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Plant and associate, genetic distances and STRUCTURE

Comparative population genetic structure in a plant–pollinator/seed predator system

ISABEL S. MAGALHAES,* GABRIELA GLEISER,*† ANNE-MARIE LABOUCHE*‡ and GIORGINA BERNASCONI*

*Department of Evolutionary Botany, Institute of Biology, University of Neuchâtel, Rue Emile-Argand 11, CH-2000 Neuchâtel, Switzerland, †Laboratorio Ecotono, INIBIOMA, Universidad del Comahue, CONICET, 8400 Bariloche, Argentina, ‡Department of Ecology and Evolution, University of Lausanne, CH-1015 Lausanne, Switzerland

Abstract

Comparative analyses of spatial genetic structure of populations of plants and the insects they interact with provide an indication of how gene flow, natural selection and genetic drift may jointly influence the distribution of genetic variation and potential for local co-adaptation for interacting species. Here, we analysed the spatial scale of genetic structure within and among nine populations of an interacting species pair, the white campion *Silene latifolia* and the moth *Hadena bicruris*, along a latitudinal gradient across Northern/Central Europe. This dioecious, short-lived perennial plant inhabits patchy, often disturbed environments. The moth *H. bicruris* acts both as its pollinator and specialist seed predator that reproduces by laying eggs in *S. latifolia* flowers. We used nine microsatellite markers for *S. latifolia* and eight newly developed markers for *H. bicruris*. We found high levels of inbreeding in most populations of both plant and pollinator/seed predator. Among populations, significant genetic structure was observed for *S. latifolia* but not for its pollinator/seed predator, suggesting that despite migration among populations of *H. bicruris*, pollen is not, or only rarely, carried over between populations, thus maintaining genetic structure among plant populations. There was a weak positive correlation between genetic distances of *S. latifolia* and *H. bicruris*. These results indicate that while significant structure of *S. latifolia* populations creates the potential for differentiation at traits relevant for the interaction with the pollinator/seed predator, substantial gene flow in *H. bicruris* may counteract this process in at least some populations.

Keywords: adaptation, insects, mutualism, parasitism, plant mating systems, population genetics – empirical, species interactions

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Introduction

Understanding the genetic structure of populations and the mechanisms that control it is a major objective in evolutionary biology. Spatial genetic structure within and among populations results from the combined action of gene flow, genetic drift and natural selection (Garant *et al.* 2007; Holderegger *et al.* 2010). Because of their limited mobility, the genetic structure of plant populations is generally correlated with the spatial distribution of individuals (Hamrick & Holden 1979; Vekemans & Hardy 2004). However, another important factor that can impact the genetic structure of populations are interspecific interactions, which may affect gene flow (e.g. through animal dispersal of seeds or pollen), or patterns of selection, including reciprocal selection and local adaptation that are expected in co-evolving systems. For instance, in plant species pollinated by animals, differences in pollination strategies and use of space by pollinator species can be a main factor in the organization of genetic structure among and within plant populations (Brunet & Holmquist 2009). Among populations, genetic structure may be

Correspondence: Giorgina Bernasconi, Fax: +41 32 718 30 01; E-mail: gb2398@columbia.edu

high if there is limited movement and local foraging, especially by small insects, while long-distance pollen dispersal and transfer may prevent divergence (Whelan *et al.* 2009). Within populations, genetic structure may be promoted for example through pollinators that are restricted to nearest-neighbour plants and therefore increase the level of inbreeding and subdivision (Herlihy & Eckert 2002; Whelan *et al.* 2009), while pollinators with high variance in pollen carryover will visit more plants and reduce subdivision (Ahmed *et al.* 2009). Most studies on spatial genetic structure of plant populations focus on pollinator behaviour and how it affects genetic diversity and structure of plant populations. Comparative analyses of spatial genetic structure of plants and their pollinators/seed predators can provide information on patterns of dispersal and pollination and on the effects of spatial distances in the genetic variation and divergence of populations of interacting species.

Here, we jointly analyse the spatial dispersion of genes within and among populations of a common plant, the white campion *Silene latifolia*, and of its pollinator and specialist seed predator *Hadena bicruris* along a latitudinal gradient across part of its native range in Europe. *Silene latifolia* and *H. bicruris* have complex interactions as the moth not only pollinates the flowers (mutualistic interaction) but is also a specialist seed predator (antagonistic interaction) (Bernasconi *et al.* 2009; see also Methods: Study species and their interaction for details). Data on visitation behaviour and pollination efficiency in experimental plant patches demonstrated that nectaring behaviour is sufficient to ensure pollen transfer (Labouche & Bernasconi 2010). Results from an experiment simulating predation showed that predated plants had increased flower productivity and decreased floral longevity, suggesting that plants reallocate resources to the production of new flowers after the destruction of fruits (Wright & Meagher 2003). Therefore, these pollinators may not only play a role in prezygotic reproductive isolation and genetic structure (Bernasconi *et al.* 2009), but also generate costs to the plant as attack rates of 25–70% of the fruits are common (Elzinga *et al.* 2005), thus potentially favouring reciprocal selection and co-evolution. Noteworthy, this type of complex interactions occurs for multiple species pairs within the two genera (Kephart *et al.* 2006) and represent an important test case for a general understanding of interspecific interactions and co-evolutionary processes because this is presumed to be a less specialized case with respect to better studied systems, such as Yucca and Yucca moths, or figs and fig wasps (Kephart *et al.* 2006).In particular, we examined the geographical variation across nine European populations of co-occurring plant and pollinator/seed predator distributed at different distances from each other along a Northern/Central Europe latitudinal transect. The sampled populations lie within the western 'race' of *S. latifolia* (Taylor & Keller 2007; Keller & Taylor 2008). We analysed nine microsatellite loci for *S. latifolia* and eight newly developed microsatellite loci for *H. bicruris* to (i) describe genetic diversity of each of the nine populations of *S. latifolia* and *H. bicruris*, and (ii) estimate larger scale spatial genetic structure in *S. latifolia* and *H. bicruris*.

