performed with the forward primer 528F and the reverse primer 1044R (Chiel et al. 2009). *Rickettsia*-harboring *B. tabaci* individuals were used as positive controls. An infection with the bacterium *Spiroplasma* was studied by using the primers Spixof and Spixor (Duron et al. 2008) and *Spiroplasma*-infected smaller European spruce bark beetle, *Pityogenes chalcographus* (L.) (Coleoptera: Curculionidae) as positive control. To study an infection with *Wolbachia*, PCR reactions were performed with the forward primer 81F and the reverse primer 691R (Braig et al. 1998; Zhou et al. 1998). *Wolbachia*-harboring cherry fruit fly, *Rhagoletis cerasi* (L.) (Diptera: Tephritidae) individuals were used as positive controls. The PCR products were separated via gel electrophoresis using a 2% agarose gel, stained with GelRed nucleic acid dye (Biotum, Hayward, CA).

Results

Mitochondrial Genotyping of *T. pityocampa*

Sampling and analyzing individuals from eight populations from the island of Evoia resulted in two already described *T. pityocampa* 'ENA clade' haplotypes (TPena5 and TPena19) (Avtzis et al. 2016) and four 'endemic' *T. pityocampa* haplotypes (Korsch et al. 2015). Two of the latter are found for the first time (Haplotype 16 and Haplotype 17). Populations located in the central parts of Evoia contained a mixture of 'endemic' and 'ENA clade' haplotypes, while southern populations harbored only *T. pityocampa* 'ENA clade' haplotypes (Table 1; Fig. 1).

Genetic data from previous work in Greece, supplemented with those from the current study, were used for the construction of Haplotype Accumulation Curves for both *T. pityocampa* 'ENA clade' and 'endemic' *T. pityocampa*. An asymptote was reached for none of them. Nevertheless, the fit of the power-law models provided two very different slopes for them, with values of 0.26 (*T. pityocampa* 'ENA clade'; Fig. 2) and 0.46 ('endemic' *T. pityocampa*; Fig. 3).

Endosymbiont Infection of *T. pityocampa* lineages

PCR screening of a total 11 individuals of *T. pityocampa* 'ENA clade' and 17 individuals of 'endemic' *T. pityocampa* did not reveal an infection with any of the four bacterial endosymbionts. None of the specimens showed a positive banding pattern in the gel electrophoresis for fragments in the size range of *Cardinium*, *Rickettsia*, *Spiroplasma*, or *Wolbachia*, targeted by taxon-specific primers.

Discussion

The detection of the *T. pityocampa* 'ENA clade' in Greece (Avtzis et al. 2016) has triggered a series of related studies on the expansion (Avtzis et al. 2018) and the taxonomic status of this new lineage (Trematerra et al. 2017). The potential of the *T. pityocampa* 'ENA clade' to expand its distribution is clearly shown in the current study although a few years ago Halkida contained only *T. pityocampa* haplotypes (Korsch et al. 2015). Therefore, the 'ENA clade' haplotypes gradually invaded these regions (Avtzis et al. 2018) and, finally, became established in certain populations in the inner parts of the island. The haplotypes that spread in Evoia (TPena5 and TPena19) are the same as the ones detected by Avtzis et al. (2018), indicating a recent demographic expansion. Interestingly, none of these haplotypes expanded northward, with both remaining in the central and southern populations of the island.

The sufficiency of a sample size is strongly dependent on the research question to be answered coupled with features of the organism to be studied and the methods applied (Lenth 2001). In the intraspecific genetic studies, the adequate sample size is directly related to a species' genetic variation and, therefore, it can vary across taxa, even across the closely related ones (Coeur d'acier et al. 2014). According to previous studies (Zhang et al. 2010), sample sizes of both *T. pityocampa* 'ENA clade' and 'endemic' *T. pityocampa* fell within the size range needed to reveal most of the genetic diversity. Interestingly, the slope for these lineages differed significantly, with 'endemic' *T. pityocampa* exhibiting a value almost twice as high compared to *T. pityocampa* 'ENA clade', corroborating that the *T. pityocampa* 'ENA clade' has only recently entered and expanded in Greece.

