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Mating patterns of the European grapevine moth, *Lobesia botrana* (Lepidoptera: Tortricidae) in sympatric and allopatric populations

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Phytophagous insects have been at the heart of investigations of ecological speciation, and it is clear that adaptation to different host plant species can promote host race formation and insect speciation. However, the evolution of host races has typically been studied at the plant species scale, using sympatric populations of insects that are specialized on particular plant species. Because many crop pest species are adapted to various plant varieties selected from a single plant species, it is of interest to establish whether reproductive barriers could evolve at this much smaller geographical scale, between individuals exploiting different plant varieties. To assess this we evaluated premating and postmating prezygotic barriers among sympatric populations of the European grapevine moth *Lobesia botrana* originated from different cultivars of the same plant species (*Vitis vinifera*), and between allopatric populations originated from different geographical sites. We found weak reproductive isolation for sympatric populations of *L. botrana*, but marked reproductive isolation among allopatric populations. In sympatric populations, the only effect was on the latency period prior to mating, which was longer for heterotypic partners that originated from different cultivars than for homotypic partners originated from the same cultivar. In allopatric populations, reproductive isolation was evident in both premating barriers and postmating prezygotic barriers. In summary, we did not find any trend for sympatric host race formation in *L. botrana*, but the occurrence of non-random mating patterns between different allopatric populations suggests the beginning of reproductive isolation, which could lead to the evolution of cryptic species of *L. botrana*. © 2016 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2016, **00**, 000–000.

KEYWORDS: allopatric speciation – cryptic species – host races – *Lobesia botrana* – mating patterns – reproductive isolation.

INTRODUCTION

Understanding the geographical context for speciation is central to evolutionary biology (Coyne & Orr, 2004). Speciation is now widely accepted as a continuous process in which genetic variation becomes segregated among populations, but the relative importance of each of the modes of speciation (allopatric vs. sympatric) remains uncertain (Turelli, Barton & Coyne, 2001). Nevertheless, the formation of new species can be inextricably linked to adaptation to the ecological environment (Nosil, 2012). Specifically, the ecological hypothesis of speciation states that reproductive isolation evolves ultimately as a consequence of divergent natural selection on traits between environments (biotic and abiotic elements of habitats), and can occur in allopatry or sympatry (Schluter, 2001; Funk, Filchak & Feder, 2002; Rundle & Nosil, 2005; Rabosky, 2016). For allopatric speciation, whereby new species arise from geographically separated populations of the same ancestral species (Mayr, 1963; Coyne, 1992; Barraclough & Vogler, 2000), both prezygotic barriers (including ecogeographic, mechanical, temporal, and behavioural isolation) and postzygotic barriers (hybrid inviability and sterility) often evolve following

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geographical isolation because of divergent selection and/or genetic drift (Ramsey, Bradshaw & Schemske, 2003; Rundle & Nosil, 2005; Gray *et al.*, 2016). In sympatric speciation, the evolution of reproductive isolation occurs under a variety of conditions without geographic barriers (reviewed by Bolnick & Fitzpatrick, 2007), and its occurrence in nature has recently been demonstrated in several studies (Malausa *et al.*, 2005; Barluenga *et al.*, 2006; Papadopulos *et al.*, 2011). The impact of environmental factors on mating traits, and their contributions to speciation, has been widely studied (Maan & Seehausen, 2011; Servedio *et al.*, 2011). For sympatric populations, the key mechanism ensuring premating reproductive isolation over time is assortative mating (i.e. patterns of non-random mating; Jiang, Bolnick & Kirkpatrick, 2013), which is therefore a pivotally important component of ecological speciation (Dieckmann & Doebeli, 1999; Via, 2001; Malausa *et al.*, 2005; Schluter & Conte, 2009).

Herbivorous insects have been at the centre of investigations of 'ecological speciation', from which it is clear that adaptation to different host plants can promote host race formation, and therefore speciation (Funk *et al.*, 2002; Matsubayashi, Ohshima & Nosil, 2010). Indeed, for many herbivorous insects the host plants are the site of most activities, including feeding, development, oviposition, pupation, and sometimes mating (Schoonhoven, Van Loon & Dicke, 2005). Because of the great intimacy between herbivores and their host plants, phytophagous insects are powerful model systems to investigate speciation mechanisms via divergent host plant specialization (Berlocher & Feder, 2002; Drès & Mallet, 2002). A central hypothesis in the study of ecological speciation is that host plant specialization in recently diverged taxa is often because of features that also produce assortative mating. For example in the pea aphid (*Acyrthosiphon pisum*) the influence of feeding behaviour (and especially the behavioural acceptance of a plant as host) on both assortative mating and resource specialization is central to the maintenance of host races of this species (Caillaud & Via, 2000). Globally, the occurrence of host races of herbivorous insects has often been used as evidence for the likelihood of sympatric speciation (Drès & Mallet, 2002). Most studies on host race formation have involved phytophagous insects feeding on different host plant genera or species (Bush, 1969; Emelianov *et al.*, 2003; Linn *et al.*, 2012). Because plants of different species or genera differ markedly in their chemical, physical, distributional, and phenological traits (Jaenike, 1990), phytophagous insects are often specialized on a limited number of host plants, and divergent ecological specialization can contribute to the buildup of reproductive isolation (Rundle & Nosil, 2005). However, some herbivorous insects, particularly crop pest species, more closely exploit host plants in cultivated areas, feeding on different varieties of the same plant species, which are thought to have more similarities than occur between different plant species. Although sympatric speciation has typically been sought among species exploiting very different plant species or genera, no study investigating the occurrence of assortative mating on a much smaller scale, within individuals adapted to different varieties of a single host plant species, have been reported.

We investigated this phenomenon using the European grapevine moth (*Lobesia botrana*), which is one of the most important grapevine pests worldwide. Its adaptation to grapes is considered to be relatively recent, as abundant populations were first noticed in vineyards during the beginning of the 20th century (Balachowsky & Mesnil, 1935). One hypothesis, based on the ecological habits and actual geographical distribution of the pest, strongly supports a Mediterranean origin, with progressive extension first towards Central and then Western Europe (Maher & Thiéry, 2006). *L. botrana* is now found throughout Europe, north and west Africa, the Middle East, and eastern Russia. It was more recently (2008–2010) introduced into Japan, Argentina, Chile, and the USA (California) (Gilligan *et al.*, 2011). While cultivars of the same species are likely to be more similar than different species, much evidence is available to support the hypothesis that *L. botrana* can locally adapt to a particular cultivar at small geographic scales. Firstly, as for many other phytophagous insects, *L. botrana* larvae have low mobility (Torres-Vila *et al.*, 1997), and the fate of the offspring is greatly affected by the mother's oviposition preference (Moreau *et al.*, 2008). Indeed, females are able to disperse within and among vineyards (although the range of this species remains unknown), and during their lifetime to distribute eggs on different plants. To achieve this, the mated females typically oviposit at dusk, laying eggs singly in response to olfactory cues (Gabel & Thiéry, 1994; Masante-Roca, Gadenne & Anton, 2002), physical characteristics of the oviposition site (Maher & Thiéry, 2003), and taste stimuli (Maher & Thiéry, 2004; Maher, Thiéry & Städler, 2006). Female choice of oviposition site can also be modulated by natal habitat preference induction (NHPI), with females from larvae raised on grapes having increased preference to lay eggs on the same cultivar on which they had experienced as larvae (Moreau *et al.*, 2008). NHPI, whereby early experience of a particular host increases the probability that the same host is chosen for egg laying following adult dispersal, can result in patterns of host-associated assortative

