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DNA methylation patterns in the tobacco budworm, *Chloridea virescens*

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ABSTRACT

DNA methylation is an important epigenetic modification that is prone to stochastic variation and is responsive to environmental factors. Yet changes in DNA methylation could persist across generations and thus play an important role in evolution. In this study, we used methylation-sensitive amplified fragment length polymorphisms (MS-AFLP) to evaluate whether DNA methylation could contribute to the evolution of the sexual communication signal in the noctuid moth *Chloridea virescens*. We found that most DNA methylation was consistent across tissues, although some methylation sites were specifically found in pheromone glands. We also found significant DNA methylation differences among families and two pheromone phenotype selection lines, and these differences correlated with genetic variation. Most DNA methylation patterns were inherited, although some sites were subject to spontaneous *de novo* DNA methylation across generations. Thus, DNA methylation likely plays a role in a wide range of processes in moths. Together, our results present an important initial step towards understanding the potential role of DNA methylation in the evolution of sexual communication signals in moths.

1. Introduction

Epigenetics is the study of the processes that regulate heritable differences in gene expression without changing the underlying DNA sequence (Bird, 2007; Richards, E. J., 2006). These processes include DNA methylation, histone modifications, as well as non-coding RNA-associated gene silencing. Cytosine methylation is the most studied type of epigenetic modification and involves the covalent addition of a methyl group to the fifth carbon atom of the cytosine ring, resulting in 5-methylcytosine. This modification occurs through a family of enzymes called DNA methyltransferases (DNMTs). DNA methylation is prone to stochastic variation (Feinberg and Irizarry, 2010) and is responsive to environmental factors (Jablonka, 2013). Moreover, changes in DNA methylation could persist across generations (Jablonka and Raz, 2009), and thus be of evolutionary significance.

DNA methylation has been well studied in plants and mammals, and in recent years more studies have focused on insects as well. In contrast to plants and mammals, many insect genomes are sparsely methylated (Bewick et al., 2017; Xiang et al., 2010), particularly in holometabolous insects (Provataris et al., 2018). For example, in the silk moth (*Bombyx mori)* genome, only 0.11% of the cytosines at CG-dinucleotides was found to be methylated (Xiang et al., 2010), compared to 70–80% in humans (Ziller et al., 2013) and 24% in the plant *Arabidopsis thaliana* (Cokus et al., 2008). Bewick et al. (2017) found DNA methylation to be present across the insect phylogeny, with the exception of Diptera, suggesting an important function for DNA methylation in insects. However, DNA methylation levels are highly variable across insect species (Bewick et al., 2017; Provataris et al., 2018), and the role of DNA methylation in insects remains unclear (Glastad et al., 2019). In insects, DNA methylation occurs almost exclusively at CG-dinucleotides within genes (Hunt et al., 2013; Suzuki and Bird, 2008; Xiang et al., 2010). In mammals, DNA methylation within genes is associated with active gene expression, while DNA methylation in promoters could have various effects (Miranda and Jones, 2007; Angeloni and Bogdanovic, 2019). Methylated genes in insects and other invertebrates are usually phylogenetically conserved “house-keeping” genes that are constitutively expressed, while inducible and tissue-specific genes have lower DNA methylation levels (Provataris et al., 2018; Sarda et al., 2012). However, it is not yet clear how intragenic DNA methylation levels translate to gene expression (Glastad et al., 2014; Hunt et al., 2013).

In Lepidoptera, the presence of DNA methylation has been demonstrated in a few studies (Bewick et al., 2017; Jones et al., 2018; Mandrioli and Volpi, 2003; Xiang et al., 2010, 2013). For example, Bewick et al. (2017) found evidence for DNA methylation in 12 out of 14 species, including the tobacco budworm, *Chloridea virescens*