Our PCR screening on the presence of *Cardinium*, *Rickettsia*, *Spiroplasma*, and *Wolbachia* does not suggest that Greek *T. pityocampa* is infected with bacteria that can manipulate the reproduction of their hosts. These results do not support the hypothesis that the above bacteria alter the reproductive outcome of this lepidopteran species resulting in the spread of the *T. pityocampa* 'ENA clade' and the replacement of 'endemic' *T. pityocampa*.

Endosymbiont infections, however, can result in reproductive advantages of infected over uninfected hosts in numerous species (Moran et al. 2008; Engelstadter and Hurst 2009). For example, the bacteria *Wolbachia* and *Cardinium* provide fitness benefits to the fly *Drosophila pandora* McEvey and Schiffer (Diptera: Drosophilidae) (Richardson et al. 2016) or the wasp *Encarsia pergandiella* Howard (Hymenoptera: Aphelinidae) (Hunter et al. 2003), respectively, by inducing CI. Such fitness effects can yield in the expansion of endosymbiont-infected individuals, associated by the so-called

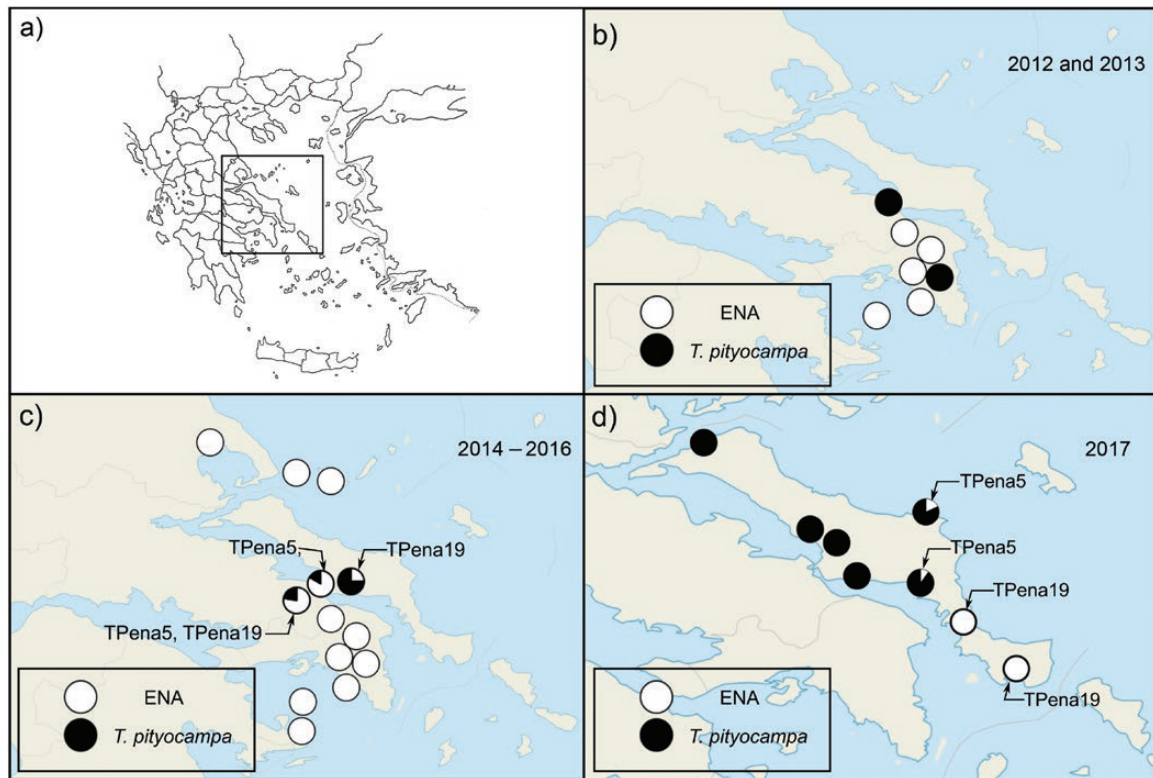


Fig. 1. (a) Map of Greece and range changes of *T. pityocampa* 'ENA clade' and 'endemic' *T. pityocampa* in the area of central Greece during (b) 2012 and 2013 (Korsch et al. 2015; Avtzis et al. 2016), (c) 2014–2016 (Avtzis et al. 2018), and (d) 2017 (current study).

