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Evidence of potential hybridization in the *Thaumetopoea pityocampa-wilkinsoni* complex

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Abstract

1 The winter pine processionary moth complex includes some major defoliating insects of *Pinus* and *Cedrus* forests in southern Europe and the Mediterranean Basin, where they also cause health problems to humans and animals.
2 The complex includes at least two species that were separated recently based on molecular and morphological evidence: *Thaumetopoea pityocampa* in the west and *Thaumetopoea wilkinsoni* in the east of the Mediterranean Basin.
3 Individuals from two populations, selected as representative of *Th. pityocampa* and *Th. wilkinsoni*, were used to test whether hybridization is possible under controlled conditions.
4 The hybrid offspring showed intermediate morphological and performance traits, whereas heterosis for pupal weight was detected in one of the hybrid lines. The genetic analysis confirmed the crosses.
5 Both species have large phenological plasticity and may come into contact at the edge of their range, where they could hybridize.
6 Based on the evidence accumulated so far, it is recommended that the current species designations are maintained, although a deeper study of the trait variability is required, especially in the contact zones.

Keywords Genetics, hybridization, morphology, phenology, pine processionary moth.

Introduction

Although not uncommon between sibling species coming into secondary contact, hybridization is usually considered costly, yielding progeny with diminished fitness and, indeed, is considered to be limited in natural systems by reproductive isolating barriers (Mallet, 2005). Barriers to reproduction can be the result of either postzygotic genetic incompatibilities or prezygotic barriers that can appear through drift alone or be enhanced by natural selection (Coyne & Orr, 2004). However, hybrids have been recorded in numerous species of insects, including Lepidoptera (Scriber & Ording, 2005; Mallet *et al.*, 2007; Descimon & Mallet, 2009). Studies of hybridization between closely-related taxa can provide important insights into potential gene flow between taxa, as well as the process of speciation (Mullen & Shaw, 2014).

The winter pine processionary moth complex includes at least two major phytophagous insect species defoliating *Pinus* and *Cedrus* forests in southern Europe and the Mediterranean Basin (Basso *et al.*, 2017). In addition, their larvae carry urticating setae that represent a threat to the health of domestic animals and humans (Moneo *et al.*, 2015), meaning that both species are pests of high socio-economic impact (Gatto *et al.*, 2009). *Thaumetopoea pityocampa* ([Denis et Schiffermüller], 1775) (the pine processionary moth *sensu stricto*) occurs from Maghreb through southern Europe to western Asia Minor, whereas *Thaumetopoea wilkinsoni* Tams, 1925 (the eastern pine processionary moth) occurs from western Asia Minor to southern Israel, as well as in Crete and Cyprus (Roques *et al.*, 2015) (Fig. 1).

Thaumetopoea wilkinsoni was separated from *Th. pityocampa* based on subtle morphological and behavioural differences found in some individuals from Cyprus (Tams, 1925). In the revisions of the genus by Agenjo (1941) and de Freina and Witt (1987), the status of *Th. wilkinsoni* as a different species was confirmed, as was also the case in the recent catalogue of Notodontidae (Schintlmeister, 2013) and in the revision of Basso *et al.* (2017). The range of *Th. wilkinsoni* has been enlarged by attributing all the Asiatic (Turkey and Near East) populations

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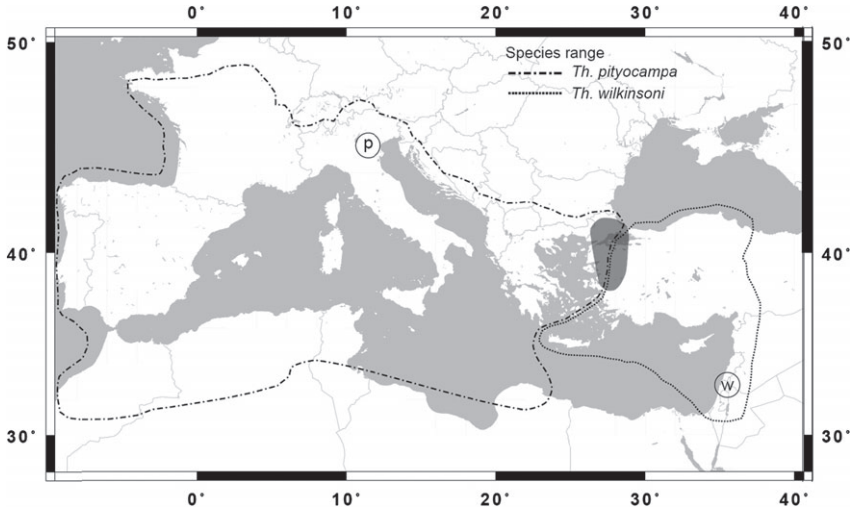

