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Incipient allochronic speciation in the pine processionary moth (*Thaumetopoea pityocampa*, Lepidoptera, Notodontidae)

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Abstract

A plausible case of allochronic differentiation, where barrier to gene flow is primarily attributable to a phenological shift, was recently discovered in Portugal for the pine processionary moth *Thaumetopoea pityocampa*. Previous results suggested that the observed 'summer population' (SP) originated from the sympatric winter population (WP). Our objectives were to finely analyse these patterns and test their stability in time, through field monitoring and genetic analyses of larvae and adults across different years. Reproductive activity never overlapped between SP and WP. Microsatellites showed a clear differentiation of the SP, consistent with a strong reduction in gene flow owing to the phenological shift. Assignment tests suggested that some individuals shift from the SP to the WP phenology, causing some hybridization. We discuss these patterns and their maintenance over time. This could be a first stage of allochronic speciation, and SP should be considered as a distinct phenological race.

Introduction

The process by which species diverge into different strains that become reproductively isolated and evolutionarily independent is well known and documented for numerous cases of spatial isolation known as allopatric speciation (e.g. Johannesson, 2001; Turelli *et al.*, 2001). Speciation in sympatry is yet a controversial subject, and the proposed models require conditions that are difficult to obtain in natural populations (Bolnick & Fitzpatrick, 2007). Nevertheless, a few case studies have shown that it could be more common than originally thought (Bush & Smith, 1998). Sympatric speciation occurs when reproductive isolation is observed while the different

populations still exist in the same area. Host or habitat specialization is one of the most documented processes leading to sympatric isolation and speciation, mostly in fishes and phytophagous insects (e.g. Berlocher & Feder, 2002; Drès & Mallet, 2002; Feder *et al.*, 2005; Peccoud *et al.*, 2009).

Yet, sympatric speciation can also occur because of separation of populations by breeding time, a process known as allochronic speciation (Alexander & Bigelow, 1960). Such a situation occurs when populations reproduce in different years as observed in periodical Cicadas (Marshall & Cooley, 2000; Simon *et al.*, 2000; Cooley *et al.*, 2001; Ritchie, 2001), in different seasons of the year (Alexander & Bigelow, 1960; Friesen *et al.*, 2007) or even in different times of the day (Miyatake *et al.*, 2002). In many insect groups, the adult stage is short-lived; thus, small differences in phenology can prevent adults occurring in the same area from mating. Reproductive isolation can occur if there are differential nonoverlapping reproductive seasons and if these phenologies are heritable. In some cases, phenological differentiations have been documented in phytophagous insects as an

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adaptation to the phenology of different hosts (Bolnick & Fitzpatrick, 2007). Very few cases of allochronic speciation owing to phenological isolation *per se* (without host plant diversification) have been studied so far (Abbot & Withgott, 2004; Santos *et al.*, 2007; Yamamoto & Sota, 2009; Ording *et al.*, 2010) probably owing to its scarcity.

The pine processionary moth (PPM) (*Thaumetopoea pityocampa* Den. & Schiff., Lepidoptera, Notodontidae) is a well-known defoliator occurring in the Mediterranean countries (Kerdelhué *et al.*, 2009). It is considered as an important forest pest owing to the damages caused by the larvae feeding on needles of several *Pinus* species, and as a pest of public concern because of the larvae's urticating hairs that cause severe allergic reactions to people and animals (Vega *et al.*, 2004). The larvae are gregarious, inhabit silk nests and pupate below ground. Adults mostly emerge during summer, reproduce and die within a few days (Démolin, 1969). In Portugal, at low altitudes where the populations studied in the present study are located, adults classically reproduce between the end of July and September and larval development occurs during fall and winter (Fig. 1a). Pupation starts in February or March, with an obligate pupal diapause, until new adults emerge in summer. A facultative

prolonged diapause can occur (Huchon & Démolin, 1970), leading to the emergence of adults one or several years later, still in the end of summer.

An exceptional phenological shift from a winter larval development to a summer larval development was observed in one single site in Portugal, in the National Forest of Leiria (Pimentel *et al.*, 2006), which raised ecological and evolutionary questions. Locally, individuals with a normal life cycle (adult emergence in summer and larval development in winter) co-occur with individuals that have a shifted life cycle, with adults emerging in spring and larvae developing during summer (Fig. 1b). Larvae from this population are exposed to different conditions from those developing in winter, as climate, host phenological stage and active natural enemies differ between seasons. This abnormal population has a limited geographical range, restricted to a 10 × 50 km forest of pure even-aged maritime pine stands (*Pinus pinaster* Ait.) named the 'Mata Nacional de Leiria' (MNL), the management of which dates back to the fourteenth century. Throughout this paper, the unique shifted population will be referred to as the 'summer population' (SP), whereas all other populations that exhibit the common and widespread phenology will be called 'winter populations' (WP). The specific Leiria