mating that restrict gene flow among populations. Secondly, a recent study of *L. botrana* demonstrated that the phenology of adult emergence was influenced by the grape variety (Thiéry, Monceau & Moreau, 2014). Differences in the phenology can lead to assortative mating between moths because the likelihood of encountering moths of the same cultivar is higher than encountering moths of a different cultivar due to a synchrony of adult emergence. Thirdly, larval food quality greatly affects male and female reproductive life history traits and reproductive success (Moreau *et al.*, 2007; Muller *et al.*, 2015), and could shape mating patterns in this species.

The aim of our study was to investigate the occurrence of reproductive isolation among sympatric populations derived from different host grape cultivars in the same vineyard, which is a very small spatial scale never previously studied. We also investigated these patterns for allopatric populations derived from different geographical sites, to serve as a control at a much larger scale. To assess the occurrence of both allopatric and sympatric speciation, we evaluated multiple premating (mating success, mating duration, and the latency period prior to mating) and postmating prezygotic (oviposition latency, and fecundity and fertility of mated females) barriers to gene exchange among three different allopatric and sympatric populations of *L. botrana*.

MATERIAL AND METHODS

Field sampling

Larvae of *L. botrana* corresponding to the first larval generation of the year were collected in the field in France during May 2014. To test for a cultivar effect on mating patterns in sympatric populations, larvae were sampled from three grape (*Vitis vinifera*) cultivars (Carignan: CAR; Grenache: GRE; and Syrah: SYR) that were located within several metres of each other in the same vineyard (N 42°44′7.063″, E 2°52′56.441″) in Perpignan (P), France. To test for a geographical effect in allopatric populations, larvae were also sampled from the same cultivar (GRE) from three geographically distinct vineyards including: Perpignan (P); Nîmes (N), which is 200 km east of P (N 43°56′49.781″, E 4°39′39.372″); and Sénas (S), which is 290 km east of P and 90 km east of N (N 43°43′54.251″, E 5°1′45.621″).

For each cultivar and geographical site, the larvae were sampled at the end of the larval cycle (fifth instar) when they were building glomerulae made of silk and flower buds (phenology 17–25; Eichhorn & Lorenz, 1977). *L. botrana* larvae complete their development in a single bunch, and each glomerulus contains only one larva (Torres-Vila *et al.*, 1997). The larvae were reared to adulthood in the laboratory in large polyethylene boxes (60 × 40 cm, height 21 cm) at 22 ± 1 °C, 60 ± 10% RH, and under ambient photoperiod conditions, and were fed *ad libitum* using grape bunches from the same cultivar and location where they were reared. The larvae were checked daily until pupation, at which time the pupae were gently extracted from glomerulae. The pupae were weighed to the nearest 0.01 mg using a Precisa 262 SMA-FR microbalance, placed individually in glass tubes (70 × 9 mm diameter) stoppered with cotton wool plugs, and stored at 22 °C under ambient photoperiod conditions. The pupae were checked each morning, and newly emerged adults were immediately sexed. Males and females were used for the mating experiments.

No-choice mating experiments

No-choice mating trials were conducted to assess assortative mating as a function of cultivar (sympatric populations) or geographical site (allopatric populations). We used no-choice tests because this most closely reflects the natural situation: females are isolated in vineyards, and male moths use sex pheromones to find a conspecific female and make contact with only one potential mate at a given time (Thiéry, 2008). This procedure has been successfully used in numerous studies that have investigated the occurrence of assortative mating in sympatric and allopatric speciation (Munoz *et al.*, 2010; Xue, Li & Yang, 2014). To test for a cultivar effect, males and females reared on the same host cultivar (CAR × CAR, GRE × GRE or SYR × SYR) or on different cultivars (the six reciprocal crosses) were tested ($N = 30$ for each combination). To test for a geographical effect, males and females reared on the same cultivar (GRE) from the same geographical site (P × P, N × N, S × S) or from different geographical sites (the six reciprocal crosses) were tested ($N = 30$ for each combination).

At dusk, a single 2-day-old virgin male originating from each test condition (cultivar or geographical site) was placed into a mating tube (100 × 15 mm diameter) stoppered with a cotton plug with a single 2-day-old virgin female originating from each test condition (cultivar or geographical site). The observations were made at dusk by one observer (K. Muller) who constantly observed the mating tubes during 4 h (corresponding to the natural mating period of the female each night) and recorded each novel mating event. Mating was considered to have been successful if: (1) mating lasted for > 30 min (which is the minimum time required for the spermatophore to be fully transferred in this species; K. Muller, personal observation); and (2) the female subsequently

laid fertile eggs. Females that engaged in mating for > 30 min and laid no eggs during their lifetimes were considered non-fertile, and were discarded from the analysis. The mating success of individuals was evaluated based on: (1) observed matings, including fertile + non-fertile matings; and (2) fertile matings (observed matings that led to the production of fertile eggs). If the pair in the mating tube had not mated after 4 h, we considered the mating to be a failure. The latency period prior to mating (the time from pairing to copulation, reflecting the reluctance or acceptance to mate) and the duration of mating (the time spent copulating) were recorded only for fertile matings.

Female reproductive output

After one successful mating, and to avoid the possibility of confusing premating isolation with postmating prezygotic interactions, we kept the female in her mating tube following copulation, and counted the number of eggs laid by that female to ensure that the mating had been successful and gave rise to fertile progeny. The females were provided with water *ad libitum*, and could oviposit freely on the inside surface of the glass tube until their death. Female survival was checked daily, and after the female died the eggs were incubated under the same conditions. During this period we recorded: (1) the period until the first egg was laid; (2) fecundity (the mean number of eggs laid per female); (3) fertility (the proportion of eggs that hatched); and (4) female longevity.

Statistical analyses

All statistical tests were performed using R Software version 3.2.0 (R Development Core Team, 2015). For each analysis we recorded the full model with insignificant interactions deleted, following the approach of Forstmeier & Schielzeth (2011). The premating and postmating prezygotic barriers to reproductive isolation were assessed by: (1) measuring the effect of cultivar by pooling individuals in homotypic pairs (partners originated from the same cultivar) vs. heterotypic pairs (partners originated from different cultivars), using a geographical control to compare within-population pairs (partners from the same geographical site) vs. between-population pairs (partners from two different geographical sites); and (2) measuring the effect of each of the nine crosses (involving individuals from different cultivars or different sites) at a much finer scale. We analyzed separately the mating success of observed matings (including fertile + non-fertile matings) and fertile matings (only those matings that led to the formation of fertile eggs). All others analyses (the latency period prior to mating, the mating duration, and female reproductive traits) were based only on fertile matings.