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(Lepidoptera, Noctuidae). Furthermore, DNA methyltransferases (DNMT1 and DNMT2) and a methyl-CpG-binding domain (MBD) have been characterized in Lepidoptera (Li et al., 2013; Mitsudome et al., 2015; Uno et al., 2005). The first indication that DNA methylation is a regulatory signal in Lepidopteran cells came from Knebel et al. (1985), who showed that in a *Spodoptera frugiperda* cell culture, the introduction of methylation at a 5′-CCGG-3′ site in a promotor sequence is capable of silencing genes. Xiang et al. (2013) provided the first direct experimental evidence of functional significance of insect DNMT1 by knocking down this transferase in *B. mori,* which led to decreased hatchability. Recently, Xu et al. (2018) demonstrated the involvement of DNA methylation in mediating tissue- and stage-specific expression of genes involved in wing development of *B. mori*. Some studies in Lepidoptera have detected DNA methylation changes in the context of host-parasite interactions (Borsatti et al., 2004; Kumar and Kim, 2017; Mukherjee et al., 2019; Uckan et al., 2007; Vilcinskas, 2016) and Bt-resistance (Mukherjee et al., 2017). To the best of our knowledge, inheritance of DNA methylation has not been investigated in Lepidoptera.

In a first attempt to determine whether DNA methylation could contribute to the evolution of the sexual communication signal in *Chloridea virescens*, we assessed DNA methylation patterns in sex pheromone glands of mothers and daughters. To attract potential mating partners, female moths emit a species-specific sex pheromone. Moth sex pheromones usually consist of long-chain fatty acid derivatives of various compound classes (such as alcohols, aldehydes, and acetate esters), which are produced in a gland at the tip of the female abdomen and released in specific ratios into the environment (Allison and Cardé, 2016a). It is generally assumed that moth pheromone composition is under stabilizing selection as the signaling female and the responding male need to be fine-tuned to each other (Allison and Cardé, 2016b; Löfstedt, 1993). However, considerable phenotypic variation in the female sexual signal exists (Allison and Cardé, 2016c; Tóth et al., 1992), even within populations (Groot et al., 2014). The most obvious source of phenotypic variation is genetic variation (Groot et al., 2016). In addition to genetic variation, environmental effects could affect phenotypic variation (Groot et al., 2010; Van Geffen et al., 2015). If this plastic variation is inherited, coevolution of signal and response can be envisioned, as both signaler and responder are exposed to the same environmental conditions.

Previously, the within-population phenotypic variation that was encountered in the female sex pheromone composition of *C. virescens* was found to be mostly genetic (Groot et al., 2009, 2014). However, in selecting for the extreme phenotypes, a spontaneous appearance of the so-called High phenotype repeatedly occurred in the Low selection line, which led us to hypothesize that the sex pheromone phenotype could also be influenced by epigenetic marks. To understand the evolutionary potential of DNA methylation variation in the evolution of sex pheromone divergence, we used the two selection lines that were constructed to have either high or low ratios of saturated versus unsaturated pheromone compounds (Groot et al., 2014). The “Low” phenotype is attractive to males, whereas the “High” phenotype is not (Groot et al., 2014, 2019). Specifically, we formulated the following questions: To what extent are DNA methylation patterns 1) tissue-specific, 2) differentiated between High and Low selection lines, and 3) inherited? To address these questions, we used methylation-sensitive AFLP analysis to assess DNA methylation patterns within and across individuals, families, pheromone phenotypes, and generations of *C. virescens*.

2. Materials and methods

2.1. Moths

For this study we used the noctuid moth *Chloridea virescens* (see Suppl. Material a. for rearing conditions). We used two artificial selection lines that were constructed to have opposing ratios between the unsaturated and saturated sex pheromone compounds [see Groot et al. (2014) for further details]. In the “High” selection line, females have high ratios of hexadecanal/(Z)-11-hexadecanal (16:Ald/Z11-16:Ald) and tetradecanal/(Z)-9-tetradecanal (14:Ald/Z9-14:Ald), while the “Low” line consists of females with low ratios of 16:Ald/Z11-16:Ald and 14:Ald/Z9-14:Ald. Pheromone compound ratios were determined by gas chromatography of hexane extracts of whole pheromone glands (see Suppl. Material b. for a detailed protocol).