Within populations of *S. latifolia*, we expect that genetic diversity will be influenced by population size, density of plants, seed dispersal mechanisms and the pollination behaviour of *H. bicruris* (Vekemans & Hardy 2004). In addition to the reduced diversity expected in smaller populations as a result of the increase in biparental inbreeding, genetic substructuring may be promoted by nearest-neighbour pollination (Herlihy and Eckert 2002; Whelan *et al.* 2009), and also as a consequence of the fact that seeds are exclusively dispersed by gravity (Richards 2000). At a broader scale, genetic structure among populations may appear as a consequence of geographical distances and also if the movement of the pollinators is spatially restricted. If the geographical distance is the most important factor for differences among populations, we would expect to find isolation by distance (Wright 1943). On the other hand, highest neutral genetic divergence may occur between populations that are not necessarily the most distant ones, if for example, local adaptation led to the evolution of genetic incompatibilities, or if there are geographical barriers to gene dispersal (natural or as a product of human activity).

In this work, we present several analyses of genetic diversity and structure of both a plant and its pollinator/seed predator, to understand the role that spatial distances play on their genetic diversification and divergence and to elucidate the patterns of effective pollination by moths. Additionally, we aim to provide background information on the potential for local adaptation and co-adaptation in these interacting species.

Materials and methods

Study species and their interaction

Silene latifolia is a short-lived perennial, dioecious and sexually dimorphic plant (Delph & Meagher 1995; Shykoff & Bucheli 1995; Witt *et al.* 1999). It occurs throughout Europe and the Mediterranean region, on calcareous and sandy soils, and is patchily distributed along disturbed and agricultural habitats (Baker 1947; Goulson & Jerrim 1997; Richards *et al.* 2003; Elzinga *et al.* 2005). Morphological and molecular variations

Table 1 Characteristics of the study populations of *Silene latifolia* and *Hadena bicruris*

			N sampled plants			*N* emerged moths		
Population	Plant density	Population size	Male	Female	Total	Male	Female	Total
S	Medium	Medium	10	10	20	1	7	8
J	High	Medium	10	10	20	4	3	7
A	High	Large	20	20	40	16	7	23
NA	Low	Medium	8	10	18	6	2	8
GK	Low	Small	10	10	20	5	4	9
K1	High	Medium	10	10	20	8	4	12
K2	High	Medium	10	10	20			
K3	High	Medium	10	10	20			
R	High	Small	10	10	20	7	5	12*
T	Low	Small	10	10	20	13	7	19*
ST1	Low	Small	10	10	20	10	2	12*
ST2	High	Medium	10	10	20			

We selected populations containing a minimum of 15 female and 15 male flowering plants. If the population was patchy (K and ST), we sampled separately in each patch. We estimated plant density qualitatively, based on the length of the transect needed to sample 15 male and 15 female flowering plants (low density: 75–100 m; medium density: 50–75 m, high density: <50 m). We estimated plant population size qualitatively (small population: 30–60 individuals; medium: 50–80 individuals; large: >100 individuals). Moths were collected as larvae and reared to adult emergence in the laboratory. In three populations (*) moths were sampled again a few weeks after the first sampling to increase sample size.
S, Sörby; J, Jönköping; A, Aarhus; NA, Norra Åsum; K, Kåseberga; GK, Gärds Köpinge; R, Renkum; T, Tolkamer; ST, Stechendorf.

suggest that *S. latifolia* found refuge in Northern Africa during the last glaciations from where it colonized its current range with the spread of agriculture (Mastenbroek & van Brederode 1986; Vellekoop *et al.* 1996). Within the European range, populations are genetically and phenotypically differentiated (Wolfe *et al.* 2004; Ironside & Filatov 2005; Jolivet & Bernasconi 2007a,b; Barluenga *et al.* 2010; Hathaway *et al.* 2009). The plant flowers from May to October and overwinters as a rosette. It builds up to 40 fruits per season, with 48–359 seeds per fruit (Baker 1947; Jolivet & Bernasconi 2007b). *Silene latifolia* fruits are sired by multiple fathers (Teixeira & Bernasconi 2007) and can be pollinated by several insects (Giménez-Benavides *et al.* 2007), but the moth *Hadena bicruris* is considered its main pollinator (Brantjes 1976; Jürgens *et al.* 1996; Goulson & Jerrim 1997; Bopp & Gottsberger 2004; Kephart *et al.* 2006). Both male and female *H. bicruris* efficiently pollinate the plant (Labouche & Bernasconi 2010), and the species has been reported in 90% of European *S. latifolia* populations surveyed (Wolfe 2002).

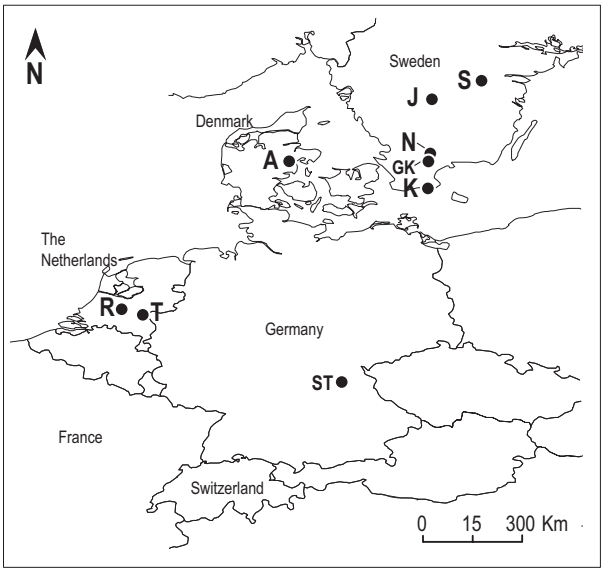

Fig. 1 Geographical location of the *Silene latifolia* and *Hadena bicruris* study populations. GPS coordinates are as follows: S (Sörby), 58°20.773N/15°46.654E; J (Jönköping), 57°46.356N/14°09.464E; A (Aarhus), 56°14.745N/10°08.522E; NA (Norra Åsum), 55°59.117N/14°09.506E;GK (Gärds Köpinge), 55°56.709N/14°08.853E; K (Kåseberga), 55°22.919N/14°03.613E; R (Renkum), 51°59.512N/05°46.353E; T (Tolkamer), 51°51.491N/06°05.157E; and ST (Stechendorf), 49°54.535N/11°19.217E.