For no-choice experiments, which were designed to detect cultivar or geographical effects, we used the program JMating to calculate the index of pair sexual isolation (I_{PSI}) for the allopatric and sympatric populations (Carvajal-Rodriguez & Rolan-Alvarez, 2006). IPSI values range from −1 to +1, where 0 represents random mating, +1 represents complete assortative mating (i.e. all matings homotypic) and −1 represents complete disassortative mating (i.e. all matings heterotypic). Statistical significance for sexual isolation was determined by bootstrapping 10 000 times in JMating. The mating successes for observed matings (the proportion of male–female pairs that had copulating within 4 h) and for fertile matings (the proportion of mated females that laid fertile eggs) were analyzed using logistic regression with a binomial distribution (success/failure), and significance was assessed using likelihood ratio (LR) tests. A cox regression was applied to assess the effect of male and female origin (cultivar and geographical site) and pupal mass on the latency period prior to mating. The effects of male and female origin on the duration of mating and the female fecundity were analyzed using analyses of covariance (ANCOVAs) (with the pupal mass of males and females as covariates, respectively). A generalized linear model (GLM) having a quasi-binomial error structure and a logit link function was used to analyze the proportion of hatched eggs produced by females mated with males of different origin.

RESULTS

Cultivar effect on reproductive isolation

Premating isolation

In total, 270 no-choice experiments (N = 30 per combination) were used to evaluate differences in the probability of mating for homotypic and heterotypic crosses between virgin males and females of *L. botrana* from three different cultivars from the same vineyard. Overall, the results provided no evidence of host cultivar-associated sexual isolation (heterotypic vs. homotypic pairs) for both observed matings (fertile + non-fertile matings; $I_{PSI} = 0.005 \pm 0.076$, $P = 0.942$) and fertile matings (only matings that led to the formation of fertile eggs; $I_{PSI} = 0.047 \pm 0.078$, $P = 0.536$). *L. botrana* moths were equally likely to mate if paired with a member of the opposite sex having the same (observed homotypic mating: 74.4%; fertile homotypic mating: 72.2%) or different

(observed heterotypic mating: 73.9%; fertile heterotypic mating: 66.1%) host cultivar of origin. The observed mating success of virgin *L. botrana* males and females did not vary based on their cultivar of origin (Table 1; male cultivar of origin: LR = 1.08, $P = 0.582$; female cultivar of origin: LR = 5.044, $P = 0.080$). However, the fertile mating success differed according to the female cultivar of origin (male cultivar of origin: LR = 0.87, $P = 0.647$; female cultivar of origin: LR = 8.08, $P = 0.018$). Males from Carignan were more likely to fertilize females from Carignan than females originated from Syrah (76.7% vs. 46.7% of successful fertile matings, respectively). However, the mating success of males from Grenache and Syrah was the same, irrespective of the female cultivar of origin (Table 1).

For fertile matings, the latency period prior to mating was significantly longer for heterotypic pairs (45.1 ± 3.2 min) than homotypic pairs (34.3 ± 3.3 min) (Fig. 1; Cox regression, $\chi^2_{1,184} = 5.76$, $P = 0.016$). Similarly, the latency period prior to mating for the nine cultivar crosses was affected by the cultivar of origin of individuals (Fig. 2; Cox regression, $\chi^2_{1,184} = 18.8$, $P = 0.015$). For example, females from Carignan copulated sooner with males from Carignan (22.2 ± 2.9 min) than with males from Grenache (53.1 ± 7.9 min) or Syrah (53.2 ± 8.3 min). However, the pupal mass did not influence the latency period prior to mating (Table 2). The time spent mating lasted an average 50–58 min and did not vary among cross types (Tables 2 and 3), for the results combined (homotypic vs. heterotypic effect: $F_{1,179} = 0.07$, $P = 0.288$) or for individual populations pairs (overall model: $F_{4,179} = 0.479$, $P = 0.751$).

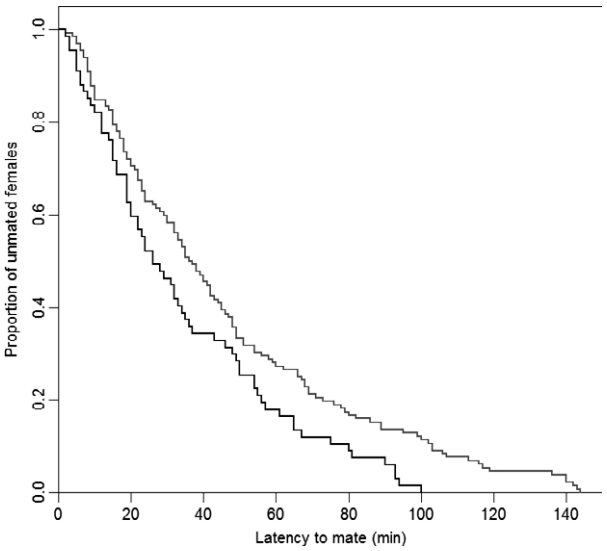

Figure 1. Proportion of unmated females according to the latency period prior to mating (min) in no-choice experiments between virgin males and females of *L. botrana* originated from the same cultivar (homotypic pairs: CAR × CAR, GRE × GRE, SYR × SYR ; black line) or from different cultivars (heterotypic pairs: CAR × GRE, CAR × SYR, GRE × CAR, GRE × SYR, SYR × CAR, SYR × GRE ; grey line) in the same vineyard. CAR, cv Carignan; GRE, cv Grenache; SYR, cv Syrah.

Postmating prezygotic isolation

Among mated females, the delay prior to the first formation of eggs (i.e. the oviposition latency) was not affected by the male or female cultivar of origin or pupal mass (Tables 2 and 3; overall model: $\chi^2_{5,184} = 6.37$, $P = 0.383$), even if the data for homotypic vs. heterotypic pairs were combined ($F_{1,184} = 0.10$, $P = 0.752$). Similarly, female fecundity did not differ among cross types, for the results combined (homotypic vs. heterotypic effect: $F_{1,180} = 0.45$, $P = 0.505$) or for the individual population pairs (Tables 2 and 3), but the number of eggs laid was positively correlated with female pupal mass (overall model: $F_{6,177} = 15.87$, $P < 0.0001$). The fertility level was high, ranging from 93.0% to 96.5% of hatched eggs, and did not vary among cross types for the result combined (homotypic vs. heterotypic effect: $F_{1,180} = 1.27$, $P = 0.521$) or for the individual population pairs (Tables 2 and 3).