Figure 1 Range of *Thaumetopoea pityocampa* and *Thaumetopoea wilkinsoni*, with the location of the sampling sites of the representative populations used in the experimental hybridization. The shaded area is the contact zone as defined by İpekdal *et al.* (2015).

of pine processionary moth (Halperin, 1990) to this species. However, Frérot and Démolin (1993) found that the species use an identical sex pheromone and claimed that fertile progeny were obtained from crossing moths of the two species under laboratory conditions, although no further details about these experiments were provided. In addition, Démolin *et al.* (1994) provided an overview of the morphological traits discriminating the two species; based on male genitalia and female tuft scales, it was concluded that the two were geographically restricted forms of the same species and that a study of the variability across the range was necessary.

The separation between the two species, however, was supported by mitochondrial and nuclear DNA markers by Salvato *et al.* (2002), Simonato *et al.* (2007), Kerdelhué *et al.* (2009) and Basso *et al.* (2017). The pattern of genetic diversity was typical of species that have experienced marked glaciation cycles with the main divergence times within the complex dating back to the end of the Miocene (Kerdelhué *et al.*, 2009). In addition, a contact zone between species was hypothesized and partly supported by molecular data from north-western Turkey (İpekdal *et al.*, 2015). Further differences could be identified in the phenology because *Th. pityocampa* emergence is generally earlier than that of *Th. wilkinsoni*, at least in the core areas, as well as in their local adaptation to certain host plant species (Battisti *et al.*, 2015).

In the present study, we tested whether experimental hybridization between *Th. pityocampa* and *Th. wilkinsoni* is possible. Individuals from populations representative of the two species were examined, under quarantine laboratory conditions, aiming to evaluate the existence of pre- or post-zygotic isolation, and to determine the heritability of adaptive traits at least for the F1 generation.

Materials and methods

Crossing experiment

Two populations representative of *Th. pityocampa* and *Th. wilkinsoni* were selected in Italy and Israel, respectively (Fig. 1). These were required to be sufficiently far apart to determine pure lines for each species. Approximately 30 colonies of the *Th. pityocampa* population were collected from a stand of *Pinus nigra* from Veneto Calbarina (45°16′N, 11°43′E, 136 m). Colonies were reared on the same host in the campus of the Padua University in outdoor cages until pupation in spring 2004. The *Th. wilkinsoni* population was from a stand of *Pinus brutia* and *Pinus halepensis* from Dishon (33°05′N, 35°31′E, 441 m), reared on these hosts in the campus of the Agricultural Research Organization at Bet-Dagan in outdoor cages until pupation in spring 2004. Both populations were obtained from man-made stands, although insects originated from areas where the pine processionary moth is established on native hosts (*Pinus sylvestris* and *Pinus brutia*, respectively). Pupae were then taken from each rearing, separated from the cocoon and sexed based on genitalia slits until a total of 350 males and 350 females was obtained for each species. The pupae of *Th. wilkinsoni* were then sent from Israel to Italy, where the pupae of both populations were kept in sand (four boxes with 350 individuals in each) under controlled conditions in a quarantine-safe laboratory.

Because each population displays a different emergence time in their natural habitat (Battisti *et al.*, 2015), a temperature manipulation was carried out to achieve, at least partly, the emergence of overlapping adults, as carried out in a similar experiment (Branco *et al.*, 2017). The pupae of *Th. pityocampa*, for which the adult emergence was expected in August, were maintained at 15 °C, which is approximatety 5 °C less than the average temperature of the soil in the original habitat, until 1 month before the expected emergence time for the *Th. wilkinsoni* population (September). The *Th. wilkinsoni* population was then subjected to higher temperatures (22–25 °C) for 2 weeks to speed up development. At the end of this treatment, the boxes with the pupae were put in two large cages (1.5 × 1.5 × 1.5 m) under a naturally fluctuating temperature from 5 August to 25 September (mean ± SD temperature, 23.8 ± 9.8 °C). In one cage, there were two boxes, one with 350 female pupae of *Th. pityocampa* and another with 350 male pupae of *Th. wilkinsoni* (and vice versa

in the other cage). Moth emergence and mating occurred in the cages. When at least 20 hybrid egg batches were obtained for each cage (see below), boxes with male and female pupae of the same species were allocated to each of two clean cages to obtain batches of the respective species for use as a control.