2.2. Experimental set-up

We investigated DNA methylation patterns in a set of independent experiments, as shown in Fig. 1 and specified below. In short, we analysed gland- and phenotype-specific DNA methylation patterns in

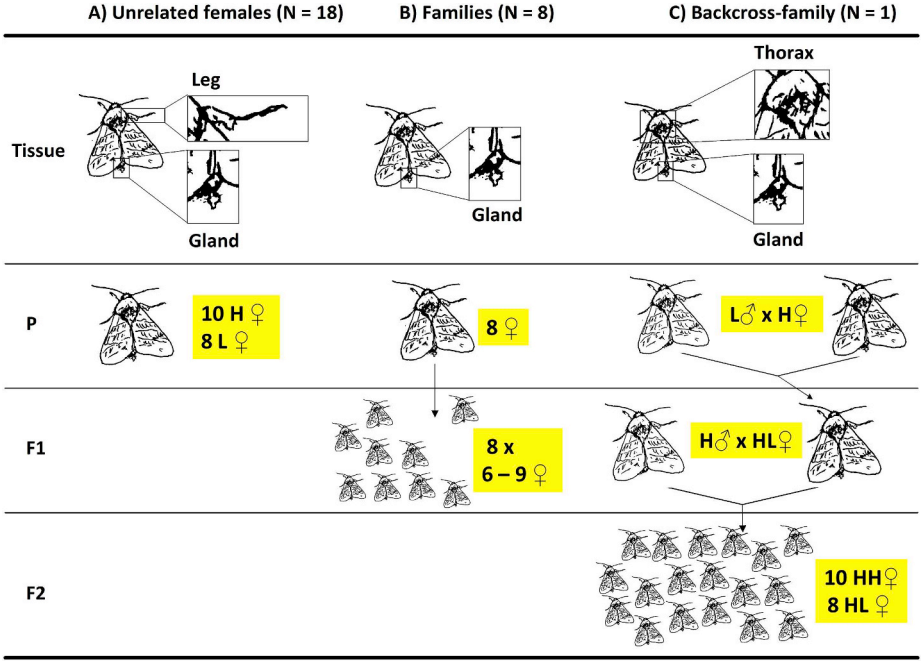

Fig. 1. Experimental setup and crossing scheme for MS-AFLP analysis of *Chloridea viresecens*. We evaluated DNA methylation patterns in A) pheromone gland and leg tissue within 10 High (H) and 8 Low (L) females; B) pheromone gland tissue across 2 generations (P and F1) in 8 families (8 mothers with 6–9 daughters each); and C) pheromone gland and thorax tissue in a backcross-family (P, F1, and F2). The 10 HH daughters had an H phenotype. The 8 HL daughters had an L phenotype.

pheromone gland and leg tissue of eight Low and ten High unrelated females (Fig. 1A). We also analysed differentiation between Low and High siblings in a backcross-family. The backcross-family was obtained by hybridizing (High [H] ♀ x Low [L] ♂) and backcrossing (HL ♀ x H ♂) males and females, resulting in 50% HL (= Low phenotype, as Low is dominant) and 50% HH (= High phenotype) offspring. From this backcross family, we analysed the pheromone gland and thorax of the maternal grandparents, parents, and 18 daughters (8 HL and 10 HH; Fig. 1C). Furthermore, we assessed the family specificity and inheritance of DNA methylation in pheromone gland tissue in 8 families, consisting of 8 mothers with 6–9 daughters each (two independent sets of 4 families: Family 1–4 and Family 5–8; Fig. 1B). To further determine whether DNA methylation patterns are heritable, and/or *de novo* DNA methylation occurs from parents to offspring, we analysed Mendelian segregation patterns of DNA methylation among 18 siblings of the backcross family (Fig. 1C), as specified below, and following Verhoeven et al. (2010).