Table 1. Percentage of successful observed (fertile + non-fertile matings) and fertile matings (mating that led to the production of fertile eggs) among the nine pairs of partners originated from the three cultivars (CAR, Carignan; GRE, Grenache; SYR, Syrah); $N = 30$ per combination

	♂ CAR, %		♂ GRE, %		♂ SYR, %	
	Observed	Fertile	Observed	Fertile	Observed	Fertile
♀ CAR	76.7	76.7 (a)	73.3	73.3	76.7	73.3
♀ GRE	76.7	70.0 (ab)	83.3	76.7	83.3	73.3
♀ SYR	60.0	46.7 (b)	73.3	60.0	63.3	63.3

Shaded squares represent homotypic pairs (partners from the same host cultivar). Squares in columns with different letters are significantly different ($P < 0.05$).

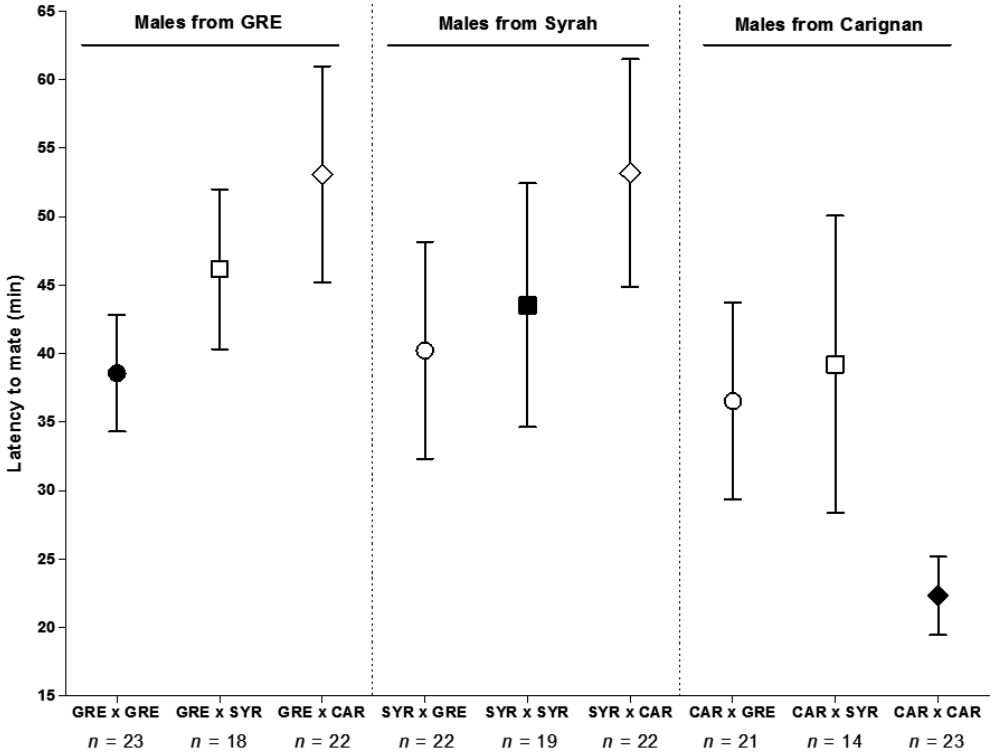

Figure 2. Latency period prior to mating in no-choice experiments between virgin males and females of *L. botrana* originated from three different cultivars. Black symbols represent homotypic pairs (partners from the same cultivar) and white symbols represent heterotypic pairs (partners from different cultivars). Symbols represent female cultivar origin (circles: Grenache; squares: Syrah; diamonds: Carignan). CAR, Carignan; GRE, Grenache; SYR, Syrah.

Geographical effect on reproductive isolation

Premating isolation

In total, 270 no-choice experiments (N = 30 per combination) were used to evaluate differences in the probability of mating between virgin males and females of *L. botrana* reared on the same cultivar (GRE) at three different geographical sites (allopatric populations). This was considered in relation to between-population crosses (partners from different geographical sites) and within-population crosses (partners from the same geographical site). Overall, the experiments revealed marked allopatric sexual isolation among the three different populations of moths (observed mating success: $I_{PSI} = 0.172 \pm 0.075$, $P = 0.029$; fertile mating success: $I_{PSI} = 0.288 \pm 0.079$, $P = 0.001$). A greater proportion of insects from a particular geographical site mated with members of the opposite sex from the same site (observed within-population mating: 80%; fertile within-population mating: 73.3%) than with mates from other geographical sites (observed between-population mating: 57.8%; fertile heterotypic mating: 41.1%). Similarly, for the nine geographical crosses the observed mating success of virgin *L. botrana* males and females varied according to their mutual geographical site of origin, with males and females from the same site have greater mating success than males and females having different geographical origins six; male geographical site × female geographical site: LR = 14.13, $P = 0.007$). This effect was more pronounced for matings that led to the production of fertile eggs (Table 4; male geographical site × female geographical site: LR = 25.76, $P < 0.0001$).

For fertile matings, the latency period prior to mating was significantly longer for between-population pairs than within-population pairs (Fig. 3; Cox regression, $\chi^2_{1,139} = 86.74$, $P < 0.0001$). Similarly, for the nine geographical crosses individually the latency period prior to mating was affected by the interaction between the male and female cultivars of origin (Fig. 4; Cox regression, male × female geographical site interaction, $\chi^2_{4,139} = 97.39$, $P < 0.0001$). Partners from the same geographical site copulated sooner than did partners from different geographical sites. However, the pupal mass did not influence the latency period prior to mating (Table 5).There was no difference in the time spent mating for between-population vs.

Table 2. Male and female cultivar and pupal mass effects on precopulatory barriers (latency period prior to mating and mating duration) and postcopulatory prezygotic barriers (female oviposition latency, fecundity and fertility)

	Precopulatory barriers				Postcopulatory barriers					
	Latency to mate*		Mating duration†		Oviposition latency*		Fecundity‡		Fertility§	
Source	Test-value	*P*	Test-value	*P*	Test-value	*P*	Test-value	*P*	Test-value	*P*
Male × female cultivar	LR = 10.33	0.035	$F_{4,173} = 7.11$	0.130	LR = 6.57	0.160	$F_{4,173} = 0.52$	0.72	LR = 2.87	0.579
Male cultivar	LR = 7.76	**0.021**	$F_{2,173} = 0.73$	0.696	LR = 4.26	0.119	$F_{2,173} = 0.14$	0.87	LR = 2.21	0.331
Male mass	LR = 0.09	0.762	$F_{1,173} = 4.52$	**0.033**	LR = 0.20	0.648	$F_{1,173} = 0.52$	0.47	LR = 1.07	0.300
Female cultivar	LR = 0.08	0.961	$F_{2,173} = 1.49$	0.474	LR = 0.25	0.884	$F_{2,173} = 0.40$	0.67	LR = 1.70	0.426
Female mass	LR = 1.92	0.166	$F_{1,173} = 3.31$	0.069	LR = 0.97	0.320	$F_{1,173} = 92.67$	**< 0.0001**	LR = 2.90	0.088

Bolded *P*-values indicate significance.
*Cox regression.
†GLM using a log transformation.
‡ANCOVA.
§GLM using a quasi-binomial error structure.

within-population crosses (population effect: $F_{1,139} = 0.03$, $P = 0.874$). For the nine crosses individually, the duration of mating ranged from 47.5 to 65.5 min, and did not depend on the male or female geographical origin (Tables 5 and 6; overall model: $F_{6,132} = 1.15$; $P = 0.339$).