To facilitate mating, several wooden sticks approximately 20 cm long were inserted into sand on the bottom of the boxes to allow emerging moths to climb, unfold wings and take off, as well as to provide a place for females to start pheromone release. Three potted mountain pines (*Pinus mugo*) approximately 1 m high were used to allow mated females to lay eggs. The pine species was selected because it is suitable for oviposition of *Th. pityocampa* (Stastny *et al.*, 2006). In addition, those female moths that did not oviposit during the first night after emergence were taken from the cages and isolated with one male from the same cage in darkness inside a 100-mL jar (6×10 cm) containing a wooden stick to facilitate moth emergence, mating and oviposition (Démolin, 1969). The numbers of moths emerged and egg batches laid were checked every day from the first emergence, counting live individuals and removing both the egg batches and dead moths. Egg batches from the four F1 lines were designated as: PW (hybrid line of *Th. pityocampa* female and *Th. wilkinsoni* male), WP (hybrid line of *Th. wilkinsoni* female and *Th. pityocampa* male), PP (pure line of *Th. pityocampa*) and WW (pure line of *Th. wilkinsoni*).

The egg batches were labelled and maintained individually at $24 \pm 2\,^{\circ}$C until hatching. When a sufficient number of eggs hatched, subsets of individuals from each batch were taken for running starvation and performance bioassays. Neonate larvae taken individually from different egg batches were used as replicates. In the starvation bioassay, 10 nonsib larvae were isolated without food in Petri dishes at $24 \pm 2\,^{\circ}$C and the number of dead larvae was recorded daily until total mortality was reached. The performance bioassay consisted of rearing two subsets of 20 larvae each in Petri dishes on needles of *P. halepensis* and *P. nigra*, as described by Stastny *et al.* (2006), for each of the four F1 lines. The larvae were weighed at hatching and soon after moulting to the second instar to estimate the relative growth rate (RGR, mg/mg/day), calculated as: $RGR = [\ln(M_f) - \ln(M_i)]/T$, where ln is the natural logarithm, M_f and M_i are the final and initial mass of larvae, and T is the elapsed time in days (Gordon, 1968). The mortality of the larvae was assessed; any tested individual was considered dead when it did not react to mechanical disturbance with a brush.

Surviving larvae of the four lines and those hatched from egg batches not used in the bioassays were reared on fresh cut branches of *P. halepensis* and *P. nigra* in eight separate cages exposed to a natural fluctuating temperature between September 2004 and March 2005. Pupae were retrieved from the soil on the bottom of the cage and samples of 15 females and 15 males for each line and host plant species were weighed individually. As a result of limitations in the capacity to maintain rearing lines under quarantine conditions, pupae belonging to the two pure lines were discarded, whereas those of the two hybrid lines were maintained in two separate cages to obtain the F2, termed PWPW and WPWP. The egg batches of the F2 were maintained under laboratory conditions, as for the F1, and, once egg hatching was observed, larval viability was tested using *P. nigra* needles. After the experiment was terminated, the insect material was disposed as requested in accordance with quarantine regulations.

Genetics

Both mitochondrial and nuclear markers were used to assess the identity of the individuals obtained from the crossing experiment (i.e. to check whether they were actual hybrids and to exclude possible errors in the sexing of pupae). Because female moths mate only once and lay all their eggs in one batch (Battisti *et al.*, 2015), one individual per colony was used. Mitochondrial markers were tested on 53 larvae of the F1 lines (16, 11, 18 and 8 from PP, WW, PW and WP, respectively) and 12 larvae of the F2 lines (9 and 3 for PWPW and WPWP, respectively). Nuclear markers were tested on 38 larvae of the F1 lines (10, 10, 10 and 8 from PP, WW, PW and WP, respectively) and nine larvae of the F2 lines (6 and 3 PWPW and WPWP, respectively). Each larva was preserved in ethanol 70% and stored at $-20\,^{\circ}$C before DNA extraction, which followed the salting-out procedure (Patwary *et al.*, 1994).

A mitochondrial DNA fragment of the COI gene from all individuals was amplified and examined through single-strand conformation polymorphism (SSCP) analysis, as described by Salvato *et al.* (2002). For each mobility class, one to five individuals were sequenced directly by dideoxy chain termination method at the BMR Genomics sequencing service (BMR Genomics srl, Italy) to check the accuracy of the SSCP analysis and to determine the corresponding haplotypes. The amplified fragment length polymorphism (AFLP) protocol (Vos *et al.*, 1995) was used with three primer combinations yielding 155 polymorphic loci on 47 individuals analyzed. Approximately 50 ng of DNA was digested with *Eco*RI and *Mse*I restriction enzymes and ligated to specific AFLP adapters. Each sample was subsequently diluted 10-fold and used as template for preselective and selective (*Eco* RI AAC/*Mse*I-CAT, *Eco*RI-AGC/*Mse*I-CAT, *Eco*RI-AAG/*Mse*I-CAC) polymerase chain reaction amplifications. AFLP products were run in an ABI PRISM 3700 DNA Analyzer (Applied Biosystems, Foster City, California). Band scoring was performed with GENOTYPER, version 3.7 (Applied Biosystems) considering bands in the range 50–400 bp. The intensity of each individual peak was normalized on the basis of the total signal intensity and the peak was considered only if its intensity exceeded a fixed threshold of 100 fluorescent units. AFLP profiles were recorded in a matrix as the presence or absence of bands for each individual.