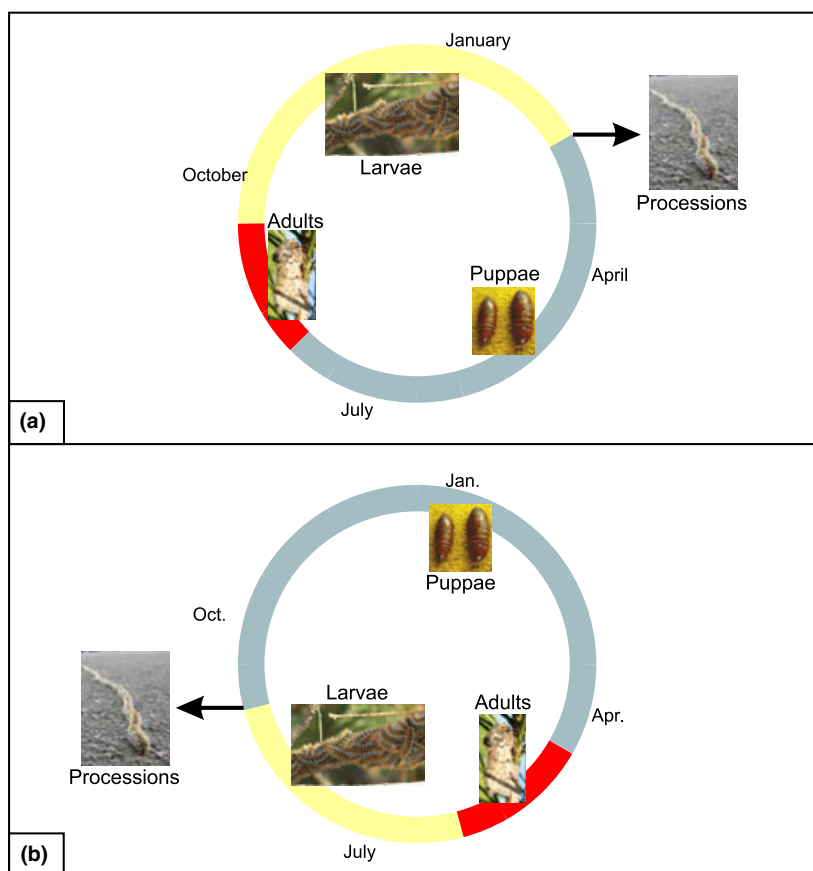


Fig. 1 Biological cycle of *Thaumetopoea pityocampa* in Portugal. (a) Normal cycle observed at low altitude including in Leiria (i.e. for Leiria WP), with a winter larval development. (b) Shifted cycle observed solely for Leiria SP, with a summer larval development. Photo credits: J. C. Martin and I. Pivolto.

WP, which is in sympatry with the SP, will be referred to as 'Leiria WP'.

Previous results suggested that allochronic differentiation was ongoing, as a direct consequence of the phenological shift. Based on mitochondrial and nuclear sequences, an earlier study hypothesized that the SP belongs to the same species as the surrounding WPs (*T. pityocampa*) and that it is not a cryptic species (Santos *et al.*, 2007). These genetic markers showed no differentiation between SP and Leiria WP, suggesting that the phenological shift is fairly recent. Conversely, five microsatellite markers revealed a strong differentiation between SP and WP, with signs of founder event in the SP. These first results thus suggested that the SP was recently established by a reduced number of individuals with early sexual reproduction and consecutive rapid larval development. However, these first conclusions should be considered with caution, as these preliminary data were based on one single sampling season per population, using a limited number of larvae, and only global, F_{ST} -based analyses of differentiation. Moreover, no adults were then included, and the genetic origins of the males trapped during monitoring were not analysed. In this study, we gathered new data (larvae, males and females collected during successive years and analysed with six microsatellite markers) and proceeded to detailed analyses of individual genetic assignment and of population differentiation to (i) precisely determine the phenology of *T. pityocampa* in Leiria, by monitoring the complete flight periods of both SP and WP populations in several years through pheromone traps; (ii) test the stability in time of the genetic structure of both SP and WP, by genotyping with six microsatellite loci individual larvae and adults sampled over three different years; and (iii) check whether all individuals, including trapped adults, could be correctly assigned to their reference populations.

Material and methods

Monitoring of males flight period

To assess whether mating disruption between both populations could be hypothesized, we tested whether the adult activity of the Leiria WP and SP could eventually overlap by monitoring the flight of adult males from early May to the end of September in 2005, 2007 and 2008, covering the whole known flight period in Leiria. To provide reference data for the normal phenology in Portugal, another site (Apostiça) located ca. 100 km south of Leiria, at similar altitude and longitude, was monitored in 2005 and 2008. Funnel traps baited with synthetic PPM pheromone dispensers (10515/BFL072, AgriSense™, Pontypridd, UK) were hung on trees at a reachable height. Twelve to fourteen traps were distributed all over the known area of the SP at 1- to 5-km intervals, and 5–12 traps were placed in Apostiça

in 20- to 50-m intervals. Pheromone dispensers were replaced every 6 weeks. All funnels had a DDVP (2,2-dichlorovinyl dimethyl phosphate, BIOSANI) tab inside, to act as a killing agent. Traps were assessed weekly from May to July and weekly or every 2 weeks from August to September.

Sampling and DNA extraction

Samples of *T. pityocampa* collected in Leiria in 2002, 2005, 2006, 2007 and 2008 were used to complete the original data set of larvae collected during 2003–2004 (Santos *et al.*, 2007) and consisted of larvae, adult males and adult females from both summer and WPs. Additional larvae were collected in September 2002 and 2005 for the WP and July 2006 and 2007 for the SP. Males and females were collected in 2007 and 2008. Concerning the populations from Leiria, each individual was coded *a priori* as belonging to either the SP or the WP based on phenotypic data (date of collect for the larvae, and date of emergence or trapping for the adults). Samples consisted of 30 (15 in 2002) first instar larvae per population and year, each originating from different nests to prevent sampling siblings. First instar larvae collected in May or June were phenotypically coded as SP, whereas those collected in September and October were coded as WP. Males were captured by pheromone trapping as described earlier (individuals captured before the end of June were *a priori* coded as SP, and individuals trapped after the end of July as WP). Females were obtained from laboratory rearing of pupae and larvae collected in the field in 2008 from both SP and WP (based on the date of emergence of adults in the laboratory) from Leiria and from a WP in Apostiça, located south of Lisbon. Sampling details are given in Table 1. All samples were collected in maritime pine stands (*Pinus pinaster*) and immediately stored in 95% ethanol.

DNA was extracted from the whole body of PPM larvae and from thoracic muscles of adults, using the GenElute mammalian Genomic DNA miniprep kit (Sigma) and eluted in 200 μ L of buffer.

Microsatellite genotyping

Six microsatellite loci were used to genotype the newly sampled individuals. Five of these loci, namely MS-*Thpit1*, MS-*Thpit2*, MS-*Thpit3*, MS-*Thpit4* and MS-*Thpit5*, are described in Rousselet *et al.* (2004), and MS-*Thpit6* is described in Santos *et al.* (2007). Moreover, MS-*Thpit2* was used to genotype the individuals studied in this latter reference, which were previously analysed using only five microsatellite markers. Fluorescent PCR products were run and detected on an ABI 3730 automatic sequencer, and product sizes were determined using the Genemapper v4.0 software (Applied Biosystems, Foster City, CA, USA). We used 17 positive controls

Table 1 Sampling localities and date of collection of the 341 genotyped individuals of *Thaumetopoea pityocampa* and number of genotyped individuals per population. Data from Santos *et al.* (2007) are indicated.