Postmating prezygotic isolation

For the three population pairs combined, the delay prior to the first appearance of eggs (i.e. the oviposition latency) was not affected by the cross type (Table 6; between-population vs. within-population effect: $F_{1,139} = 0.76$, $P = 0.384$). However, among mated females in the nine crosses the delay prior to the first appearance of eggs was affected by the female geographical origin but not by the male geographical origin or the male or female pupal mass (Tables 5 and 6; overall model: $\chi^2_{6,139} = 17.33$, $P = 0.008$). Females from N mated with males from P took less time to lay their first eggs than females from P and S (Table 6). In some combinations the fecundity and fertility were reduced for females in between-population vs. within-population crosses. Firstly, among the three population pairs combined the female fecundity was significantly less for between-population (52.1 ± 1.8 eggs) vs. within-population crosses (71.4 ± 2.6 eggs) (overall model: $F_{3,135} = 31.1$, $P < 0.0001$; between-population vs. within-population effect: $F_{1,135} = 42.26$, $P < 0.0001$; female mass effect: $F_{1.135} = 43.17$, $P < 0.0001$). Among mated females in the nine crosses, this effect was detected for two of the three groups of allopatric populations (Fig. 5A; global model: $F_{10,128} = 5.93$, $P < 0.0001$; male site × female site interaction: $F_{4,128} = 10.04$, $P < 0.0001$), with the within-population pairs from N and S producing more eggs than the between-population pairs. Secondly, female fertility was less in the between-population pairs ($88.7 \pm 0.8\%$ of hatched eggs) relative to the within-population pairs ($94.9 \pm 0.5\%$ of hatched eggs), for the results combined (between-population vs. within-population effect: $F_{1,135} = 139.59$, $P < 0.0001$) and for individual population pairs (Fig. 5B; interaction site male × site female: $F_{4,135} = 143.72$, $P < 0.0001$).

DISCUSSION

This study is the first to have assessed the occurrence of reproductive isolation in sympatric and allopatric populations of *L. botrana*, and is also the first to have assessed the effect of the plant cultivar, particularly at a very small geographical scale. Our results provided little evidence for sympatric isolation between moths from the same population originated from different host cultivars. We found no

Table 3. Precopulatory (number of fertile matings and mean ± SEM of mating duration) and postcopulatory prezygotic barriers (mean ± SEM of oviposition latency, fecundity and fertility) to reproductive isolation for the nine crosses of males and females from the three cultivars (CAR, Carignan; GRE, Grenache; SYR, Syrah) from the same vineyard (P); $N = 30$ for each combination

Cultivar crosses		Precopulatory barriers		Postcopulatory barriers		
Male	Female	Number of fertile matings	Mating duration (min)	Oviposition latency (days)	Fecundity (number of eggs laid)	Fertility (percentage of eggs hatched)
CAR	CAR	23	56.8 ± 3.1	2.4 ± 0.2	60.6 ± 5.0	94.2 ± 1.3
CAR	GRE	21	53.6 ± 2.4	2.5 ± 0.3	55.7 ± 4.8	93.4 ± 1.3
CAR	SYR	14	46.6 ± 2.6	2.8 ± 0.3	58.1 ± 5.2	95.0 ± 0.9
GRE	CAR	22	53.9 ± 2.9	3.6 ± 0.7	62.4 ± 4.1	94.9 ± 0.8
GRE	GRE	23	54.6 ± 3.4	2.7 ± 0.3	60.5 ± 4.3	96.5 ± 0.7
GRE	SYR	18	58.2 ± 4.6	2.4 ± 0.2	63.1 ± 4.9	93.0 ± 1.6
SYR	CAR	22	57.1 ± 3.6	2.1 ± 0.2	61.2 ± 4.7	95.2 ± 0.9
SYR	GRE	22	50.5 ± 2.0	2.4 ± 0.2	63.4 ± 4.1	95.3 ± 1.1
SYR	SYR	19	56.9 ± 3.6	2.4 ± 0.2	60.3 ± 3.4	94.6 ± 1.6
Homotypic		65	56.1 ± 1.9	2.5 ± 0.1	60.4 ± 2.5	95.2 ± 0.7
Heterotypic		119	53.6 ± 1.3	2.6 ± 0.2	60.8 ± 1.9	94.5 ± 0.5

Shaded rows indicated homotypic pairs (from the same cultivar).

Table 4. Percentage of successful observed (fertile + non-fertile matings) and fertile matings (mating that led to the production of fertile eggs) between the nine pairs of partners from the three geographical sites (Perpignan, Nîmes, and Sénas) on the same cultivar (Grenache); $N = 30$ per combination

	♂ Perpignan (%)		♂ Nîmes (%)		♂ Sénas (%)	
	Observed	Fertile	Observed	Fertile	Observed	Fertile
♀ Perpignan	25 (83.3) a	23 (76.7) a	21 (70.0) a	12 (40.0) b	16 (53.3) b	10 (33.3) b
♀ Nîmes	16 (53.3) b	13 (43.3) b	24 (80.0) a	20 (66.7) a	24 (80.0) a	17 (56.7) ab
♀ Sénas	13 (43.3) b	11 (36.7) b	14 (46.7) b	11 (36.7) b	23 (76.7) a	23 (76.7) a

Shaded squares represent homotypic pairs (partners from the same host cultivar). Values in the same column having different letters are significantly different ($P < 0.05$).

evidence in our experiments for sexual isolation between pairs originated from different cultivars, or for postmating isolation. However, we found that the latency period prior to mating was longer for heterotypic partners originated from different cultivars than for homotypic partners originated from the same cultivar. In contrast to this weak sympatric effect on mating patterns, we detected strong premating and postmating reproductive isolation between *L. botrana* pairs where the partners came from populations in different geographical locations. Premating isolation was evident for both assortative mating (higher mating success for within-population pairs: 73.3% vs. 41.1%) and the latency period prior to mating (much longer for between-population pairs). Moreover, homotypic pairs had more eggs and sired more viable offspring than heterotypic pairs, which indicates partial reproductive incompatibility among individuals from the different populations.