Morphology

The morphological traits considered were the male genitalia, the scales of female moth abdominal tuft and the wing pattern according to Basso *et al.* (2017). Genitalia of 10 males from each pure and hybrid lines were analyzed. The abdomen was removed and placed in 10% KOH solution for 20–25 min. Subsequent to maceration, abdomen and genitalia were cleaned and mounted on slides to be examined under the microscope in accordance with the protocol described by Robinson (1976). Scales of female abdominal tuft were gently picked up with forceps from the central part of the tuft and mounted on slides

for microscopic observation. Images and measurements of male genitalia and female scales were captured using an AxioCam (MRc5) mounted on a Lumar.V12 stereoscrope (Carl Zeiss, Oberkochen, Germany) and with AXIOVISION SE64, version 4.9.1.

Statistical analysis

Analysis of variance (ANOVA) was used to compare the fresh weight of neonate larvae, the RGR of the first instar and pupal weight. If available, sex was used as a factor, as well as the host plant. ANOVA was also used to compare the ratio length/width of the tuft scales of the female moths. Tukey's test was used for pairwise comparison of means. The Kaplan–Meier product limit survival curve (log rank test) was used to examine the resistance to starvation in fasting bioassays. $P < 0.05$ was considered statistically significant. Separate generalized linear models (quasibinomial distribution, logit link) were used to analyze the effect of lines and host on larval mortality in the performance bioassay. For the genetics data, the COI sequence chromatograms were visualized and aligned with *Th. pityocampa* and *Th. wilkinsoni* COI haplotypes retrieved from Salvato *et al.* (2002) and Simonato *et al.* (2007), using MEGA, version 7 (Kumar *et al.*, 2016). The AFLP dataset was analyzed via principle coordinate analysis (Huff *et al.*, 1993) implemented in GENALEX, version 6.1 (Peakall & Smouse, 2012).

Results

Crossing experiment

The 350 pupae for each sex and species used to start the experiment yielded variable numbers of moths: 33.4% (males of *Th. pityocampa*), 44.9% (males of *Th. wilkinsoni*), 52.2% (females of *Th. pityocampa*) and 59.6% (females of *Th. wilkinsoni*), whereas the remaining pupae died. The moths of the two species overlapped in their emergence time (from 10 August to 20 September) and could mate and lay eggs (Table 1). The egg-laying yield clearly depended on the availability of males and females of the two species that emerged on any particular day (see Supporting information, Appendix S1).

Larvae hatched from all of the egg batches. The larvae of WW were significantly heavier than those of PP (Fig. 2A), with the hybrid WP identical to WW and the hybrid PW at an intermediate level between the pure lines ($F_{3,56} = 10.11$, $P < 0.001$). The starvation bioassay showed a significant difference, with a higher resistance to starvation of hybrid WP and pure line WW than that of hybrid PW and pure line PP ($P < 0.001$, log rank test) (Fig. 2B).

The relative growth rate of the first instar larvae was significantly higher for WW, on both host plants, with hybrid lines showing intermediate values ($F_{3,110} = 5.716$, $P < 0.01$) (Fig. 3). The host plant did not affect the RGR of the first-instar larvae ($F_{1,112} = 0.39$, $P = 0.43$). The mortality at the end of the first instar in the performance bioassay was very low (i.e. less than 2.7%), with no significant difference among lines and between host (LR test in a generalized linear model, quasibinomial error, log link, $F_{4,104} = 7.02$, $P = 0.21$ for the line and $F_{1,107} = 7.46$, $P = 0.36$ for the host plant). Pupal weight, however, was higher for individuals reared on *P. nigra* than on *P. halepensis* (female $F_{1,114} = 41.64$, $P < 0.001$; male $F_{1,114} = 19.32$, $P < 0.001$) and the hybrid WP was heavier for both females ($F_{3,113} = 15.57$, $P < 0.001$) and males ($F_{3,114} = 21.52$, $P < 0.001$), providing evidence of heterosis in this line (Fig. 4).

The adults of the F1 hybrid lines emerged in the second half of August 2005. A limited number of moths was obtained (six females and 12 males for PW; three females and two males for WP) and they were given the chance to mate and lay eggs, which resulted in five and three egg batches for PWPW and WPWP, respectively. All of the egg batches hatched and the larvae were able to feed on *P. nigra* needles, and then the experiment was terminated.