Population code	Population type	Development stage	Country	Locality	Geographical coordinates	Year of collect	# Genotyped individuals	Genotypes for MS- <i>Thpit</i> 1, 3, 4, 5 & 6 from:	Genotypes for MS- <i>Thpit</i> 2 from:
LSP03	Summer	L1 larvae	Portugal	Leiria	39°47'N 8°58'W	2003	29	Santos <i>et al.</i> , 2007	Present paper
LSP06	Summer	L1 larvae	Portugal	Leiria	39°47'N 8°58'W	2006	30	Present paper	Present paper
LSP07	Summer	L1 larvae	Portugal	Leiria	39°47'N 8°58'W	2007	30	Present paper	Present paper
LSP07-M	Summer	Adult males	Portugal	Leiria	39°47'N 8°58'W	2007	16	Present paper	Present paper
LSP08-F	Summer	Adult females	Portugal	Leiria	39°47'N 8°58'W	2008	19	Present paper	Present paper
LWP02	Winter	L1 larvae	Portugal	Leiria	39°47'N 8°58'W	2002	15	Present paper	Present paper
LWP04	Winter	L1 larvae	Portugal	Leiria	39°47'N 8°58'W	2004	36	Santos <i>et al.</i> , 2007	Present paper
LWP05	Winter	L1 larvae	Portugal	Leiria	39°47'N 8°58'W	2005	30	Present paper	Present paper
LWP07-M	Winter	Adult males	Portugal	Leiria	39°47'N 8°58'W	2007	15	Present paper	Present paper
LWP08-F	Winter	Adult females	Portugal	Leiria	39°47'N 8°58'W	2008	2	Present paper	Present paper
Apostiça-F	Winter	Adult females	Portugal	Apostiça	38°34'N 9°07'W	2008	17	Present paper	Present paper
Alcácer	Winter	L1 larvae	Portugal	Alcácer	38°23'N 8°31'W	2002	31	Santos <i>et al.</i> , 2007	Present paper
Viseu	Winter	L1 larvae	Portugal	Viseu	40°40'N 7°54'W	2002	24	Santos <i>et al.</i> , 2007	Present paper
Guadarrama	Winter	L1 larvae	Spain	Guadarrama	40°41'N 4°02'W	2002	24	Santos <i>et al.</i> , 2007	Present paper
Granada	Winter	L1 larvae	Spain	Granada	37°47'N 3°46'W	2002	23	Santos <i>et al.</i> , 2007	Present paper

from our previous work to ensure that alleles were correctly identified, and this new data set was merged with all data from Santos *et al.* (2007).

For MS-*Thpit*2, exceptionally long alleles were found in the SP (see Results). Four individuals having long alleles were amplified and cloned using the Strataclone PCR cloning kit (Stratagene, Santa Clara, CA, USA). Eight to ten clones were sequenced per individual using the BigDye terminator v3 cycle sequencing kit (Applied Biosystems) and carried out with an ABI 3100 automatic sequencer. This procedure ensured that both alleles were sequenced for each individual and permitted to check whether the long sizes of these alleles were actually attributable to a higher number of repetition of the microsatellite motif or to an insertion.

Data analyses

File format conversions were all performed using CONVERT (Glaubitz, 2004). Allelic richness and frequencies, as well as observed and expected heterozygosities

were calculated for each locus using GENETIX v. 4.04 (Belkhir *et al.*, 1996–2004). Histograms of allelic frequencies were built using R (R Development Core Team, 2008). Hardy–Weinberg equilibrium (HWE) was tested using ARLEQUIN 3.11 (Excoffier *et al.*, 2005) for each locus and population, with a significance level of 95%, using 1000 permutation steps and 100 000 steps in the Markov chain. Linkage disequilibrium (LD) was tested in each population for all pairs of loci with 10 000 permutations using ARLEQUIN. Null allele frequencies were estimated for each locus using the expectation maximization (EM) algorithm of Dempster *et al.* (1977) as performed in the FRENA package (Chapuis & Estoup, 2007).

Analyses were conducted using first instar larvae and then repeated with all sampled individuals (i.e. including males and females). Larval populations were considered as 'reference populations', both because their location can be clearly identified and because only embryonic mortality has occurred. On the other hand, adult populations may have been strongly affected by selection,

and the individuals may have flown and been trapped far from their locality of origin.

Two loci, namely *MS-Thpit2* and *MS-Thpit6*, showed significant departures from HWE (see Results). Subsequently, all analyses were first performed using all loci and then repeated excluding these two loci to check for consistency of the results.

Population genetic structure

Population structure was first analysed using pairwise F_{ST} (Weir & Cockerham, 1984) estimated using the excluding null alleles (ENA) correction implemented in *FRÉNA* to correct for the positive bias induced by the presence of null alleles (Chapuis & Estoup, 2007). The 95% confidence intervals of corrected pairwise F_{ST} values were obtained by bootstrapping 1000 times over loci.

The principal component analysis (PCA) was then used to analyse the microsatellite data set. One advantage of the PCA is that it is free from strong assumptions about an evolutionary model (e.g. HWE), which is particularly valuable to analyse poorly known biological systems (Patterson *et al.*, 2006; Jombart, 2008; Jombart *et al.*, 2009). PCA was complemented by a statistical procedure called the between-class test to assess the between-population divergences. The procedure compares the observed between-class inertia (i.e. variance) to the corresponding values stemming from randomizations (Dolédéc & Chessel, 1987). Both PCA and randomization tests were performed using the R packages *adegenet* (Jombart, 2008) and *ade4* (Chessel *et al.*, 2004). A PCA was first performed with the larvae sampled from the SP (all years), from Leiria WP (all years) and from the four additional sites sampled in Portugal and Spain. The between-population test was conducted using 1000 randomizations. A complementary analysis was then performed using all sampled individuals, including larvae, males and females.

Individual assignments

We used larvae and adults sampled at different dates in Leiria to assign individuals to clusters based on their multilocus genotypes using a Bayesian inference method implemented in *STRUCTURE* 2.3.3 (Pritchard *et al.*, 2000). We fixed the number K of clusters to 2, as the objective was to test whether individuals could be correctly assigned to either the SP or the Leiria WP. We used 100 000 burn-in steps followed by 100 000 MCMC simulation steps with a model allowing admixture. To assess the consistency of results, we performed 25 independent runs and carefully compared the obtained individual Q-matrices. The results were graphically displayed using *DISTRUCT* 1.1 (Rosenberg, 2004).