Plant collection

We sampled leaves from nine geographically separated *S. latifolia* populations along a north–south transect across Central Europe (Table 1, Fig. 1), ranging from Sweden [Sörby (S), Norra Åsum (NA), Jönköping (J), Kåseberga (K) and Gärds Köpinge (GK)], Denmark [Århus (A)], the Netherlands [Renkum (R) and Tolkamer (T)] to Germany [Stechendorf (ST)]. The greatest distance as the crow flies between north (Sörby) and south (Stechendorf) was of about 1000 km. This transect

was chosen so as to sample a maximum divergence along the south–north gradient, while avoiding to have differences as are known between the eastern and western races (Hathaway *et al.* 2009), as we sampled only populations from the western race (Taylor & Keller 2007; Keller & Taylor 2008). Populations were identified based on GPS coordinates provided by colleagues (see Acknowledgements). We considered a patch with at least 30 plants with no other groups of plants in the direct vicinity (i.e. no other *S. latifolia* individuals could be detected by sight) as a population. Populations were sampled simultaneously for plants and insects from 10 August 2007 to 4 September 2007 (Table 1). In each population, we sampled leaves from 10–30 female and 8–30 male flowering plants. From each plant, we collected a single leaf, which was dried and stored in silica gel for later DNA extraction.

In the smallest populations (GK, R and T, Table 1), we sampled most of the flowering individuals. In those of intermediate size (J, S and NA), we sampled flowering plants that were at least 2 m apart from each other along a randomly laid transect. In the largest population (A), we sampled plants in two subpatches separated by 150 m (A1 and A2). Within these patches, we performed the same transect-sampling design than intermediate-size population. Finally, the Kåseberga and Stechendorf populations consisted of several subpopulations of small or intermediate size (Table 1), separated by several hundred metres. In Kåseberga, we sampled three (K1, K2 and K3) and in Stechendorf two subpopulations (ST1 and ST2).

Insect collection and rearing

We collected eggs and larvae of *H. bicruris* in the sampled populations of *S. latifolia*. To this end, we inspected flowering female plants for larva presence (developing fruits with excrements or holes, or ripe fruits sheltering a large larva). Per population, we sampled between three and 110 larvae (Table 1). We reared all eggs and larvae until adult emergence (see Supporting Information for detailed methods in Data S1–S4). All of the emerged adults were confirmed to belong to *H. bicruris* by Dr Ladislaw Reser, Natural History Museum of Lucerne (Switzerland). In addition, in K, NA and GK, we collected nocturnal moths using a light trap (Table S1, Supporting Information for Methods and the list of observed species).

DNA extraction and microsatellite amplification

Genotyping of Silene latifolia *individuals.* We extracted genomic DNA from dried leaves from 258 field-collected individuals using the Qiagen Biospring DNA kit. Genotypes were obtained for nine nuclear microsatellite regions previously isolated in *S. latifolia*: Sil 1, Sil 4, Sil 6, Sil 8 (Teixeira & Bernasconi 2007) and Sillat 06, Sillat 07, Sillat 08, Sillat 25 Sillat 28 (Austerlitz *et al.* 2011). Forward primers were labelled with fluorescent dyes. PCR was carried out in a final volume of 10 μL that contained 5–10 ng of template DNA, 2 μM of each of the forward and reverse primers, and 1 × Qiagen HotStarTaq *Plus* Master Mix. PCR amplification was performed in a Biometra thermal cycler, following the conditions described for each locus in Austerlitz *et al.* 2011). PCR products were then separated on an ABI PRISM 3100 genetic analyzer (Applied Biosystems), and fragment sizes were assigned to bins with the software TANDEM (Matschiner & Salzburger 2009).

Isolation and characterization of microsatellite markers for Hadena bicruris*, and genotyping of individuals.* Genotypes of the *H. bicruris* moths were obtained for eight newly developed nuclear microsatellites; these microsatellite regions were isolated by Ecogenics GmbH (Zurich, Switzerland; see Supporting Information for methods on Data S3; Table S2). These markers were used to genotype 111 adult moths collected in the nine *S. latifolia* populations mentioned above. The DNA of these moths was extracted with the DNeasy Blood &Tissue kit (Qiagen) from one insect leg. The tissue was frozen inside an Eppendorf with liquid nitrogen; the isolation protocol was immediately performed after grinding the frozen tissue with a pestle. DNA was finally diluted in a volume of 60 μL of TE buffer. The PCR mix had a final volume of 10 μL and consisted in 5–10 ng of template DNA, 2 μM each of the forward and reverse primers, and 1 × Qiagen HotStarTaq *Plus* Master Mix. The amplification reactions were carried out in a Biometra thermal cycler; the PCR programme consisted on an initial activation step of 15 min at 95 °C, followed by *n* cycles composed of 30 s of denaturation at 94 °C, 90 s of annealing at *T*, 60 s of extension at 72 °C and a final extension step of 30 min at 60 °C (see Table S2, Supporting Information for the *n* and *T* values for each locus). The analysis of the amplified fragments and allele binning methodology was the same that has been previously described for the samples of *S. latifolia*.

Genetic data analyses. To create input files for other programs, we used CONVERT (Glaubitz 2004). We checked for the possibility of large allele dropouts, stuttering and null alleles for each loci and population with MICRO-CHECKER 2.2.3 (Van Oosterhout *et al.* 2004). For the analyses of departures from Hardy–Weinberg equilibrium (HWE) to evaluate the significance of inbreeding that occurred in each locus, in each population and in overall loci, we used ARLEQUIN 3.11 (Excoffier *et al.* 2005). We

used the same program to calculate observed (H_O), expected (H_E) heterozygosities and to perform tests for genotypic linkage disequilibrium (LD). To estimate allelic richness corrected for differences in sample size (R_S) for each locus and population, we used HP-Rare 1.0 (Kalinowski 2005). To estimate the multilocus inbreeding coefficient (F_{IS}) and its 95% confidence intervals, H_O and H_E for each population, we used GENETIX 4.05 (Belkhir *et al.* 1996). We used FSTAT 2.9.3 (Goudet 2001) to estimate the significance of the multilocus F_{IS} values. For populations where plants had been collected from patches separated by 150 m (A) or more (K, ST), we estimated the F_{IS} of subpopulations to look at within-population structure. We adjusted the statistical significance in the above tests for multiple comparisons using sequential Bonferroni adjustments (Rice 1989).