Table 1 Number of moths emerging from the pupae used for the experiment (350 males and 350 females for each of the two species) and number of egg batches obtained for the four lines of the F1 generation

Insect stage	PP	WW	PW	WP
Female × male moths	91 × 80	28 × 27	92 × 130	181 × 37
Egg batches	41	8	39	22
Egg batches yield (%)	45.1	28.6	42.4	12.2

First letter indicates female. P, *Thaumetopoea pityocampa*; W, *Thaumetopoea wilkinsoni*.

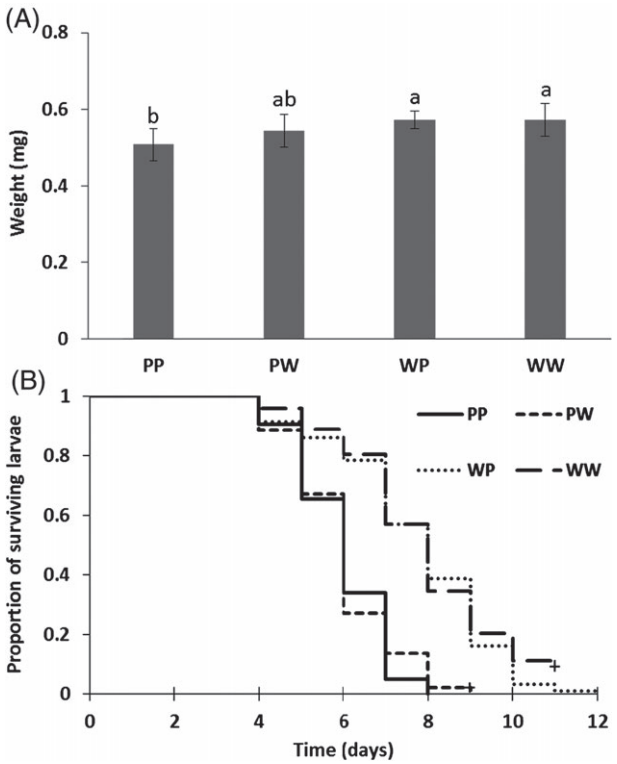

Figure 2 (A) Individual fresh weight (mean ± SE) of neonate larvae of the four F1 lines. Different letters indicate significant differences in a pairwise comparison of means (Tukey's test, $P < 0.05$). (B) Larval survival during the fast bioassay with first instar larvae of the four F1 lines. First letter indicates female (P, *Thaumetopoea pityocampa*; W, *Thaumetopoea wilkinsoni*).

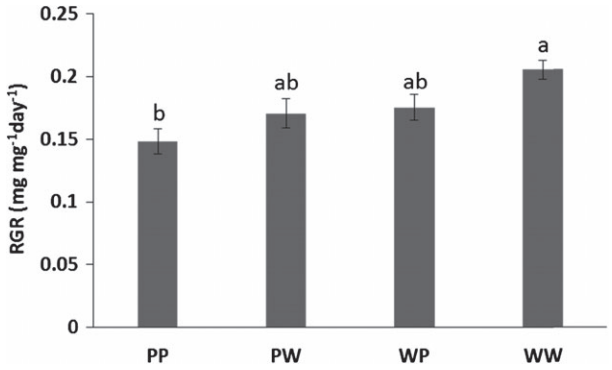

Figure 3 Relative growth rate of the first instar larvae of the four F1 lines in the performance bioassays (mean ± SE). Different letters indicate significant differences in a pairwise comparison of means (Tukey's test, $P < 0.05$). First letter indicates female. P, *Thaumetopoea pityocampa*; W, *Thaumetopoea wilkinsoni*.

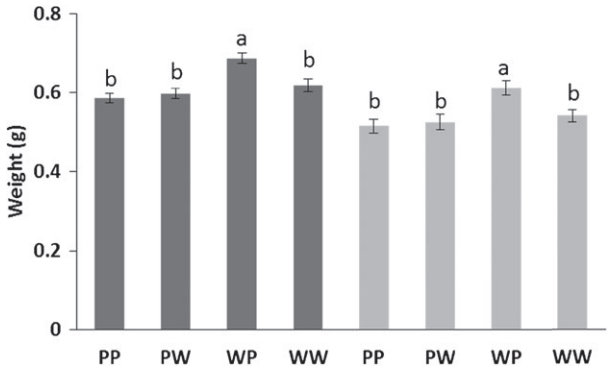

Figure 4 Fresh weight of female pupae of the four F1 lines (mean ± SE), in black fed with *Pinus nigra* and in grey with *Pinus halepensis*. Different letters indicate significant differences in a pairwise comparison of means within host plants (Tukey's test, $P < 0.05$). First letter indicates female. P, *Thaumetopoea pityocampa*; W, *Thaumetopoea wilkinsoni*.