2.3. Methylation-sensitive AFLP protocol

Methylation Sensitive Amplified Fragment Length Polymorphisms (MS-AFLP) were analysed following an adjusted AFLP procedure as described in Sheck et al. (2006) and Ruiz-Garcia et al. (2010) (for detailed protocol, see Suppl. Material c.). In short, genomic DNA was extracted from moth tissues and digested with *Eco*R1/*Msp*I and *Eco*RI/*Hpa*II (New England Biolabs, Ipswich, MA, USA). Subsequently, the restricted DNA was subjected to two rounds of selective PCR (core primer and adapter sequences are given in Suppl. Table 1, and primer extensions and combinations used in each experiment are given in Suppl. Table 2), after which the banding patterns were visualized on a polyacrylamide gel. *Eco*RI/*Hpa*II and *Eco*RI/*Msp*I fragments in the size range of 50–700 bp were scored for presence (1) and absence (0), using a semiautomatic image analysis program designed specifically for AFLP analysis (Quantar PRO 1.0; KeyGene Products, Wageningen, The Netherlands).

Presence-absence scores were processed in Microsoft Excel (2010) and analysed using the R package "*msap*" [Version 1.1.9 (Pérez-Figueroa, 2013) in R studio (Version 3.1.2); R Core Development Team)]. Presence of both *Eco*RI/*Hpa*II and *Eco*RI/*Msp*I fragments (pattern 1/1) denotes an unmethylated state, presence of either the *Eco*RI/*Hpa*II fragment or the *Eco*RI/*Msp*I fragment (pattern 0/1 or 1/0) denotes a methylated state, and absence of both *Eco*RI/*Hpa*II and *Eco*RI/*Msp*I fragments (pattern 0/0) is considered either as uninformative or as a DNA sequence difference at the 5′-CCGG-3′ restriction site (Suppl. Table 3). After comparing the data from both enzyme combinations, *msap* determines if each locus is susceptible to DNA methylation (defined as loci that show evidence of DNA methylation above a genotyping error rate of 5%), or if there is no evidence for DNA methylation. Methylation-Susceptible Loci (MSL) represent DNA methylation variation, and Non-Methylated Loci (non-ML) represent sequence variation (Suppl. Table 3).

Classifying MS-AFLP loci as MSL or non-ML to compare genetic to epigenetic variation has been done in many population epigenetic studies (Baldanzi et al., 2017; Cervera et al., 2002; Herrera and Bazaga, 2010; Liu et al., 2012; Watson et al., 2018). While MSL provide a good estimation of epigenetic variation, non-ML may miss some genetic variation, as *Hpa*II and *Msp*I may not cut due to absence of a CCGG-site, but due to full methylation of the external cytosine or both cytosines or by hemimethylation of both cytosines (Schulz et al., 2013). Even though there is no evidence that these types of DNA methylation exist in moths (Xiang et al., 2010), we additionally scored conventional AFLP markers on the same individuals in the backcross family to confirm that non-ML provide a good estimate of genetic variation.

2.4. Conventional AFLP analysis protocol

For conventional AFLP analysis, we used the DNA methylation insensitive restriction enzyme combination *Eco*RI/*Mse*I (See Suppl. Tables 1 and 2 for primer and adapter sequences). Conventional AFLP analysis was performed on thorax DNA extracts of the backcross family, following the same protocol as in the MS-AFLP analysis (Sheck et al., 2006).

2.5. Data analysis

2.5.1. Reproducibility of DNA methylation detection by MS-AFLP

The reproducibility of methylation detection by MS-AFLP was tested by repeating the complete procedure on the same set of 7 DNA extracts (6 pheromone glands and 1 leg). Scores of the two sets of MS-AFLP analyses were compared in Microsoft Excel (2010).

2.5.2. Tissue-specific DNA methylation patterns

We compared DNA methylation patterns at MSL in sex pheromone glands with leg tissue within 18 unrelated females (Fig. 1A), and with

Table 1
Epigenetic (using Methylation-Susceptible Loci) and genetic (using Non-Methylated and AFLP loci) differentiation between High and Low pheromone phenotype selection lines of *Chloridea virescens* (n = 10 High and 8 Low).