We used two different Bayesian model-based programs to test for the optimal number of genetic groups (clusters) and assignment of individuals from all populations to those clusters. First, we used STRUCTURE 2.3.3 (Pritchard *et al.* 2000) that does not assume predefined genetic delimitation of populations and allows to test for a minimum optimal number of clusters of one. Then we used the spatially explicit software TESS 2.3 (Chen *et al.* 2007; Durand *et al.* 2009), which takes into account the spatial organization of individuals, but that allows to test a minimum number of clusters of two. In STRUCTURE, the number of clusters (denoted by K hereafter) representing the data was explored by performing 50 replicates of each simulation from $K = 1$ to $K = 10$, with a burn-in of 50 000 steps followed by 500 000 Markov chain Monte Carlo (MCMC) iterations under the admixture model and the assumption of correlated allele frequencies among populations. Individuals were assigned to clusters based on their highest membership coefficient to a particular cluster averaged over the fifty independent runs. In TESS, we used three values for the spatial interaction parameter, which represents the strength of the spatial component ($ø = 0.6$; 0.75 and 0.9; Fedy *et al.* 2008; Ball *et al.* 2010). For each of these parameters, the data were explored by performing 50 replicates of each simulation from $K = 2$ to $K = 10$, with a burn-in of 50 000 steps followed by 500 000 MCMC iterations to identify which K values produced the highest likelihood runs (K_{max}). Results from STRUCTURE and TESS were visualized using the programs STRUCTURE HARVESTER (http://taylor0.biology.ucla.edu/struct_harvest/) and CLUMPP 1.1.2 (Jakobsson & Rosenberg 2007). The assignment of individuals to genetic populations performed by both STRUCTURE and TESS does not account for the fact that individuals may arise from unsampled populations, which can lead to misassignment of individuals. To account for the probability that individuals are migrants from other, sampled or unsampled populations, we have used GENECLASS 2.0 (Cornuet *et al.* 1999). We selected the detection of first-generation migrants that identifies individuals that were not born in the population where they were sampled. We estimated the 'L_home', the likelihood of finding a given individual in the population in which it was sampled, which is the most appropriate estimation to use when not all potential source populations have been sampled (Paetkau *et al.* 2004). We used a frequencies-based method (Paetkau *et al.* 1995) and Monte Carlo resampling of 10 000 individuals per locality (Paetkau *et al.* 2004).

We used F_{ST} values as measures of neutral genetic differentiation between populations. We estimated single-locus and multilocus F_{ST} values and their significance with 10 000 bootstrap replicates (Weir & Cockerham 1984) using ARLEQUIN 3.11 (Excoffier *et al.* 2005). For the geographically substructured populations (A, K and ST), we also estimated F_{ST} values between subpopulations.

Sampling small natural populations may result in the collection of closely related individuals, which would lead to nonindependent genotypes and violation of HWE and LD assumptions. Therefore, we used MLRELATE (Kalinowski *et al.* 2006) to calculate which of four levels of relatedness (unrelated, half-sib, full-sib and parent–offspring) had the highest likelihood for each pair of individuals within populations. When estimating relatedness, this program allows to accommodate null alleles, which were detected at several loci (see Results section). Despite initial analysis indicating deviations from HWE and high F_{IS} values, the per-population number of full-sib half-sib and parent–offspring families within our samples (presumably also as a result of the sampling procedure, see Materials and methods) was very low with an average of 93.5% (±2.9%) of paired individuals of *S. latifolia* and 92.6% (±4.6%) of *H. bicruris* being assigned as unrelated (Table S3, Supporting Information). Therefore, we have used the complete data set in the analyses.

Correlation of geographical and genetic distances. We assessed spatial genetic structure at the population level. We tested for isolation by distance (IBD hereafter) by estimating the correlation between the matrix of genetic distance between pairs of populations ($FST/(1 - F_{ST})$) and the matrix of geographical distances, and tested the significance of the correlation using a Mantel test with 10 000 permutations as implemented in ARLEQUIN. We computed the geographical distance matrix based on the latitude and longitude of each sampling location using GDMG 1.2.3 (http://biodiversityinformatics.amnh.org/open_source/gdmg/documentation.php). Finally, we also tested for a

possible significant correlation between the genetic distances of *S. latifolia* and those of *H. bicruris* by performing a Mantel test with 10 000 permutations in ARLEQUIN.

Results

Neutral genetic variation within populations

Silene latifolia. We genotyped a total of 258 individuals (18–60 per population) at nine microsatellite loci. Over all loci and populations, we found 374 alleles. Locus Silat28 had the lowest number of alleles (18) and locus Sil6 had the highest (70). MICRO-CHECKER did not find large allele dropout or stuttering in our data set, but it detected the possibility of null alleles in several loci in most populations because of an excess homozygotes in most allele size classes (Table S4a, Supporting Information). Despite this, all populations were rather polymorphic for most microsatellite loci. Among populations, the lowest number of alleles was found in Sörby with 68 alleles found over nine loci and Kåseberga had the highest number of alleles, 197. Allelic richness was on average highest in Aarhus and lowest for Sörby (Table 2).

We found significant linkage disequilibrium (LD) in six populations (Table S5, Supporting Information). After sequential Bonferroni correction, out of 36 pairwise tests per population, one test (2.8%) was significant in Gärds Köpinge, two (5.5%) in Kåseberga, three tests (8.3%) were significant in Aarhus, two (5.5%) in Renkum, four (16.7%) in Tolkamer and six (13.9%) in Stechendorf. However, only two pairwise tests involving the same two loci were significant for more than one population (pair Sil1, Sil6 in Aarhus and Tolkamer, and pair Sil6, Silat07 in populations Aarhus and Kåseberga).

The H_O ranged between 0.13 (silat28 in Stechendorf) and 1.000 (Sil6 in Norra Åsum, Silat06 in Tolkamer and Silat25 in Gärds Köpinge). There were many significant differences between observed and expected heterozygosities. We observed deviations from HWE in all populations but one (Sörby), with the H_O being significantly lower than the H_E at several loci, even after sequential Bonferroni correction (Table S5, Supporting Information). Sörby, the most northern population, also presented the lowest inbreeding coefficient ($F_{IS} = -0.034$), while the most southern population (Stechendorf) had the highest ($F_{IS} = 0.208$; Table 3). We also found that the estimates of inbreeding coefficient of each population were all significant ($P < 0.001$) except in population Sörby ($P = 0.855$). This explains the existence of heterozygote deficit in eight of the populations that were under study. In populations that were subdivided, each subpopulation presented significant values of F_{IS}. In Aarhus, patch 1 and 2 had F_{IS} values of 0.130 ($P < 0.01$) and 0.104 ($P < 0.01$), respectively. In Kåseberga, subpopulation 3 had the lowest F_{IS} ($F_{IS} = 0.069$, $P < 0.05$), followed by subpopulation 1 ($F_{IS} = 0.090$, $P < 0.01$) and finally 2($F_{IS} = 0.149$, $P < 0.01$). In Stechendorf, subpopulation 1 had a F_{IS} of 0.132 and subpopulation 2 had a F_{IS} of 0.175 (both $P < 0.05$).