We also performed assignment tests using *GENECLASS2* (Piry *et al.*, 2004) to specifically test whether reference populations of larvae could be assigned or excluded as origin of the trapped adults based on their genotypes. We used four reference populations containing only larvae,

namely Leiria SP (all years), Leiria WP (all years), Portugal (Alcácer and Viseu) and Spain (Guadarrama and Granada) and assigned all males and females trapped in Leiria and in the nearby site of Apostiça. We used the Bayesian method described in Rannala & Mountain (1997). For each individual, the score of assignment to each reference population was computed as defined in Piry *et al.* (2004).

Test of a founder effect

The allelic richness was compared between Leiria SP and Leiria WP to test whether it was significantly reduced in the SP. The test was conducted for larvae from LWP02, LWP04 and LWP05 for Leiria WP, and LSP03, LSP06 and LSP07 for Leiria SP (see Table 1 for details). We used the two-sample Wilcoxon test (also known as the Mann–Whitney test) to compare the median of the average allelic richness (i.e. the allelic richness divided by the sample size) in WP vs. SP. We used the function Wilcoxon test from the R software (R Development Core Team, 2008). The procedure was carried out for each locus separately and with all loci together. As a result of the peculiar results obtained with *MS-Thpit2*, the test was repeated with five loci only.

Results

Monitoring of males' flight period

In Leiria, for the 3 years of study, the males started to emerge between the 3rd and 12th of May and were mostly trapped between the end of May and the end of

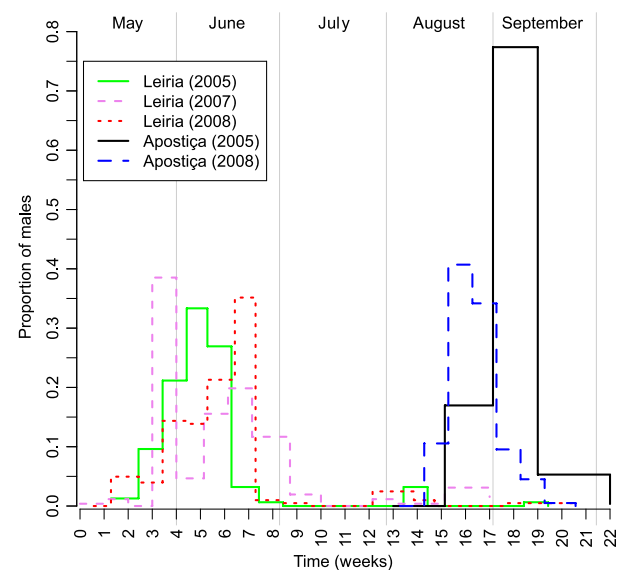


Fig. 2 Males caught with pheromone traps from May to September in Leiria (2005, 2007 and 2008) and Apostiça (2005 and 2008). For each date, data are expressed as the proportion of males caught by site and by year.

June. No male was captured between the end of June and the end of July, and then a small number of males were captured in August and September. In all years of monitoring, the shortest interval between male captures (i.e. the time during which no males were trapped) in Leiria was 24 days (in 2007) and the longest was 41 days (2005). In the reference WP of Apostiça, the flight season occurred between August and the end of September in all years (Fig. 2).

Microsatellite results

The new data obtained here were merged with the data available from Santos *et al.* (2007) to obtain a final data set of 341 individuals genotyped with six markers.

The total number of alleles per locus varied from eight in locus *MS-Thpit5* to 40 in locus *MS-Thpit6*. The total number of alleles per population ranged from 27 to 32 in Leiria SP, from 33 to 47 for Leiria WP, and from 53 to 57 in the other Portuguese and Spanish WPs (Table 2) except for the females collected in Apostiça (Table 1).

Distribution of allelic frequencies in Leiria WP and SP is shown for all markers in Fig. 3, and the detailed allelic frequencies are given in Supporting Information (Table S1). Except for locus *MS-Thpit2*, all the alleles found in Leiria SP were also present in Leiria WP, whereas the opposite was not true. In several cases, the allelic frequencies of the SP were distorted compared to the frequencies of the Portuguese WPs. For example, *MS-Thpit1* is almost fixed for allele 165 (96%) in the SP, whereas this allele is never above 61% in any Portuguese

Table 2 Number of individuals (*N*), observed (*H_o*) and expected (*H_e*) heterozygosities, percentage of null alleles (%NA) per locus estimated using FREENA and total number of alleles found in each *Thaumetopoea pityocampa* population. Population codes are given in Table 1.

Population code	<i>N</i>	<i>H_o</i>	<i>H_e</i>	%NA	%NA	%NA	%NA	%NA	%NA	Total number of alleles
				<i>MS-ThPit1</i>	<i>MS-ThPit2</i>	<i>MS-ThPit3</i>	<i>MS-ThPit4</i>	<i>MS-ThPit5</i>	<i>MS-ThPit6</i>	
LSP03	29	0.48	0.51	0.000	0.041	0.000	0.102	0.000	0.181	30
LSP06	30	0.46	0.48	0.000	0.045	0.000	0.011	0.000	0.121	29
LSP07	30	0.46	0.49	0.000	0.055	0.000	0.000	0.000	0.227	32
LSP07-M	16	0.47	0.48	0.000	0.000	0.000	0.000	0.021	0.000	27
LSP08-F	19	0.44	0.52	0.000	0.000	0.002	0.000	0.076	0.354	27
LWP02	15	0.52	0.63	0.000	0.219	0.010	0.000	0.089	0.116	33
LWP04	36	0.52	0.61	0.000	0.150	0.000	0.000	0.067	0.261	47
LWP05	30	0.51	0.62	0.027	0.148	0.015	0.004	0.000	0.279	43
LWP07-M	15	0.58	0.70	0.000	0.172	0.101	0.000	0.000	0.000	35
Apostiça-F	17	0.44	0.55	0.097	0.000	0.000	0.037	0.000	0.435	23
Alcácer	31	0.60	0.75	0.006	0.105	0.019	0.051	0.000	0.291	50
Viseu	24	0.68	0.76	0.023	0.049	0.009	0.000	0.000	0.120	56
Guadarrama	24	0.64	0.73	0.000	0.065	0.000	0.000	0.000	0.234	57
Granada	23	0.69	0.81	0.000	0.128	0.000	0.000	0.053	0.262	53