Reproductive isolation in allopatric populations

We detected marked allopatric reproductive isolation between different geographical pairs of populations of *L. botrana*. Reproductive isolation in allopatric populations has been demonstrated in numerous species to be a result of multiple barriers arising from genetic divergence between geographically separated populations (Mendelson, Imhoff & Venditti, 2007; Nosil, 2007; Jennings, Snook & Hoikkala, 2014). At the premating level we found reduced copulation success among heterotypic pairs compared with homotypic pairs, which was primarily because of a lack of

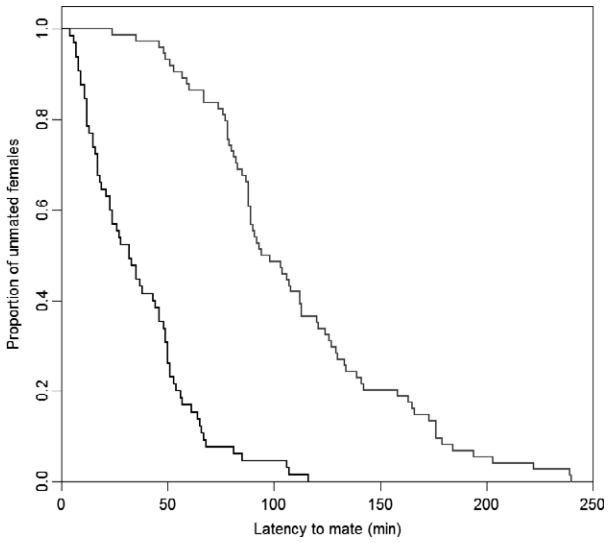

Figure 3. Proportion of unmated females according to the latency period prior to mating (min) between virgin males and females of *L. botrana* originated from the same geographical site (homotypic pairs: N × N, P × P, S × S, black line), or at different geographical sites (heterotypic pairs: N × P, N × S, P × N, P × S, S × N, S × P; grey line) on the same cultivar (Grenache). Geographical sites were Perpignan (P), Nîmes (N), and Sénas (S).

motivation to mate. Among the potential barriers leading to reproductive isolation in moths, mate selection behaviour is one of the most important (Dopman, Robbins & Seaman, 2010). The difference between allopatric populations in terms of mating probability may be largely because of differences in the probability of initiating courtship and the motivation to mate (i.e. the calling position in females), as has been shown for *Drosophila* species (Price & Boake, 1995; Jennings *et al.*, 2011) and in the cabbage beetle, *Colaphellus bowringi* (Liu *et al.*, 2014). Indeed, among successful matings between heterotypic partners, the latency period prior to mating was nearly three times longer than for homotypic partners, suggesting a greater aversion of males and/or females to mate heterotypically. In Lepidoptera, the pheromones produced by females generally comprise a blend of compounds, major components of which are long-chain derivatives of fatty acids that specifically attract males of the same species, which reduces the chance of heterotypic mating. In some cases the pheromone blend can differ depending on the geographical distribution of a species (Boo, 1998; Kawazu *et al.*, 2000). The sex pheromone of *L. botrana*, which has been studied since the 1970s, consists mainly of the compound (7E,9Z)-7,9-dodecadienyl acetate (E7,Z9-12Ac), plus some other minor compounds (Roelofs *et al.*, 1973; El-Sayed *et al.*, 1999; Witzgall *et al.*, 2005). We hypothesize that differences in the ratios and combinations of pheromone minor components in *L. botrana* females from different allopatric populations explain the low success rate of heterotypic partners. Further studies in wind tunnels testing the orientation of males to such females should produce interesting data.

In our study, mating of *L. botrana*, even for > 30 min, did not necessary lead to the production of fertile eggs, especially in heterotypic matings. Non-fertile mating can result from a failure to transfer spermatophores. When moths copulate the male stays attached to the female, even in the absence of successful insemination (Muller, personal observations). Mating success is usually high in butterflies and moths, with most species exceeding 75% (usually 95%) success for mated females (Rhainds, 2010). This success is much greater than we observed for heterotypic crosses. At the postmating prezygotic level, heterotypic pairs produced significantly fewer progeny than homotypic crosses, and we hypothesize that mating success may be reduced by reproductive cytoplasmic incompatibility in crosses involving different allopatric populations. In our experiment we did not determine at what stage (e.g. sperm transfer/storage, cryptic female choice, sperm–egg interaction, embryonic development) the observed hybrid dysfunction occurred, but we speculate on the following explanations. Firstly, the reduced fecundity observed in heterotypic pairs could reflect reduced fertilization rates, with only fertilized eggs being laid (Gregory & Howard, 1994). Secondly, it is possible that heterotypic males transfer less sperm than homotypic males to females, and those females reduce their oviposition rates when sperm is limited. Moreover, only a fraction of heterotypic sperm could be stored in female spermathecal, this being inefficient (Price *et al.*, 2001). Thirdly, the sperm of heterotypic males might contain seminal proteins that are unable to stimulate oviposition by females (Herndon & Wolfner, 1995), explaining why we found non-fertile matings. Moreover, the longer oviposition latency of heterotypic partners could reflect cryptic female choice, with for example, females blocking or delaying sperm transfer. Gametic incompatibility, in which sperm is (for instance) physiologically incapable of fertilizing the eggs, has occasionally been reported in Lepidoptera (Dopman *et al.*, 2010). Further laboratory studies are needed on the mechanisms underlying the sexual isolation between allopatric populations in *L. botrana*.

Our results provide reproduction-based evidence supporting the suggestion that the three populations of *L. botrana* studied potentially belong to three reproductively isolated cryptic species. Cryptic species exhibit few if any differences in morphology, but can have very distinct mating signals (e.g.