Hadena bicruris. We genotyped a total of 111 *H. bicruris* individuals (8–24 individuals per population) at eight microsatellite loci. MICRO-CHECKER did not find large allele dropout or stuttering in our data set, but it detected the possibility of null alleles in several loci in most populations because of an excess of homozygotes in most allele size classes (Table S4b, Supporting Information).

Table 2 Number of individuals sampled (N_s for *Silene latifolia* and N_h for *Hadena bicruris*), observed heterozygosities (H_o), mean gene diversities (H_e), mean allelic richness (R_s) and inbreeding coefficient (F_{IS}) and its statistical significance for nine *S. latifolia* and *H. bicruris* populations

	S. latifolia					*H. bicruris*				
Population	N_s	H_o	H_e	R_s	F_{IS}	N_h	H_o	H_e	R_s	F_{IS}
S	20	0.762	0.719	4.34 ± 1.20	−0.034 ns	7	0.625	0.669	3.97 ± 1.20	0.143**
J	20	0.674	0.821	5.50 ± 0.91	0.203***	7	0.642	0.725	4.27 ± 1.02	0.189n.s.
A	40	0.762	0.887	6.63 ± 1.21	0.153***	24	0.511	0.708	4.05 ± 0.87	0.299***
NA	18	0.79	0.866	6.26 ± 1.04	0.116***	8	0.520	0.697	4.30 ± 1.29	0.316***
GK	20	0.807	0.864	6.24 ± 1.28	0.093***	9	0.539	0.659	4.03 ± 1.47	0.240***
K	60	0.754	0.885	6.36 ± 1.41	0.156***	12	0.699	0.748	4.42 ± 0.98	0.109**
R	20	0.7	0.851	5.92 ± 1.00	0.204***	13	0.605	0.7	4.06 ± 1.18	0.174***
T	20	0.717	0.860	6.18 ± 1.38	0.192***	20	0.620	0.749	4.51 ± 1.27	0.197***
ST	39	0.715	0.888	6.43 ± 1.02	0.208***	12	0.559	0.743	4.39 ± 0.94	0.289***

***$P < 0.001$; **$P < 0.01$; *$P < 0.05$; ns, not significant.

S, Sörby; J, Jönköping; A, Aarhus; NA, Norra Åsum; K, Kåseberga; GK, Gärds Köpinge; R, Renkum; T, Tolkamer; ST, Stechendorf.

Table 3 Pairwise *F*-statistics (F_{ST}) between populations of (a) *Silene latifolia* and (b) *Hadena bicruris*

	S	J	A	NA	GK	K	R	T	ST
(a) *S. latifolia*									
S		***	***	***	***	***	***	***	***
J	0.132		***	***	***	***	***	***	***
A	0.098	0.07		***	***	***	***	***	***
NA	0.099	0.073	0.038		***	***	***	***	***
GK	0.099	0.088	0.032	0.027		***	***	***	***
K	0.085	0.076	0.032	0.022	0.026		***	***	***
R	0.135	0.08	0.061	0.077	0.069	0.068		***	***
T	0.100	0.086	0.028	0.057	0.062	0.056	0.079		***
ST	0.103	0.057	0.033	0.052	0.055	0.05	0.057	0.039	
(b) *H. bicruris*									
S		n.s.	**	*	n.s.	*	*	*	*
J	0.021		n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
A	0.061	0.039		n.s	*	*	n.s.	*	*
NA	0.056	0.022	0.01		n.s.	n.s.	n.s.	n.s.	n.s.
GK	0.040	0.011	0.037	0.023		n.s.	*	*	n.s.
K	0.051	0.012	0.03	0.007	0.02		n.s.	n.s.	n.s.
R	0.049	0.037	0.024	0.012	0.049	0.025		n.s.	n.s.
T	0.036	0.013	0.023	0.006	0.034	0.007	0.012		n.s.
ST	0.058	0.033	0.036	0.018	0.021	0.027	0.025	0.014	

F_{ST} values in the lower matrix and significance (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, n.s. not significant) in the upper matrix.
S, Sörby; J, Jönköping; A, Aarhus; NA, Norra Åsum; K, Kåseberga; GK, Gärds Köpinge; R, Renkum; T, Tolkamer; ST, Stechendorf.

Over all loci and populations, we found 130 alleles. Locus Hadbic12 had the lowest number of alleles (7) and locus Hadbic01 had the highest (24). Among populations, the lowest number of alleles was found in Sörby, with 41 alleles found over nine loci, while Tolkamer had the highest number of alleles, 79. The same populations had the lowest and highest allelic richness, respectively.

We found that after sequential Bonferroni correction, only five tests of linkage disequilibrium (LD) were significant, all in different populations and involving different pairs of loci (Table S5, Supporting Information).

The observed heterozygosity ranged between 0.11 (Hadbic24 in Gärds Köpinge) and 1.00 (Hadbic02 in Kåseberga, Hadbic10 in Sörby and Hadbic29 in Kåseberga, Gärds Köpinge and Jönköping). However, similar to *S. latifolia*, there were many significant differences between observed and expected heterozygosities. With the exception of Renkum, all other populations presented significant deviations from Hardy–Weinberg equilibrium (HWE) with the observed heterozygosity being consistently lower than the expected heterozygosity in at least one locus, even after sequential Bonferroni correction (Table S6, Supporting information). Inbreeding coefficients had a narrow range among populations: values ranged from 0.109 in Kåseberga to 0.321 in Norra Åsum (Table 3). Permutation tests revealed that the estimates of inbreeding coefficient of each population were all significant ($P < 0.05$), even after sequential Bonferroni correction. This further supports the evidence of heterozygote deficit in all nine populations of *H. bicruris* under study. Also, similar to *S. latifolia,* values of F_{IS}, H_o and H_e were not affected by density and size of plant populations ($P > 0.05$).

Neutral genetic divergence between populations

Silene latifolia. The population assignment test performed with STRUCTURE on the nine sampled populations supported the existence of six genetic groups (estimated ln probability of data = −12863.8, $P > 0.99$; Fig. 2a). When we used TESS and took into consideration the spatial autocorrelation between populations (Fig. 2b), the most likely number of clusters was reduced and $K_{max} = 5$ for all levels of spatial correlations assayed (0.6, 0.75 and 0.9). In both analyses, the genetic clusters were not necessarily composed of the populations that were geographically closer together, and several individuals were assigned to more than one cluster.