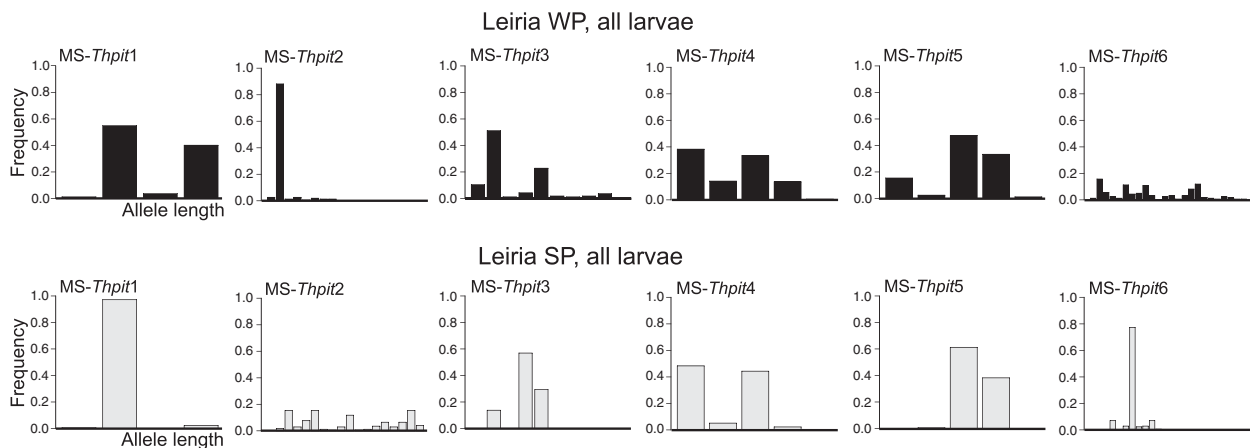


Fig. 3 Allelic frequencies found for larvae sampled in Leiria WP and Leiria SP for the six studied microsatellite markers. In each population, the frequencies were calculated with individual larvae sampled from all years.

WP. Likewise, for locus MS-*Thpit3* the allele 239 has very low frequencies in WP (values between 2% and 8%) whereas it is the most frequent in SP, with values ranging between 46% and 67% depending on the year of sampling. Results obtained from locus MS-*Thpit2* were completely different, as it was the single locus for which the number of alleles in the SP was higher than in the WP. Longer alleles were found only in the SP for this locus. In all WPs (including Leiria), allele sizes ranged from 143 to 163 bp, whereas alleles up to 183 bp were found in the SP. The 'long alleles' (> 165 bp) were found for each sampled year of the SP in similar proportions, and the corresponding chromatograms were unambiguous. Cloning and sequencing indicated that these alleles showed an increase in the number of repetitions, consistent with the observed size, and no mutation in the flanking regions or interruptions owing to insertions in the microsatellite motif. Consequently, we can confidently assume that these long alleles and the peculiar results obtained for marker MS-*Thpit2* are not an artefact.

All populations were in HWE for MS-*Thpit1*, MS-*Thpit3*, MS-*Thpit4* and MS-*Thpit5*, except population LSP03 for MS-*Thpit5* and Granada for MS-*Thpit1* and MS-*Thpit4*. On the contrary, no population was in HWE for MS-*Thpit6* except the male samples (LSP07-M and LWP07-M). Finally, most populations were not in HWE for MS-*Thpit2* (all populations but LSP07, LSP07-M, LSP08F, Apostiça-F and Viseu). Consistently, using the EM algorithm available in FREEANA, these two loci (MS-*Thpit2* & MS-*Thpit6*) were estimated to have high proportions of null alleles in several populations (Table 2). Interestingly, all populations except the male samples (LSP07-M and LWP07-M) had over 10% of null alleles for MS-*Thpit6*, and the two female samples (LSP08-F and Apostiça-F) even reached 35% and 43%. Moreover, all females were homozygous for this locus. Concerning MS-*Thpit2*, the estimations were over 10%

of null alleles for all the samples of the Leiria WP (LWP02, LWP04, LWP05 and LWP07-M), as well as for Alcácer and Granada. No pairs of loci were in LD in more than two populations, except for the pairs MS-*Thpit1* – MS-*Thpit6* and MS-*Thpit2* – MS-*Thpit3* that were in LD in three populations. Hence, the microsatellite loci used were considered independent.

Population genetic structure

The matrix of pairwise F_{ST} obtained with the ENA correction for the presence of null alleles (Chapuis & Estoup, 2007) is given in Table 3. These indices were significant between any pair of populations except when comparing different years of the SP (pairwise $F_{ST} < 0.011$, whatever the development stage – larvae, males or females) or different years of Leiria WP when considering only larvae (pairwise $F_{ST} < 0.012$). Interestingly, the pairwise differentiation indices were slightly higher when comparing male Leiria WP to larvae from the same locality (F_{ST} ranging between 0.021 and 0.042, this latter value being significant). The differentiation between any year of SP and any year of Leiria WP was always above 18.0% and reached 26.6% except when comparing the Leiria WP males to any Leiria SP samples (F_{ST} were in this case between 0.075 and 0.123), indicating that sympatric SP and WP are significantly and strongly differentiated. F_{ST} values between Leiria SP and the populations sampled in other Iberian localities were comprised between 13.2% with Alcácer and 32.4% with Apostiça. The pairwise F_{ST} obtained between pairs of geographically distant WPs were significant but lower than those obtained between any SP and any WP. Differentiation indices between Leiria WP and any other WP were comprised between 1.6% (with Viseu) and 15.1% (with Guadarrama). When omitting MS-*Thpit2* and MS-*Thpit6* from the analyses, we obtained very similar F_{ST} estimates (Table S2, Supporting information).

Table 3 Pairwise F_{ST} matrix obtained using all microsatellite loci after applying the ENA correction for null alleles using FREEANA; significant indices are shown in bold.