	Analysis[a]	Source of variation	Methylation-Susceptible Loci (MSL)				Non-Methylated Loci (non-ML)				Mantel test		
			No. of loci	% var.	Φ_{ST}	P-value	No. of loci	% var.	Φ_{ST}	P-value	r	R^2	P-value
Unrelated females	**GH x GL**	Among phenotypes	**126**	20.22	0.2022	0.0003	**321**	18.40	0.1840	<0.0001	0.2733	0.0747	0.0020
		Within phenotypes		79.78				81.60					
		Total		100				100					
	SH x SL	Among phenotypes	**118**	25.22	0.2523	0.0001	**329**	19.96	0.1996	0.0001	0.3285	0.1079	0.0020
		Within phenotypes		74.78				80.04					
		Total		100				100					
Backcross-family	**GH x GL**	Among phenotypes	**136**	14.54	0.1454	0.0001	**688**	6.128	0.0613	0.0056	0.4590	0.2107	0.0010
		Within phenotypes		85.46				93.87					
		Total		100				100					
	SH x SL	Among phenotypes	**112**	20.93	0.2093	0.0003	**712**	8.168	0.0817	0.0009	0.3152	0.0994	0.0020
		Within phenotypes		79.07				91.83					
		Total		100				100					
Conventional AFLP loci													
Backcross-family	**High x Low**	Among phenotypes					**819**	7.12	0.0712	0.0028			
		Within phenotypes						92.88					
		Total						100					

[a] AMOVA, GH = Gland High, GL = Gland Low, SH = Systemic High, SL = Systemic Low. Systemic loci are those gland loci that were also found in leg or thorax.

Table 2
Mendelian segregation ratios of Methylation-Susceptible Loci, Non-Methylated Loci, and AFLP loci of *Chloridea virescens*.

Type	Expected ratio	No. of loci					Fisher's exact test P-values			
		Total	Mendelian	% Mendelian	Deviating[a]	% Deviating	vs. AFLP loci	vs. non-ML (systemic)	vs. non-ML (gland)	vs. MSL (systemic)
AFLP loci										
M0P0	1	77	77	100	0	0.0				
M1P1	0.75	118	107	90.7	11	9.3				
M0P1/M1P0	0.5	212	202	95.3	10	4.7				
Non-Methylated Loci (non-ML)										
Systemic[b]										
M0P0	1	80	80	100	0	0.0	ns			
M1P1	0.75	53	49	92.5	4	7.5	ns			
M0P1/M1P0	0.5	109	100	91.7	9	8.3	ns			
Gland										
M0P0	1	84	84	100	0	0.0	ns	ns		
M1P1	0.75	53	50	94.3	3	5.7	ns	ns		
M0P1/M1P0	0.5	110	101	91.8	9	8.2	ns	ns		
Methylation-Susceptible Loci (MSL)										
Systemic[b]										
M0P0	1	77	74	96.1	3	3.9	ns	ns	ns	
M1P1	0.75	15	14	93.3	1	6.7	ns	ns	ns	
M0P1/M1P0	0.5	37	25	67.6	12	32.4	<0.001	<0.001	<0.001	
Gland										
M0P0	1	84	73	86.9	11	13.1	<0.001	<0.001	<0.001	0,050
M1P1	0.75	42	39	92.9	3	7.1	ns	ns	ns	ns
M0P1/M1P0	0.5	60	40	66.7	20	33.3	<0.001	<0.001	<0.001	ns

[a] Number of loci that significantly deviate from Mendelian segregation. Exact chi square goodness-of-fit tests, $\alpha = 0.05$.
[b] Systemic non-ML and systemic MSL are only those gland loci that are reproducible in thorax.

thorax tissue within 18 sisters of a backcross family (Fig. 1C). For each female, we calculated the percentage DNA methylation in sex pheromone glands that is systemic (i.e. reproducible across tissues), and the percentage that is gland specific. Calculations were done in Microsoft Excel (2010).