GENECLASS identified 20 individuals as first-generation migrants ($P < 0.01$). Only the Sörby population did not have any migrants. In the rest of the populations, the number of migrants ranged from one (Renkum) to five (Kåseberga). The highest percentage of migrants was found in Norra Åsum (11%) and the lowest was found

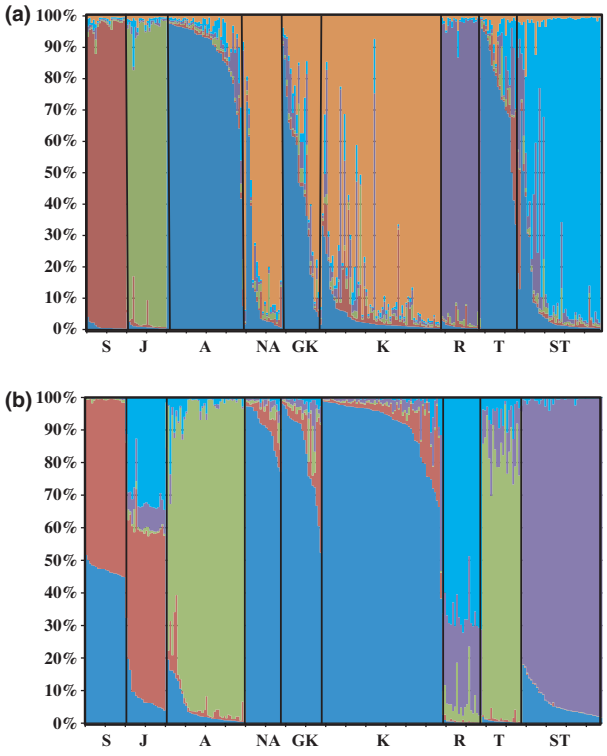

Fig. 2 Bayesian assignment probabilities of individuals of *Silene latifolia* to lineages estimated using (a) STRUCTURE 2.3.3 and (b) TESS 2.3. Each vertical line represents an individual, and colours indicate the proportion of an individual's genotype assigned to a particular lineage for the nine populations. In both graphs, lineage one is represented by dark blue, lineage two by red, lineage three by green, lineage four and five by purple and light blue, respectively, and lineage six by orange.

in Renkum (5%). Out of the 20 migrants, 11 had low assignment probabilities for all localities, suggesting that these individuals represent migrants from unsampled populations. Out of the other nine migrants, two individuals from Gärds Köpinge and one from Tolkamer were assigned to Aarhus, two individuals from Aarhus and Norra Åsum were assigned to Gärds Köpinge, two individuals from Aarhus and Kåseberga were assigned to Renkum and finally two individuals from Kåseberga were assigned to Norra Åsum.

Neutral genetic divergence between populations was always highly significant ($P < 0.001$; Table S4a). The lowest level of neutral genetic divergence was between Norra Åsum and Kåseberga ($F_{ST} = 0.022$). Pairwise genetic comparisons of Kåseberga, Norra Åsum and Gärds Köpinge, which are the closest populations in geographical space, presented the lowest F_{ST} values. The highest neutral genetic divergence was between the populations Sörby and Renkum ($F_{ST} = 0.135$). Sörby, the population that is furthest north, was the most genetically differentiated population and had the highest F_{ST} values in general. Single-locus F_{ST} values (Table S7a, Supporting information) were generally significant and had distributions that ranged between 0 (locus Sillat08 between Norra Åsum and Gärds Köpinge) and 0.338 (Sil8 between Sörby and Renkum).

Between subpopulations, F_{ST} values were also always significant and sometimes as high as or higher than between populations. Between subpopulations of Aarhus and between subpopulations of Stechendorf, F_{ST} values were 0.015 and 0.017 ($P < 0.05$ both), respectively. Genetic divergence between subpopulations from Kåseberga was lowest between subpopulations 1 and 2 ($F_{ST} = 0.043$), followed by divergence between subpopulations 1 and 3 ($F_{ST} = 0.048$) and between 2 and 3 ($F_{ST} = 0.051$) (all $P < 0.001$).

Hadena bicruris. The population assignment test performed with STRUCTURE recognized the highest probable genetic structure between the nine sampled populations as $K = 1$ (estimated ln probability of data = −3399.6, probability > 0.99). Results from TESS indicated that the highest score for the minimum K was 2, but this is likely to be a consequence of TESS not being able to evaluate the likelihood of one genetic cluster as a better model fit.

GENECLASS identified 10 individuals as first-generation migrants ($P < 0.01$). Only the Kåseberga population did not have any migrants. In the rest of the populations, the number of migrants was two in Sörby and Stechendorf and one in all the others. The highest percentage of migrants was found in Sörby (28%) and the lowest was found in Aarhus (5%). The two individuals from Stechendorf identified as migrants had low assignment probabilities for all other localities, suggesting that these individuals represent migrants from unsampled populations. Out of the other eight migrants, three individuals from Sörby and Aarhus and Norra Åsum were assigned to Tolkamer, two individuals from Gärds Köpinge and Tolkamer were assigned to Aarhus, one individual from Sörby was assigned to Jonkoping, one from Jonkoping was assigned to Renkum and one individual from Renkum was assigned to Sörby.

Neutral genetic divergence between populations was also very low and often not significant (Table S4b). The lowest level of neutral genetic divergence was between Norra Åsum and Kåseberga ($F_{ST} = 0.007$, $P > 0.05$), just as for *S. latifolia*. The highest level of neutral genetic divergence was between Sörby and Aarhus ($F_{ST} = 0.061$, $P < 0.01$). Similar to what was found for *S. latifolia*, *H. bicruris* from Sörby appear to be the most genetically differentiated population with the highest F_{ST} values in general. Overall, only 24 of 288 single-locus F_{ST} values were significant. The single-locus F_{ST} values ranged from 0 for many pairwise comparisons to 0.229