	LSP03	LSP06	LSP07	LSP07-M	LSP08-F	LWP02	LWP04	LWP05	LWP07-M	Apostiça-F	Alcácer	Viseu	Guadarrama
LSP06	0.010												
LSP07	0.003	0.003											
LSP07-M	0.010	-0.001	-0.001										
LSP08-F	-0.004	0.014	-0.002	0.011									
LWP02	0.223	0.266	0.261	0.264	0.217								
LWP04	0.201	0.239	0.237	0.243	0.194	0.006							
LWP05	0.188	0.225	0.224	0.232	0.180	0.012	-0.007						
LWP07-M	0.086	0.120	0.120	0.123	0.075	0.042	0.028	0.021					
Apostiça-F	0.276	0.318	0.312	0.324	0.271	0.038	0.036	0.026	0.083				
Alcácer	0.153	0.181	0.173	0.190	0.132	0.087	0.093	0.077	0.046	0.110			
Viseu	0.161	0.201	0.190	0.200	0.143	0.048	0.048	0.038	0.016	0.056	0.026		
Guadarrama	0.182	0.212	0.204	0.222	0.167	0.151	0.143	0.119	0.096	0.139	0.034	0.047	
Granada	0.232	0.260	0.252	0.249	0.213	0.113	0.126	0.120	0.100	0.145	0.071	0.061	0.075

A PCA was run including Leiria SP and WP as well as geographically distant WPs. The two first axes explained 19.7% and 10.1% of the total inertia, respectively. Axis one separated Leiria SP from all other WPs, and axis 2 separated the different WPs (Fig. 4). The between-class test was significant ($P < 0.001$) revealing strong genetic differences. Similar results were obtained without *MS-Thpit2* and *MS-Thpit6*. When the PCA was performed using all individuals (i.e. larvae, males and females), the axes explained 19.13% and 8.65% of the total inertia, respectively, and the between-class test was also highly significant. Consistently, males and females sampled from the Leiria SP clearly grouped with the larvae of the same population, but only 12 males trapped in Leiria WP (i.e. males trapped in Leiria between the end of July and September) grouped with the corresponding larvae, whereas three clustered with the larvae of the SP (Fig. S1, Supporting information). The females sampled from Apostiça were very close to the Leiria WP.

Individual assignments

Larvae and adults from Leiria, sampled in different seasons and years, were analysed using the Bayesian method implemented in STRUCTURE 2.3.3 with a number of clusters $K = 2$ to check whether the genetic assign-

ment matches the *a priori* phenotypic populations (SP and WP). The individual genetic assignments were very similar across the 25 runs performed. The individual Q-matrix of membership coefficients is graphically displayed in Fig. 5. The two main genetic clusters obtained actually mostly corresponded to the 'phenotypic' SP and WP. For clarity, the two clusters will be called 'genetic SP' and 'genetic WP', whereas the phenotypic groups (determined *a priori* on the basis of phenological phenotypes, i.e. on the date of hatching for larvae and the date of emergence for males and females) will be called 'phenotypic SP' and 'phenotypic WP'. For 196 of the 222 individuals (88%), the genetic cluster fully corresponded to the phenotypic group, with membership coefficients Q over 0.90 [this value reaching 205 individuals (92%) for a threshold of 0.80 for the membership coefficient]. For 10 individuals, the genetic cluster corresponded to the phenotypic group but with Q membership coefficients comprised between 0.59 and 0.80. For one 'phenotypically WP' male, the genetic assignment was intermediate (membership coefficients of 0.49 for WP and 0.51 for SP). On the other hand, for three larvae, the genetic and the phenotypic groups did not match, but membership coefficients were moderate (one 'phenotypically SP' larva was genetically assigned to WP with $Q = 0.58$, and two 'phenotypically WP' larvae were

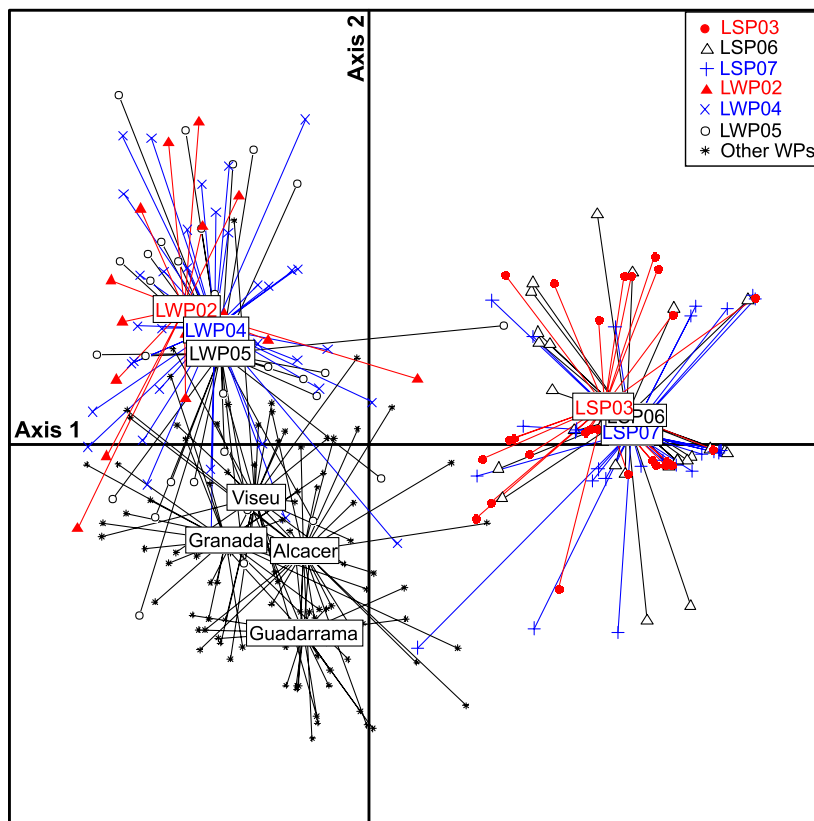


Fig. 4 Typology of pine processionary moth larvae obtained by principal component analysis of all six microsatellite data, projection of individuals upon the plane defined by components 1–2. Lines link individuals to the corresponding population (codes are given in Table 1). The analysis included all larvae sampled in Leiria (WP and SP) in different years and individuals sampled in different localities of Spain and Portugal.

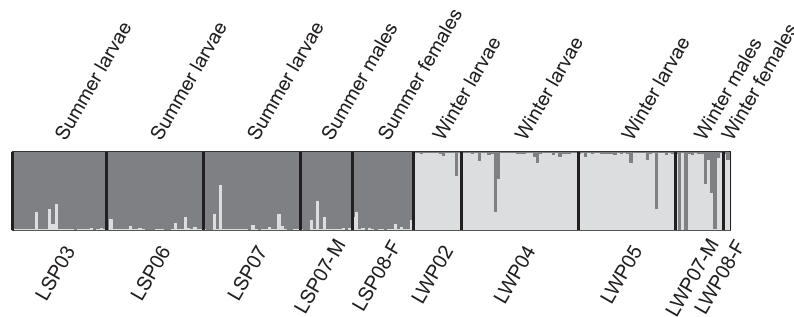


Fig. 5 Graphical representation of the individual Q-matrix (mean over 25 runs) obtained for $K = 2$ and 222 individuals (larvae, males and females) sampled from Leiria in different years and different seasons.

genetically assigned to SP with $Q = 0.76$ and $Q = 0.73$, respectively). Finally, it is worth noting that three males trapped during the normal flight season of the WP (thus 'phenotypically WP') were assigned to the 'genetic SP' with very high coefficients (0.99). Those three males were also clustered with the SP individuals in the PCA.