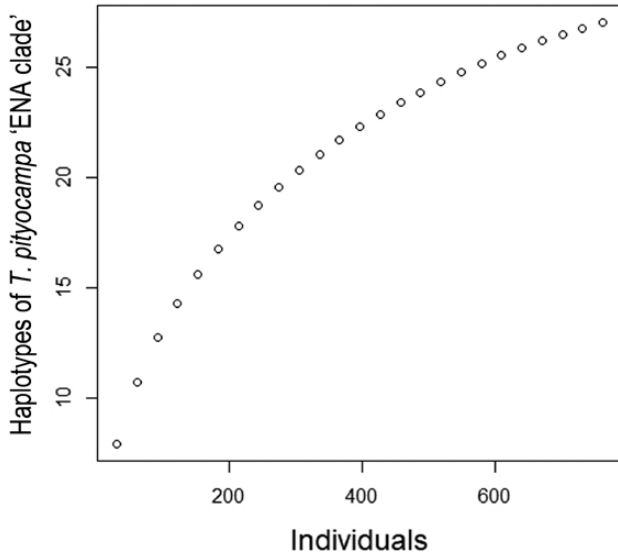


Fig. 2. Haplotype accumulation curve of *T. pityocampa* 'ENA clade'.

'hitchhiking'. This means that the mitochondrial haplotype, which is associated with the endosymbiont infection, will spread in parallel through a host population (Hurst and Jiggins 2005). Such patterns have been described, for instance, for the great eggfly, *Hypolimnas bolina* (L.) (Lepidoptera: Nymphalidae) (Charlat et al. 2009), the common house mosquito, *Culex pipiens* L. (Diptera: Culicidae) (Rasgon et al. 2006), or *R. cerasi* (Schuler et al. 2016).

Instead, other factors might explain the recent spread of the *T. pityocampa* 'ENA clade'. For instance, thermal conditions

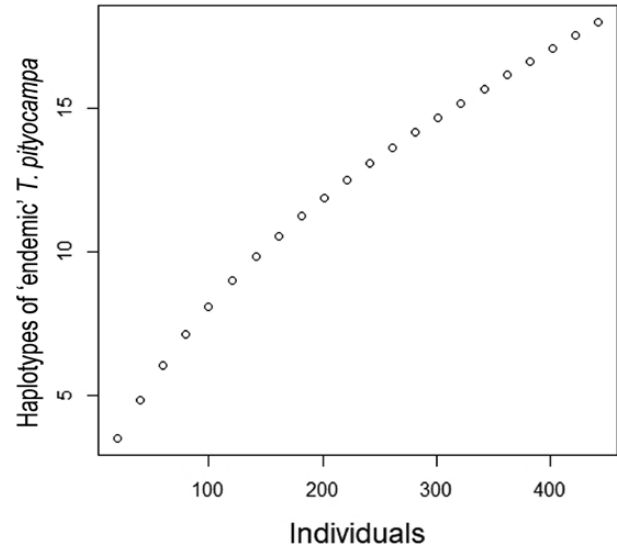


Fig. 3. Haplotype accumulation curve of 'endemic' *T. pityocampa*.

were found to be a main driver of range expansion in this species (Battisti et al. 2005, 2006). This suggests that certain lineages of *T. pityocampa*, like the 'ENA clade', might have thermal preferences that facilitate its spread. However, further studies on the thermal performance of various *T. pityocampa* lineages may reveal the mechanisms that explain the range patterns of this noxious moth species.

In conclusion, our results provide new information on the expansion history of the *T. pityocampa* 'ENA clade' on the Greek island

of Evoia and also estimate the sampling effort needed to assess the genetic diversity of each mitochondrial lineage. Most importantly, however, we found that bacterial endosymbionts do not play an active role in the observed expansion-replacement pattern, leaving one hypothesis less in the effort to explain the mechanism behind the spread of the *T. pityocampa* 'ENA clade'.

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