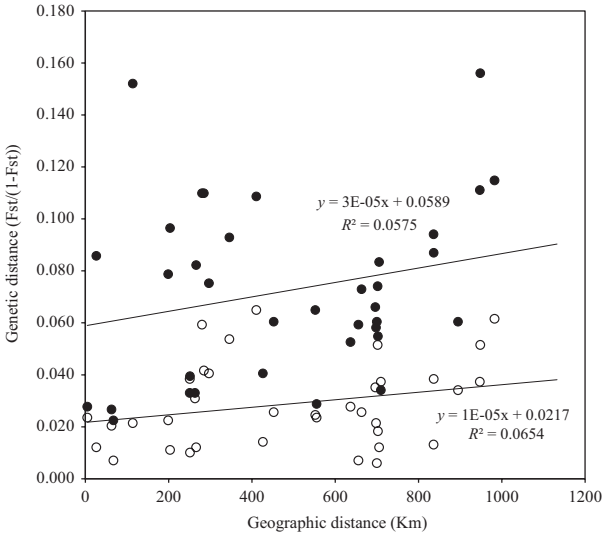

Fig. 3 Bivariate plot of pairwise genetic distances ($F_{ST}/(1 - F_{ST})$) vs. geographical distances (in kilometer) for nine populations of *Silene latifolia* (filled circles) and *Hadena bicruris* (empty circles). Filled line: linear regression for *S. latifolia* ($y = 0.00003x + 0.059$, $R^2 = 0.057$, $P = 0.162$); dashed line: linear regression for *H. bicruris* ($y = 0.00001x + 0.022$, $R^2 = 0.065$, $P = 0.131$).

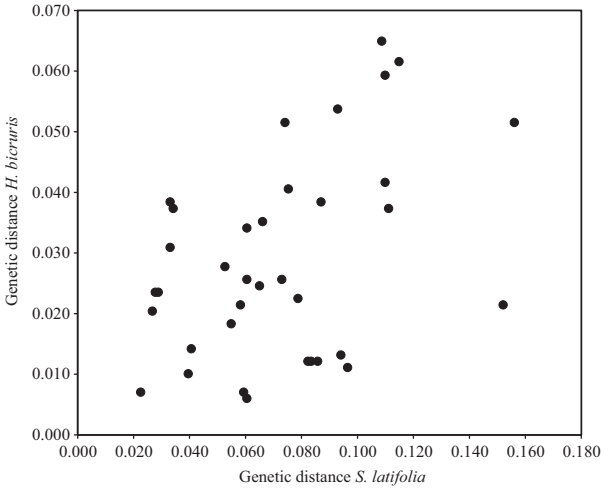

Fig. 4 Bivariate plot of pairwise genetic distances ($F_{ST}/(1 - F_{ST})$) of *Silene latifolia* vs. pairwise genetic distances ($F_{ST}/(1 - F_{ST})$) of *Hadena bicruris*.

(Hadbic19 between Renkum and Gärds Köpinge, Table S7b, Supporting information).

To eliminate the possibility that the genetic homogeneity found was attributed to type II error (false negative for population differentiation), we carried out simulations using the POWSIM software package v. 4.0 (Ryman & Palm 2006) based on the microsatellite data. Simulations were carried out for effective population sizes $N_e = 1000$ and $N_e = 10\,000$ to yield F_{ST} values of 0.005, 0.01 and 0.025 and 0.05. We ran 1000 replicates, and the power of the analysis was indicated by the proportion of tests that were significant at $P < 0.05$ using the respective allele frequencies at the eight loci studied. The simulation studies suggested that a type II error because of low power of the markers used was unlikely. The microsatellite data were able to detect F_{ST} values of as low as 0.01 in at least 98% of the time for a $N_e = 1000$ and in at least 66% of the time for $N_e = 10\,000$ (Table S8, Supporting information).

Distribution of genetic variation over geographical space

The Mantel tests revealed that neither *S. latifolia* (correlation coefficient (r) = 0.238, $P = 0.153$) nor *H. bicruris* ($r = 0.256$, $P = 0.112$) showed significant associations of genetic distances with geographical distances (Fig. 3).

We found that a weak positive trend existed in the relationship between genetic distances of *S. latifolia* and those of *H. bicruris* ($r = 0.432$, $P = 0.096$; Fig. 4).

Discussion

Population size, genetic variation and inbreeding of the plant and its pollinator/seed predator

Our study analysed a set of nine populations of *Silene latifolia* along a 1000-km north–south transect within the native range of the species western race (Taylor & Keller 2007). These populations vary in size, density and distance from each other. Despite differences in size and density, all populations presented similar levels of observed heterozygosity and inbreeding, suggesting that heterozygosity in this case is not, or not strongly, affected by population density and size. Our results can then be explained by other factors such as historical development of population size (Ouborg & Van Treuren 1995) and the degree of isolation from other populations.

Silene latifolia has several pre- and postpollination mechanisms that reduce biparental inbreeding, as suggested by multiple paternity of fruits, and evidence of postpollination selection as a function of genetic similarity between mates (Teixeira & Bernasconi 2007; Teixeira *et al*. 2009; Austerlitz *et al*. 2011). Despite this, we found high levels of biparental inbreeding for *S. latifolia* in all but one population (Sörby). An explanation for this might be that effective pollen dispersal in plants pollinated by foraging insects is known to occur predominantly between neighbouring plants (Bateman 1947; Levin & Kerster 1969). Plant populations in which this

pattern of pollination is predominant typically have leptokurtic distribution of pollen dispersal (Wright & Meagher 2004; Austerlitz *et al.* 2011) and are expected to have high levels of inbreeding, homozygosity and patchiness in the spatial distribution of genotypes. Indeed, an experimental study found that the pollen carried by moths after visiting a *S. latifolia* male flower is deposited on the next three female flowers visited (Labouche & Bernasconi 2010), supporting the idea that pollination by *Hadena bicruris* is restricted mainly to the nearest neighbour. Additionally, the fact that seeds disperse by gravity increases the risk of inbreeding (Richards 2000). So most likely pollen of male plants of *S. latifolia* is often deposited on neighbouring female plants and seeds afterwards fall close to the parental plants resulting in the patterns of inbreeding we observe even in large populations like Aarhus. In addition, *S. latifolia* mainly grows in disturbed, agricultural environments (Baker 1947), following a metapopulation dynamics, which can lead to drastic reductions in population size or even local extinctions (Richards *et al.* 2003). If after a local extinction, the number of individuals recolonizing the area is small and the probability of common origin of the colonizers is large compared to the number of migrants between populations, genetic differentiation between populations is expected to increase (Whitlock & McCauley 1990). *Silene latifolia* follows a metapopulation dynamics, whereby new colonizations may result from related individuals (e.g. seeds from the same fruit) (Richards *et al.* 2003). Such events can also explain the observed deviations from HWE, which is common in naturally occurring plant populations subjected to nonrandom mating processes.