Genetics

The SSCP analysis clearly distinguished two haplotypes for the COI fragment, which were then confirmed by sequencing. All the individuals analyzed for the PP and PW lines were assigned to the COI haplotype 1, which is the most common COI haplotype of *Th. pityocampa* in Italy (Salvato *et al.*, 2002), whereas the individuals sampled for the WP and WW lines were assigned to the haplotype 5, which is the only COI haplotype of *Th. wilkinsoni* found in Israel (Simonato *et al.*, 2007).

A total of 155 polymorphic bands was obtained by AFLP analysis. Among these, 43 and 40 bands were specific for *Th. pityocampa* and *Th. wilkinsoni*, respectively, whereas 57 bands were shared between the two species. Principal coordinate analysis, whose first three components accounted for 28.2% of the total genetic variability in the dataset, showed that the lines PP and WW were well differentiated, and that all the F1 and F2 lines fell between the parental lines, with F2 (PWPW and WPWP) showing a higher diversity than the F1 lines (PW and WP) (Fig. 5).

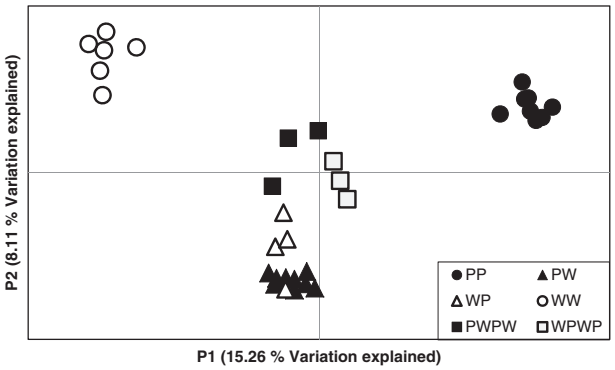

Figure 5 Principal coordinates analysis clustering of the amplified fragment length polymorphism genotypes analyzed for the four F1 lines and the two F2 lines (PWPW, WPWP). First letter indicates female. P, *Thaumetopoea pityocampa*; W, *Thaumetopoea wilkinsoni*.

Morphology

The two species were distinguishable based on the male genitalia and the abdominal tuft scales of female moths presented in Fig. 6 and the hybrids could be discriminated from the pure lines and from each other. A general description of the hybrid moths is provided in the Supporting information, Appendix S2. The male genitalia of the hybrid lines showed traits that were intermediate between the two species. The shape of socii was as in WW, although the lateral edge was less wrinkled. Valvae, comprising the most distinctive trait used for species discrimination, were also intermediate. Indeed, ribbing on the entire length of the valvae, more defined in WW and characteristic of this species (Tams, 1925), was vague in hybrid lines and present only in the first third of the valvae. A careful analysis of genitalia discriminated between hybrid types. A difference between PW and WP specimens was identified in the upper margin of valvae and in the apex of socii. Indeed, in PW specimens, valvae had an extremely convex angle just before the cucullus, and socii were a little wider than in WP specimens, with a ribbing on the apex that was lacking in WP specimens (Fig. 6, arrows).

The ratio (length/width) of the abdominal tuft scales of female moths showed a significant difference among lines ($F_{3,51} = 11.99$, $P < 0.01$). The scales were larger and shorter in WW (mean ± SE, 1.64 ± 0.041) than in PP (2.08 ± 0.054), whereas the shape of both hybrids was intermediate, with a ratio of 1.70 ± 0.05 in PW and 1.90 ± 0.12 in WP (Fig. 6). The pairwise comparison was statistically significant only between parental lines.

Discussion

Laboratory conditioned individuals from two populations representative of *Th. pityocampa* and *Th. wilkinsoni* produced fertile hybrids, as already described by Frérot and Démolin (1993) (although they did not supply details of their experimental procedure). Our successful breeding occurred despite a marked genetic divergence between the species (Salvato *et al.*, 2002; Simonato *et al.*, 2007, 2013; Kerdelhué *et al.*, 2009, 2015) and morphological differences in male genitalia and female tuft scales that have been used to discriminate the species (Tams, 1925; Wilkinson,