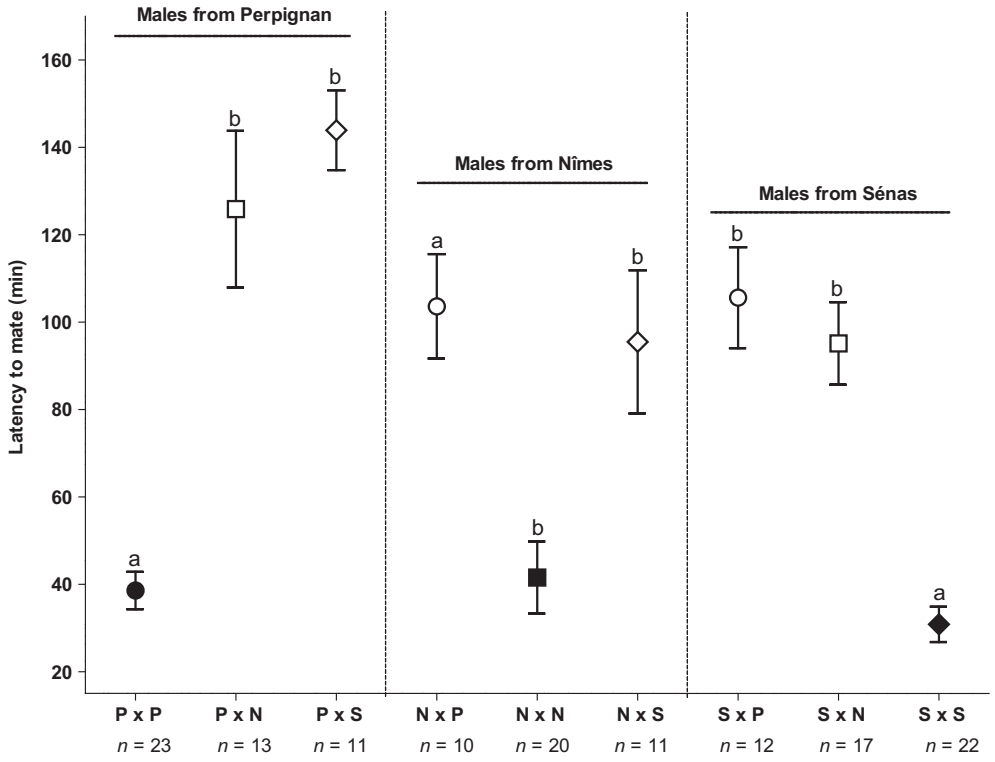

Figure 4. Latency period prior to mating in no-choice experiments between virgin males and females of *L. botrana* originated from different geographical sites (Perpignan: P; Nîmes: N; Sénas: S). Black symbols represent within-population pairs (from the same population) and white symbols represent between-population pairs (from different populations). Symbols represent the female geographical origin (circles: P; squares: N; diamonds: S). Values for mating pairs with the same letter are not significantly different ($P > 0.05$).

differences in pheromone composition) leading to ambiguous mate recognition and partial sexual isolation. Future studies should investigate the genetic differentiation of *L. botrana* moths derived from different allopatric populations over a wide geographical area, and the sex pheromone composition of females. Our study revealed the potential occurrence of speciation in allopatric populations, but what about sympatric populations, at a local scale?

Reproductive isolation in sympatric populations

Many studies have reported the presence of sympatric host races among phytophagous insects using different host plant species, including the larch budmoth (*Zeiraphera diniana*; Emelianov *et al.*, 2003), the European corn borer (*Ostrinia nubilalis*; Malausa *et al.*, 2005), and the ladybird beetle (*Henosepilachna diekei*; Matsubayashi, Kahono & Katakura, 2013). However, we found no evidence for sympatric host race formation in *L. botrana* species originated from different grape cultivars separated by several meters in the same vineyard. Indeed, the index of sexual isolation between moths from different cultivars was non-significant, suggesting completely random mating occurred between males and females from the different cultivars. In our experiment the moth larvae came from several cultivars of only one host plant species (*V. vinifera*) grown in close proximity. Thus, males and females were likely to encounter and recognize potential mates from the various cultivars, because of their geographical and chemical proximity. This is different from the studies cited above, in which host race formation was demonstrated at a much larger scale, using insects that are highly specialized on particular host plants belonging to different species; in this situation the degree of specialization may be greater than for insects exploiting closely related cultivars of host plants.

However, we demonstrated that heterotypic partners had a longer latency period prior to mating than did homotypic partners. This may reflect a greater reluctance of males and/or females to mate heterotypically than is indicated by the index of sexual isolation (Baur & Baur, 1992). These findings may also help explain reproductive isolation in nature, because heterotypic courtships last longer

Table 5. Effects of male and female geographical site, and male and female pupal mass on precopulatory (latency period prior to mating and mating duration) and postcopulatory prezygotic (female oviposition latency, fecundity and fertility) barriers

	Precopulatory behaviours				Female reproductive success					
	Latency to mate*		Mating duration†		Oviposition latency*		Fecundity‡		Fertility§	
Source	Test-value	*P*	Test-value	*P*	Test-value	*P*	Test-value	*P*	Test-value	*P*
Male × female site	LR = 96.93	**< 0.0001**	$F_{4,128} = 0.73$	0.570	LR = 3.38	0.496	$F_{4,128} = 11.05$	**< 0.0001**	LR = 58.68	**< 0.0001**
Male site	LR = 0.95	0.622	$F_{2,128} = 2.73$	0.069	LR = 1.54	0.462	$F_{2,128} = 5.07$	**0.008**	LR = 3.16	0.206
Male mass	LR = 2.04	0.153	$F_{1,128} = 1.09$	0.299	LR = 0.61	0.436	$F_{1,128} = 0.01$	0.933	LR = 0.7	0.392
Female site	LR = 1.69	0.429	$F_{2,128} = 0.04$	0.957	LR = 11.80	**0.002**	$F_{2,128} = 2.02$	0.137	LR = 0.82	0.665
Female mass	LR = 2.39	0.122	$F_{1,128} = 0.01$	0.957	LR = 2.56	0.109	$F_{1,128} = 25.96$	**< 0.0001**	LR = 4.98	**0.026**

Bolded *P*-values indicate significance.
*Cox regression.
†GLM using a log transformation.
‡ANCOVA.
§GLM using a quasi-binomial error structure.

Table 6. Precopulatory (number of fertile matings and mean ± SEM of mating duration) and postcopulatory prezygotic (mean ± SEM of oviposition latency) barriers to reproductive isolation for the nine crosses of males and females from the three geographical sites (Perpignan: P; Nîmes: N; Sénas: S)

Geographical site crosses		Precopulatory barriers		Postcopulatory barriers
Male	Female	Number of fertile matings	Mating duration (min)	Oviposition latency (days)
P	P	23	54.6 ± 3.5	2.7 ± 0.3 a
P	N	13	53.3 ± 4.5	1.5 ± 0.2 b
P	S	11	50.2 ± 3.2	2.2 ± 0.4 a
N	P	10	59.4 ± 4.8	2.4 ± 0.4 a
N	N	20	57.3 ± 5.5	2.2 ± 0.3 a
N	S	11	65.5 ± 5.6	1.9 ± 0.2 a
S	P	12	47.5 ± 2.1	2.2 ± 0.2 a
S	N	17	55.2 ± 5.3	1.9 ± 0.1 a
S	S	23	51.5 ± 2.8	1.7 ± 0.1 a
Within-population		66	54.4 ± 2.3	2.2 ± 0.1
Between-population		74	54.9 ± 1.9	2.0 ± 0.1

Shaded rows indicate within-population pairs (from the same geographical site). Values having different letters are significantly different ($P < 0.05$).

and are more likely to be interrupted. This may reflect ecologically mediated divergence in mating signals between individuals derived from different cultivars. In our study the random mating pattern could be in part a result of the no-choice experimental design used. Subjects in no-choice tests are unlikely to reject a mating opportunity because the likelihood of other opportunities is unknown, so the risk of remaining unmated is high. Thus, in the no-choice experimental design individuals may be less likely to exhibit mating preference, and be more likely to mate randomly (Barry & Kokko, 2010; Booksmythe, Jennions & Backwell, 2011). The strength of mating preferences can vary greatly under different experimental designs (Dougherty & Shuker, 2014), and several studies have shown that choice experiments lead to higher estimates of sexual isolation than no-choice experiments (Coyne, Elwyn & Rolán-Alvarez, 2005; Jennings *et al.*, 2011). Further studies using choice design and measuring more accurately individual precopulatory behaviours may be useful in this moth species for detecting mating preferences based on host plant cultivar, and to assess the occurrence of assortative mating under natural conditions.