Similarly, the positive F_{IS} values for *H. bicruris* from all populations suggest some inbreeding, even though *H. bicruris* seems to have high levels of gene flow between geographically distant populations and some individuals are first-generation migrants assigned to very distant populations. Samples sizes could provide a possible explanation for this result, as the sample sizes were rather small, the sampled individuals are a remnant one of a much greater population. Another possible explanation is that some level of dispersal (and subsequent reproduction) of moths may happen after initial egg laying in the population where adult moths have emerged. Within a season, *H. bicruris* was found to be able to travel and colonize populations over 2 km away from the population where they emerged as adults (Elzinga *et al.* 2007). If one adult moth can lay eggs and mate over a longer period of its adult life, this may produce a pattern of long range dispersal coupled with local inbreeding.

Comparative analysis of genetic structure of the plant and its pollinator/seed predator

The degree of geographical isolation of the nine populations of *S. latifolia* analysed was high. The distance between pairs of populations ranged between 4 km (between GK and NA) and 982 km (Between S and ST). Genetic differentiation among the studied populations was also pronounced and significant as shown by F_{ST} values and assignment tests. Considerable differentiation occurred not only between populations at hundreds kilometre apart, but also between subpopulations separated by only a few hundred metres. This is not surprising, as fine-scale genetic structure has been shown for populations of *S. latifolia* in patches separated by as little as 80 m (Barluenga *et al.* 2010). However, contrary to results from fine-scale genetic analysis, our analyses of large-scale spatial genetic structure within and among *S. latifolia* populations indicate no IBD. Genetic differentiation between populations appears to be more dictated by geographical position, rather than by distance. The most northern population, Sörby, is the most differentiated from all others, followed by Jönköping, the second most northern one.

In contrast, the *H. bicruris* populations were not significantly subdivided. Among populations, we observed low and mostly not significant values of genetic differentiation. This was confirmed by the Bayesian estimation of clusters showing an optimal number of clusters of one. However, for weak population structure and low number of markers, STRUCTURE is known to often fail to detect existing populations structure (Evanno *et al.* 2005). So, although there is a small probability that this is the result of the number of markers used and high effective population size, it may also constitute evidence of pronounced migratory behaviour within the study area for *H. bicruris,* which is supported by the number of first-generation migrants found. If the latter is true, this indicates the movement of individual insects over long distances and defines gene flow distances of more than 100 km. Noctuid moths of similar size and shape have been shown to be strong fliers capable of dispersing hundreds of kilometre (Förare & Solbreck 1997; Schneider 1999).

Hadena bicruris is a pollinator and also a parasite that relies on *S. latifolia* for egg laying and as food for its larvae. Adaptation to local plant communities may decrease gene flow among populations of pollinators. Additionally, heterogeneity among host populations can lead to heterogeneity among their parasites (Thompson 1994). For both reasons, it is expected that heterogeneity among *S. latifolia* populations would lead to heterogeneity among *H. bicruris* populations. However, our results reveal that the magnitude of gene flow of *H. bicruris*

between populations is greater than that among populations of *S. latifolia*. This is most likely because although *H. bicruris* appears to be mobile, pollen is not, or only rarely, carried over between populations and thus genetic structure is maintained among most plant populations at this geographical scale. This pattern can also be explained if we consider how ephemeral *S. latifolia* populations can be. Decline or disappearance (at least temporary, as seed banks may persist, Peroni & Armstrong 2001) of a local *S. latifolia* population may promote migration of adult moths to other plant populations, resulting in greater movement of the moth than of its host plant. Such large-scale migration events, combined with occasional migration between subpopulations, should maintain genetic differentiation among populations low, while strong selection might otherwise isolate populations. This could also explain the weak positive correlation found between genetic structure of plant and pollinator/seed predator. Selection for local adaptation would have to be quite strong to counteract the effect of large-scale migrations generated by extinction of plant patches and also to leave a trace on neutral genetic markers.

Analyses on the neutral genetic structure of parasite–host populations vary greatly in their outcome ranging from results similar to ours (Michalakis *et al.* 1993; Dybdahl & Lively 1996) to gene flow of the same order of magnitude in both host and parasite (Mulvey *et al.* 1991). Altogether, these studies suggest that interactions between species can be complex and are highly determined by the genetic structure of the plant/host population and dispersal of the pollinator/parasite. Our results indicate that while significant neutral genetic structure of *S. latifolia* populations creates the potential for differentiation at traits relevant for the interaction with the pollinator/seed predator, substantial gene flow in *H. bicruris* may counteract this process at least in some of the populations. Additional comparative studies on other *Silene* species that are known to also interact with moths of the genus *Hadena* (Kephart *et al.* 2006) are necessary to provide deeper understanding on these systems and to verify whether similar patterns as unveiled by our study are a general feature and how patterns of coupled gene flow impact the dynamics of these potentially co-evolved systems.

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I.S.M research focuses on mechanisms that generate and maintain biodiversity, particularly in the presence of gene flow. G.G. is interested in population genetics and the evolution of plant reproductive and mating systems. A.-M.L. is conducting her PhD research on the interactions between the plant *S. latifolia* and its pollinator and seed predator, *H. bicruris* and collected the samples investigated in this study. G.B. is interested in the ecology and evolution of reproductive traits in plants, with a focus on dioecious species and plant/pollinator interactions.

Data accessibility

GenBank accession numbers of the eight microsatellite loci isolated in *Hadena bicruris* can be found in Table S2, Supporting Information. Individual-by-individual sampling locations and microsatellites for both moth and plant can be found at the end of the Online Supporting Information file.

Supporting information

Additional supporting information may be found in the online version of this article.

Table S1 Noctuid species collected.

Table S2 Summary table of characterization of microsatellite markers for *H. bicruris*.

Table S3 Pairwise level of relatedness of sampled individuals.

Table S4 Presence of null alleles.

Table S5 *P*-values of the tests of linkage disequilibrium for nine microsatellite loci.

Table S6 Allelic variability at nine loci for *S. latifolia* and eight loci for *H. bicruris*.

Table S7 Single locus and multilocus F_{ST} values between populations.

Table S8 Statistical power for detecting varying levels of F_{ST}.

Data S1 Insect rearing.

Data S2 Description of the light trap and its use.

Data S3 Isolation and characterization of microsatellite markers for *H. bicruris*.

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