The results obtained with GENECLASS2 to assign adults to the reference larval populations were largely consistent with those obtained with STRUCTURE. All SP males and females were genetically assigned to the larval populations of Leiria SP with assignment scores (as defined in Piry *et al.*, 2004, p. 538) close to 100, except for one female that was preferentially assigned to the other Portuguese populations, with a score of 87. For comparison, using STRUCTURE, this particular female was assigned to the 'genetic SP' with a membership coefficient of 0.87. Concerning the males trapped during the WP flight period, three individuals were assigned to the SP larvae with maximal scores (100); consistently, these individuals were also assigned to the 'genetic SP' using STRUCTURE. All other WP males were assigned to either the Leiria WP larval population or nearby Portuguese populations. The same was true for all the females collected in Apostaça.

Test of a recent founder effect

Statistical analysis (Wilcoxon test) of allelic richness locus by locus revealed that Leiria WP had a significantly higher allelic richness for loci MS-*Thpit1* ($P = 0.036$), MS-*Thpit3* ($P = 0.05$), MS-*Thpit5* ($P = 0.05$) and MS-*Thpit6* ($P = 0.05$) but that no significant statistical differences were observed between Leiria SP and Leiria WP for locus MS-*Thpit4* ($P = 0.25$). On the contrary, locus MS-*Thpit2* had a significantly higher allelic richness in the SP than in the sympatric WP ($P = 0.05$). Concerning analyses conducted with all loci together, SP had a significantly reduced global allelic richness compared to WP ($P = 0.05$). The results were similar when MS-*Thpit2* was excluded from the analysis ($P = 0.05$).

Discussion

Some preliminary considerations

Our study is based on a comprehensive sampling of larvae and adults of *T. pityocampa*, mostly from Leiria, that was genotyped using six microsatellite markers. Yet, two of these markers had significant proportions of null alleles and showed deviations from Hardy–Weinberg equilibrium. Even though the results were robust enough to be consistent even when omitting these two markers, it is important to understand how reliable they are. Concerning MS-*Thpit6*, our data show that male samples are in HWE for this locus and have a proportion of null alleles estimated to zero. On the contrary, the female samples are not in equilibrium, with a high proportion of null alleles. Moreover, all females (i.e. 38 individuals) are systematically homozygous for this locus in spite of the high number of alleles found and the high corresponding expected heterozygosity. The most plausible explanation is thus that this microsatellite marker is located on the sexual Z chromosome (in Lepidoptera, females are heterogametic (Z–W), whereas males are Z–Z).

On the other hand, the results concerning MS-*Thpit2* are puzzling, as it is the single locus showing a set of alleles that were unique from the Leiria SP. These 'long' alleles (see Fig. 3) were not detected elsewhere, neither in Leiria WP nor in the other Iberian populations. They were also not observed in a previous study based on six populations scattered all over France (Kerdelhué *et al.*, 2006). Yet, the estimations of null allele frequencies suggest that many null alleles occur for this locus in all Leiria WP samples, whereas it is not the case for the SP, nor for most other Iberian populations. Similarly, a low proportion of null alleles were observed in the French populations mentioned earlier (not shown). We can thus confidently consider that the genotypes observed in Leiria SP are reliable and that the long alleles are actually absent from other regions of Portugal, Spain and France. Yet, when discussing and interpreting our data, we have

to keep in mind that the results obtained for *MS-Thpit2* in Leiria WP are biased owing to the presence of null alleles and amplification problems.

Phenology suggests complete reproductive isolation...

In 4 years of monitoring, we always observed a bimodal curve of adult emergence in Leiria, whereas typical curves of male trapping in the PPM are unimodal (Démolin, 1969). A period of 26–42 days without any male catch was observed each year around July. One peak of male activity is observed from the beginning of May to early July (with a maximum reached in June, see Fig. 2), and the other from the end of July to late September, which is consistent with earlier data (Pimentel *et al.*, 2006). The second peak corresponds to the normal period of male activity for the PPM in Portugal at low altitudes, as is exemplified by the data we obtained in Apostiça as well as previously published data (Barrento *et al.*, 2008). The most plausible hypothesis is thus that the first peak corresponds to the emergence of the SP adults and the second one to the WP adults (but see below). The low number of male captures within the normal emergence periods of WP males in Leiria is also consistent with field observations showing that the population size of Leiria WP is low (H. Santos & M. Branco, ISA, Lisbon, pers. obs.). Whether this is related to the existence of the SP or attributable to other environmental conditions or to natural changes in population dynamics remains an open question. Our data strongly suggest that a time lag of ca. 1 month occurs each year between the latest emergences of the SP and the earliest emergences of the sympatric WP. As adults do not feed, and live up to a maximum of 3–4 days (Démolin, 1969; Zhang & Paiva, 1998), we could conclude that SP and WP individuals cannot mate and that sexual reproduction between the two sympatric populations is limited, or even nonexistent. Phenology effectively leads to prezygotic isolation.

... and genetic data show a clear differentiation, but with a limited flow of individuals from SP to WP

Data on male captures alone do not allow ruling out the hypothesis that genetic exchange still occurs between populations. After larval development, an obligate diapause occurs during the pupation stage and diapause termination determines the dates of adult emergence (Huchon & Démolin, 1970). Gene flow between both populations could thus occur via individuals shifting from one phenology to the other through the mechanisms underlying adult emergence. In other words, some 'winter larvae' could emerge as 'summer adults' after pupal diapause and vice versa. If this was the case, genetic evidence of gene flow between populations should be found, and we could expect the within-

population genetic structure to vary in time as a consequence of gene flow.