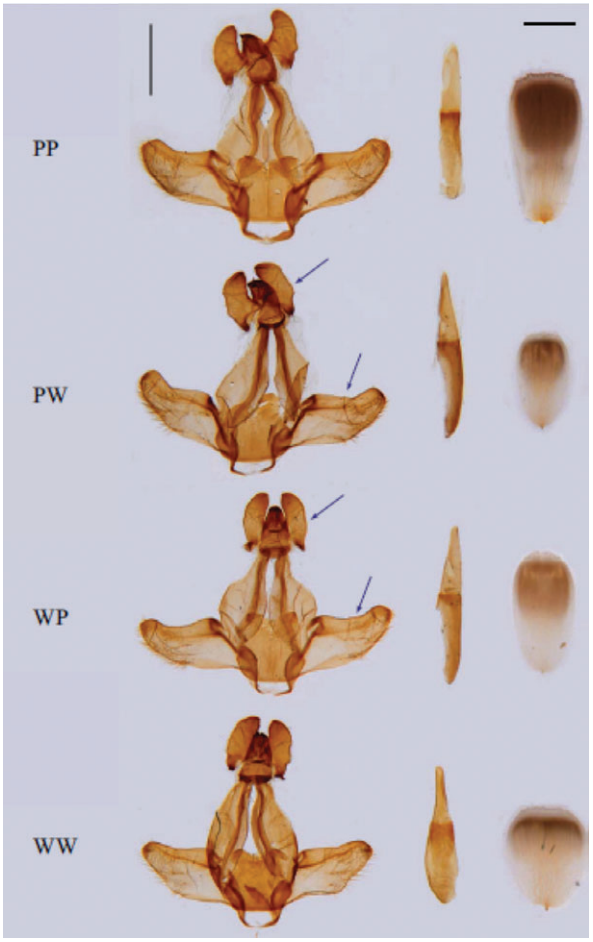

Figure 6 Male genitalia and abdominal tuft scales of female moths of the four F1 lines. Arrows indicate the discriminating traits between the hybrid lines discussed in the text. Scale bar = 1 mm. First letter indicates female. P, *Thaumetopoea pityocampa*; W, *Thaumetopoea wilkinsoni*.

1926; Agenjo, 1941; de Freina & Witt, 1987; Démolin *et al.*, 1994; Basso *et al.*, 2017). However, it was necessary to manipulate the emergence time of the two populations representative of *Th. pityocampa* and *Th. wilkinsoni* because they barely overlap in nature (Battisti *et al.*, 2015).

Hybrid zones are common in insects (Zhao *et al.*, 2005; Mallet *et al.*, 2007; Descimon & Mallet, 2009; Gompert *et al.*, 2010) and can entail complex interactions between the species (Hewitt, 1988; Bridle & Vines, 2007). In the winter pine processionary moth complex, the occurrence of hybrid zones has been hypothesized by Kerdelhué *et al.* (2009) in the contact zones between the three major genetic clades (i.e. *Th. pityocampa* and *Th. wilkinsoni* in western Turkey and between the two main *Th. pityocampa* mitochondrial clades in Algeria). Recently, İpekdal *et al.* (2015) and El Mokhefi *et al.* (2016) explored these contact zones in Turkey and Algeria, respectively, identifying signs of introgression.

Hybrids, as obtained in the present study via laboratory temperature manipulation to enable the simultaneous occurrence of adults, are known for a number of insect taxa, such as the bush cricket *Eupholidoptera* (Allegrucci *et al.*, 2014) and mealybugs *Planococcus* spp. (Kol-Maimon *et al.*, 2014), and do not invalidate the species designations *per se*. There could be a number of reasons for the species maintaining reproductive compatibility, at least in no-choice condition, despite a long separation over time and space. Incompatibility can evolve because of drift alone as a result of the independent evolution of the species in allopatry, and this is expected to be reinforced when speciation occurs in sympatry or if the lineages come into secondary contact. Based on mitochondrial DNA evidence, *Th. pityocampa* and *Th. wilkinsoni* diverged a few million years ago on the opposite sides of the Aegean Sea. They came into contact only relatively recently (Kerdelhué *et al.*, 2009, 2015) and reinforcement is unlikely to have occurred in our sampling sites far from the contact zone, which can explain the reproductive compatibility reported in the present study. It might also have been facilitated as a result of the two species sharing the same sex pheromone (Frérot & Démolin, 1993).

Although differences in genitalia, usually considered as a pre-zygotic barrier (Shapiro & Porter, 1989), were not fully covered in the present study (e.g. lock-and-key internal parts such as carina and endophallus), they did not appear to be determinant in our experimental conditions. In addition, the endophallus does not show significant variability in *Thaumetopoea* (Basso *et al.*, 2017). In any case, the male genitalia could be used as a morphological tool to identify hybrid lines from material collected in the field, especially in the contact zone. The abdominal tuft scales also displayed an intermediate shape between the pure lines; however, the high variance observed at a regional level in both species (Frérot & Démolin, 1993) suggests that this trait is unreliable for hybrid identification (İpekdal *et al.*, 2016).