2.5.3. Differentiation between High and Low selection lines

To assess genetic and epigenetic differentiation between the High and Low selection lines, we analysed non-ML, gland MSL, and systemic MSL of unrelated females (10 High and 8 Low; Fig. 1A), and of sisters within the backcross family (10 High and 8 Low; Fig. 1C). For the same individuals of the backcross family, we also analysed an independent set of conventional AFLP loci. We tested for the significance of differentiation between High and Low by analysis of molecular variance (AMOVA). We visualized the differentiation by Principal Coordinate Analysis (PCoA). To determine the correlation between genetic and epigenetic loci, a Mantel test (1000 permutations) was performed. All analyses were done using the R package *msap* (Pérez-Figueroa, 2013).

2.5.4. Inheritance of DNA methylation

To assess the relationship between genetic and epigenetic variation, we analysed MSL and non-ML in gland tissue among 8 families (two independent datasets of 4 families: Fam. 1–4, and Fam. 5–8) with 6–9 daughters each (Fig. 1B). We used the R package *msap* (Pérez-Figueroa, 2013) to perform PCoA, AMOVA, and a Mantel test, as described above. Furthermore, *msap* determined the percentage non-polymorphic MSL, i.e. those MSL for which a detectable CCGG-site was always methylated, and polymorphic MSL, i.e. those MSL for which the methylation state at detectable CCGG-sites was variable. Furthermore, for each family we determined the inheritance of DNA methylation. We defined inheritance as the percentage DNA methylation states at detected CCGG-sites that remained unchanged from mother to daughter glands. Percentages of all possible fates of DNA methylation states were plotted in stacked bar graphs, using SigmaPlot Version 13. Differences in total DNA methylation inheritance among families were tested among all families (Fam. 1–8) using One-Way ANOVA. In addition, for polymorphic MSL, we used a Welch's *t*-test to determine if the similarity between daughters and their own mothers was significantly higher than the similarity between daughters and the mothers from different families.

We analysed Mendelian segregation patterns of conventional AFLP loci, non-ML, and systemic and gland-specific MSL in 18 daughters of the backcross family (Fig. 1C). All loci were divided over 4 classes, based on fragment presence or absence in the parents. For each class, the expected Mendelian segregation ratio was determined as follows. Fragments that were present in the mother, but absent in the father (class M1P0), either did not segregate (when the mother was homozygous), or segregated at a 1:1 ratio (when the mother was heterozygous). Fragments that were absent in the mother, but present in the father (class M0P1), either did not segregate (when the father was homozygous) or segregated at a 1:1 ratio (when the mother was heterozygous). Fragments that were present in both parents (class M1P1) either did not segregate (when one or both parents were homozygous) or segregated at a 3:1 ratio (when both parents were heterozygous). Fragments that were not present in either parent (class M0P0, both parents were homozygous) did not segregate (ratio 1). For each class, we determined whether loci deviated from the expected Mendelian segregation ratio by using exact chi-square tests for goodness-of-fit (Verhoeven et al., 2010). We subsequently tested if MSL deviated significantly more than conventional AFLP loci and non-ML, using Fisher's Exact test for independence (Verhoeven et al., 2010). For M0P0 (both parents homozygous), significant deviation from 1 could not be calculated. Therefore, all loci in this class showing a deviation were considered significant. We also tested for differences in Mendelian segregation ratios between gland-specific and systemic MSL. All analyses were conducted in Microsoft Excel (2010).

3. Results

3.1. Reproducibility of DNA methylation detection by MS-AFLP

To verify that MS-AFLP scoring is reliable and reproducible in *C. virescens*, we first conducted the analysis on the same set of 7 DNA extracts. We minimized any potential scoring errors by using a well-