Yet, the genetic data we obtained for different years showed both a clear and extreme differentiation between SP and WP and the stability in time of the genetic structure observed in Leiria. The pairwise F_{ST} matrix, the PCA and the Bayesian analyses run with **STRUCTURE** all show that the SP is significantly differentiated from all studied WP. Individuals sampled in different years from the SP clearly fall in the same genetic cluster (yet with the exception of one larva from LSP07), and a similar pattern was mostly observed for Leiria WP (with only two mis-assigned larvae). Differentiation between different localities of WP is significant but always lower than the SP vs. WP indices. The results were robust, as similar conclusions were found even when omitting the two microsatellite markers *MS-Thpit2* and *MS-Thpit6*. Thus, our main conclusion is that the Leiria SP forms a very distinct genetic cluster, which is highly stable in time. The most plausible force responsible for the genetic isolation is allochronic differentiation, as the SP and the local WP apparently differ only by their timing of emergence and sexual reproduction. As a consequence, we suggest that the Leiria SP could now be considered as a highly differentiated 'phenological race', in the same manner that host races have been proposed for phytophagous or parasitoid insect lineages using different hosts (e.g. Drès & Mallet, 2002; Stireman *et al.*, 2005; Peccoud *et al.*, 2009). Individuals from the SP now experience unique selective pressures compared to the other PPM populations, such as temperature and hygrometry during metamorphosis, embryogenesis and the first stages of larval development, quality of consumed foliage and natural enemies. Natural selection is thus likely to favour the evolution of adaptations to these peculiar conditions, which could lead to reinforcement of the differentiation. Allochronic speciation has been hypothesized in a few cases, as a mechanism to explain a past speciation event between fully separated taxa (Cooley *et al.*, 2001; Ritchie, 2001; Abbot & Withgott, 2004; Danley *et al.*, 2007). We discovered here an exceptional case of ongoing allochronic differentiation that could be seen as a first stage of an incipient sympatric speciation without host shift.

Interestingly though, the Bayesian analysis of population structure and the assignment tests conducted with the adults clearly show that a limited amount of gene flow can occur between the SP and the WP. Among the 222 individuals, three larvae were assigned to the wrong genetic cluster compared to their phenotypic group, with membership coefficients Q close to 0.75, two males *a priori* 'phenotypically' WP had intermediate Q (0.48 and 0.59 for the WP, respectively) and three males trapped during the WP normal flight period were even genetically assigned to the SP with $Q = 0.99$. These results will need to be confirmed by increasing the number of males analysed, but they

strongly suggest that a proportion of individuals can shift from the SP to the normal 'winter' phenology. We hypothesize that individuals from the SP can occasionally experience a longer diapause and emerge as adults in July or August rather than April or May. They can then reproduce with WP individuals, causing a limited amount of gene flow between populations. This is probably a rare event, as it was never observed in laboratory conditions (MB & HS, pers. obs.). This would explain both the existence of 'phenologically WP but genetically SP' males and the occurrence of larvae that are identified by STRUCTURE as genetic hybrids. The opposite shift (from a normal WP phenology to the SP) is apparently very rare, as the SP adults were all unambiguously assigned to the SP genetic cluster, and a large majority of the SP larvae were correctly assigned to the SP genetic cluster. It is thus puzzling that a strong genetic differentiation is maintained between SP and WP in spite of flows of individuals. This result could suggest that shifting individuals and hybrids could be counter-selected because of a mal-adaptation to environmental conditions and hence do not significantly contribute to the next generations. Laboratory experiments will also be necessary to test whether SP and WP individuals can cross and give fertile offspring.

Local origin of the SP?

Different scenarios can be proposed to explain the existence of a PPM population with a shifted phenology. Based on previous sequencing and genotyping data, Santos *et al.* (2007) favoured a scenario involving a recent local origin of the SP from a sudden phenological 'mutation' and a bottleneck effect. The new data presented here are mostly consistent with this hypothesis. Except for MS-*Thpit2*, we observed a lower number of alleles in the SP than in the WP for all sampling seasons, with all alleles found in SP also present in Leiria WP (but not the opposite) and a distortion of the allelic frequencies. However, the data obtained for MS-*Thpit2* are contradictory, as its alleles are more numerous in the SP than in the WP and many alleles present in the SP were not found elsewhere. In particular, we found a whole group of long-sized alleles that were not observed even in the other Iberian populations. It is very unlikely that the new alleles appeared locally after the differentiation of the SP from mutation alone, both because it would be possible only if the SP was founded thousands of years ago (if we consider that the average mutation rate for microsatellite markers is ca. 10^{-3} , Goldstein & Pollock, 1997) and remained undetected, which is not likely for this well-known and conspicuous species, and because no new alleles were detected for the other five loci used. These data can be consistent with the scenario of local phenological shift and foundation of the SP from few individuals of the sympatric WP if one supposes that all the long alleles

detected in SP were rare but present in the WP but remain undetected owing to sampling or technical biases (null alleles, see 'technical considerations' above). More markers will be necessary in the future, even though developing microsatellites in Lepidoptera is not straightforward (Zhang, 2004), to determine whether MS-*Thpit2* is the only locus showing this kind of pattern and whether all other loci confirm the scenario involving a founder effect.

Our results clearly show the occurrence of a sympatric allochronic differentiation process. The most plausible scenario is that a phenologically shifted population has recently been founded by individuals originating from the local genetic pool and that very limited gene flow now occurs between both populations. This could be the first stage of allochronic speciation, which is a rarely documented process. The SP can be seen as a 'phenological race', exposed to different ecological pressures and constraints that could cause further divergence and maintain the genetic differentiation. Ecological consequences and factors potentially limiting the expansion of the SP should now be analysed. Moreover, laboratory rearing and experimental crosses will be necessary to better understand the differentiation (and possibly the taxonomic status) of both populations.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Typology of all pine processionary moth individuals (larvae, males and females) obtained by principal component analysis of all six microsatellite data, projection of individuals upon the plane defined by components 1–2. Lines link individuals to the corresponding population (codes are given in Table 1). (a) All individuals. (b) Only the dots corresponding to adult females are shown. (c) Only the dots corresponding to adult males are shown. (d) Only the dots corresponding to larvae are shown.

Table S1 Allelic frequencies for each microsatellite locus and each population.

Table S2 Pairwise F_{ST} matrix obtained using only four of the microsatellite loci, after applying the ENA correction for null alleles using FREENA; the excluded loci are MS-*Thpit2* and MS-*Thpit6*. Confidence intervals were not estimated due to the low number of loci used.

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