No signs of outbreeding depression were observed, and most hybrid fitness measures were intermediate between the parental lines, suggesting a co-dominant transmission of the corresponding traits; the heritability of most studied traits showed maternal imprinting and only one showed heterosis (weight of pupae was higher in the WP hybrid). Mortality in the hybrid lines reared to obtain the F2 was very high and probably was related to the rearing conditions, as observed in other studies with pure lines (Berardi *et al.*, 2015), or to the lethality sometimes observed in hybrids (Maheshwari & Barbash, 2011). Differences in starvation assays of the first-instar larvae were found between the two pure lines, and they may be linked to variation in egg size as a result of different ecological conditions existing in the original sites. For example, the larger eggs of *Th. wilkinsoni* may produce larvae that cope better with the semi-arid conditions of its range, and especially with tough needles, as was observed for a population of *Th. pityocampa* (Ruschioni *et al.*, 2015). A larger egg size involves a cost in terms of fecundity, which is generally lower in *Th. wilkinsoni* (Avcı *et al.*, 2015), in the southern populations of *Th. pityocampa* (Pimentel *et al.*, 2010; Zamoum *et al.*, 2015) and in a phenologically-shifted population of *Th. pityocampa* in Portugal (Rocha *et al.*, 2017). Although our representative populations originated from different latitudes, the weight of hybrid neonate larvae, which can be taken as a proxy of body and egg size, was higher in the WW pure line and the WP hybrid line than in the PP pure line, whereas the other hybrid PW showed an intermediate value. Although the RGR at the end of the first instar of the two hybrid lines was intermediate, the overall performance of the larvae, as demonstrated by the pupal weight, was higher

in the hybrid WP than in all the other lines, indicating that the initial differences alone did not determine weight differences in the successive developmental stages, and providing further evidence of the plasticity of the species. Surprisingly, *Th. wilkinsoni* did better on *P. nigra* than on its native host *P. halepensis*, confirming that host specialization is not a major obstacle in this species (Kerdelhué *et al.*, 2006). Indeed, *Th. wilkinsoni* occurs on *P. nigra* in its native range (Avcı *et al.*, 2015; İpekdal *et al.*, 2015).

The clustering of pure and hybrid lines revealed by developmental and morphological analyses was confirmed by the neutral genetic markers, which shows that genetic tools are useful for identifying hybrids in the field. It would be interesting to determine what happens in the contact zones, for both developmental and morphological traits, to infer the amount of introgression. Based on the evidence accumulated so far on several traits of the *Th. pityocampa-wilkinsoni* complex and the latest information concerning the debate on species definition (Mullen & Shaw, 2014), it is recommended that the current species is maintained as designated (Lushai *et al.*, 2005). To date, a low frequency of hybrids between the *Th. pityocampa-wilkinsoni* has been observed in the putative contact zone in W Turkey (İpekdal *et al.*, 2015). A more intensive study of trait variability would be required, especially in the contact zones, for the evaluation of the possible presence of reproductive barriers apart from phenology. Whether these hybrids can become important in pest management issues remains uncertain, although such a scenario should be taken into account because the pine processionary moth is expanding its range naturally (Battisti *et al.*, 2005) and also as a result of humans accidentally introducing it with plant material from sites located far away (Robinet *et al.*, 2012; Avtzis *et al.*, 2016). Range expansion has been observed mainly in *Th. pityocampa* to date, although it was recently suggested that it may occur in *Th. wilkinsoni* as well (İpekdal *et al.*, 2015). Hybridization can facilitate the colonization success of expanding or invasive species through adaptive introgression, leading to new allelic combination giving rise to potentially new adaptive phenotypes (Rius & Darling, 2014). Moreover, new and more aggressive pest species may emerge as a result of hybridization (Arnold, 2004). Therefore, more studies regarding the processionary moth hybrids and their invasiveness are required.

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Supporting information

Additional Supporting information may be found in the online version of this article under the DOI reference: 10.1111/afe.12224

Appendix S1. Emergence and oviposition of moths of *Thaumetopoea pityocampa* and *Thaumetopoea wilkinsoni* under controlled laboratory conditions during summer 2004, as well as their combinations, to obtain pure and hybrid lines. The first letter indicates female; P, *Th. pityocampa*; W, *Th. wilkinsoni*.

Appendix S2. Morphological description of the adult moths obtained from crossing *Thaumetopoea pityocampa* and *Thaumetopoea wilkinsoni.* The two hybrid lines PW and WP were distinguishable only based on male genitalia (Fig. 6). The first letter indicates female; P, *Th. pityocampa*; W, *Th. wilkinsoni*.

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