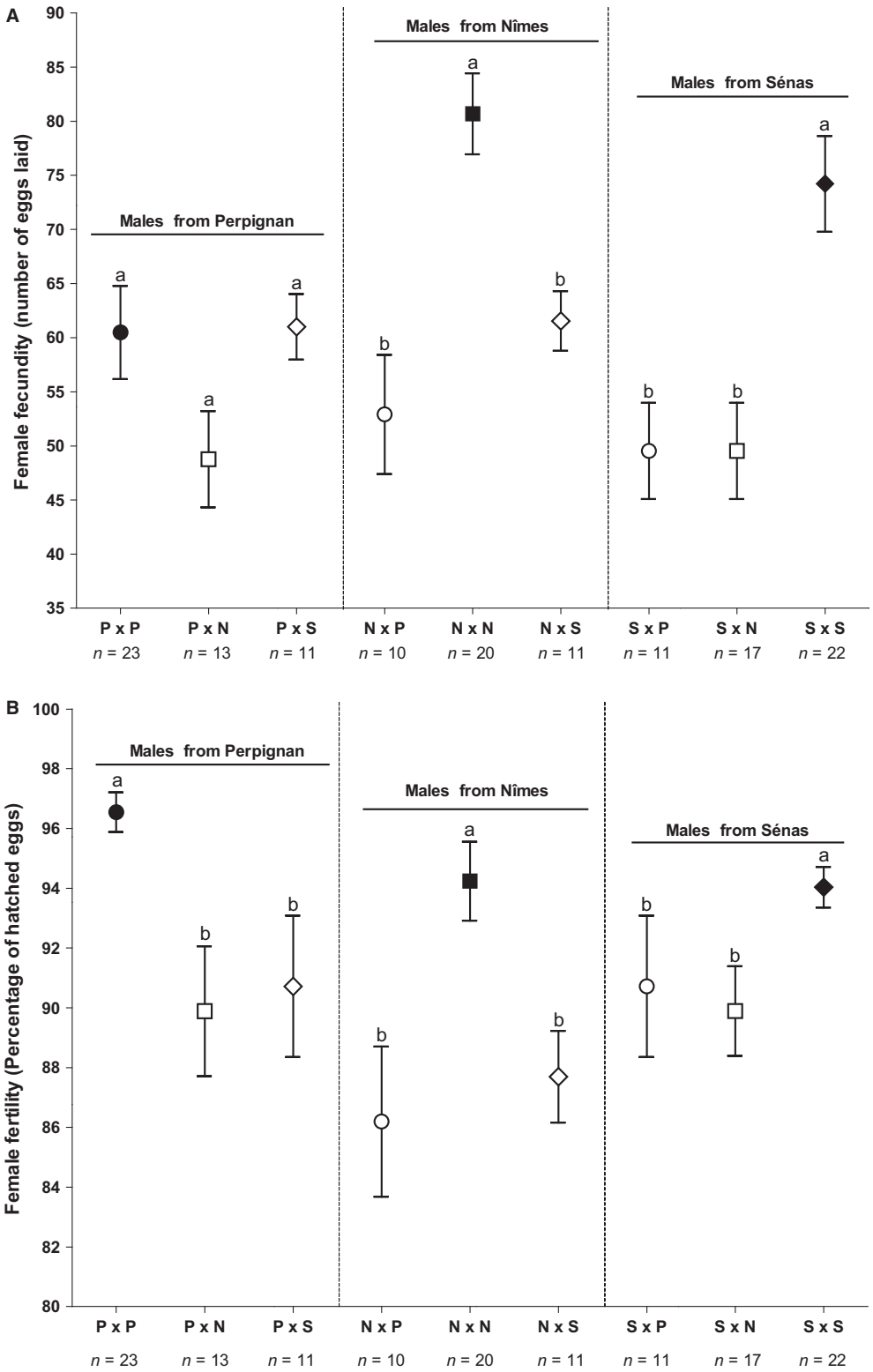

Figure 5. A, Fecundity (mean ± SEM of number of eggs laid) and (B) fertility (mean ± SEM of percentage of eggs hatched) for females mated with males from different geographical sites (Perpignan: P; Nîmes: N; Sénas: S). Black symbols represent within-population pairs (from the same population) and white circles represent between-population pairs (from different populations). The number at the bottom of each column represents the sample size. [a,b]Values for mating pairs with the same letter are not significantly different ($P > 0.05$). Symbols represent the female geographical origin (circles: P; squares: N; diamonds: S).

At the premating level, our results did not enable detection of the occurrence of ecological speciation at the cultivar scale, but we found evidence for the beginning of premating isolation through the latency period prior to mating, which was longer for heterotypic than homotypic pairs, particularly for individuals from the cultivar Carignan. Moreover, males from Carignan had a higher mating success with females from Carignan than with females from Syrah. It is possible that specialization on certain cultivars (e.g. Carignan) may be more advanced than others, and our results suggested that the degree of assortative mating in *L. botrana* species could be mediated by host cultivar. Moreover, we know that females preferred to lay eggs on the same cultivar on which they were reared as larvae (i.e. NHPI) (Moreau *et al.*, 2008). However, despite the occurrence of NHPI, some cultivars were intrinsically more attractive than others, suggesting that plant quality *per se* could influence mating patterns and assortative mating in this species.

At the postmating level we found no postmating prezygotic effect, as the fecundity and fertility did not vary according the different pairs of individuals. This suggests that postcopulatory mechanisms in *L. botrana*, including mechanical isolation, gametic incompatibility, and hybrid mortality, are not strongly developed between pairs of individuals from different host cultivars. The absence of postcopulatory isolation is common in sympatry (Elzinga, Mappes & Kaila, 2014), and prezygotic barriers are usually the most important in the early stages of speciation (Ramsey *et al.*, 2003; Husband & Sabara, 2004; Mendelson *et al.*, 2007; Schwander *et al.*, 2008).

In summary, we did not find sympatric host race formation in *L. botrana*, despite a longer period of latency prior to mating in heterotypic matings, which may reflect a greater reluctance of males and/or females to mate. However, we demonstrated the occurrence of non-random mating patterns between different allopatric populations, suggesting the beginning of reproductive isolation, which could lead to the evolution of cryptic species of this pest. Further studies of the genetic differentiation of *L. botrana* derived from different allopatric populations over a wide geographical area are required.

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