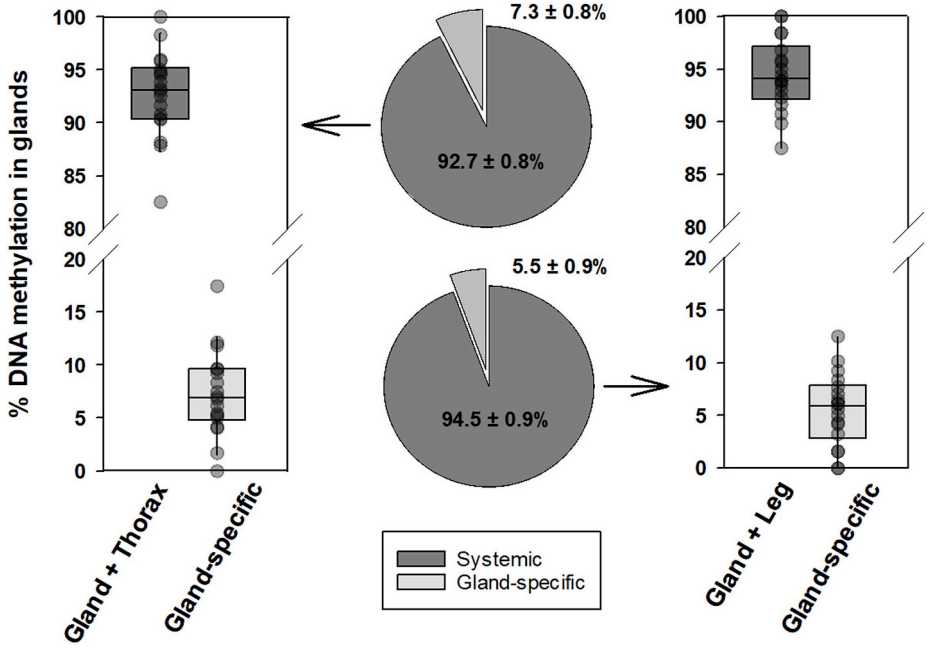

Fig. 2. Gland-specific DNA methylation of *Chloridea virescens*. Percentage (±SEM) of DNA methylation in glands that is gland-specific and systemic, i.e. also methylated in thorax tissue (left, backcross-family: n = 18) and in leg tissue (right, unrelated females: n = 18).

developed protocol [adapted from Sheck et al. (2006) and Ruiz-Garcia et al. (2010)], negative controls, a semiautomatic gel scoring program (Quantar PRO 1.0; KeyGene Products, Wageningen, The Netherlands), and an automated scoring script (*msap*) with a 5% genotyping error-rate threshold (Pérez-Figueroa, 2013). As the two sets of DNA methylation scores were nearly identical, i.e., 179 loci out of 180 (99.4%) were reproducible (Suppl. File 1, Suppl. Figure 1), we conclude that our protocol was reliable.

3.2. Tissue-specific DNA methylation patterns

We compared Methylation-Susceptible Loci (MSL) between pheromone glands and legs (N = 18 females, 125 MSL), and between pheromone glands and thoraces (N = 18 females, 180 MSL). We found that 94.5 ± 0.8% (mean ± SEM) of the DNA methylation sites in the glands was also present in legs, and 92.7 ± 0.9% was also present in thoraces (Fig. 2). We further found 5.5 ± 0.8%–7.3 ± 0.9% of the DNA methylation sites to be gland-specific DNA methylation (Fig. 2). Leg- and thorax-specific DNA methylation was also present, but not further analysed.

3.3. Differentiation between High and Low selection lines

When we compared genetic variation (non-ML) with epigenetic variation (MSL) between "High" and "Low" pheromone phenotype selection lines, we found that non-ML were significantly differentiated between unrelated females with High and Low phenotypes (AMOVA; Table 1, Fig. 3), as well as between siblings from a backcross family with High and Low phenotypes (AMOVA; Table 1, Fig. 3). Furthermore, we found that the results of non-ML were consistently similar to AFLP loci (Table 1, Suppl. Figure 2). Gland MSL and systemic MSL were also significantly differentiated between High and Low pheromone types (AMOVA; Table 1, Fig. 3). Even though there were clear general differences in DNA methylation between High and Low phenotypes, we found no specific MSL that were always linked to High or Low pheromone types. Furthermore, we found that the MSL and non-ML profiles were significantly correlated (Mantel Test; Table 1).

3.4. Inheritance of DNA methylation

To explore if DNA methylation patterns were family specific, we analysed MSL and non-ML of eight families (two sets of four families, 6–9 daughters each). Between families, we found significant genetic differences (AMOVA; Fam. 1–4: $\Phi(3,26) = 0.491$, $P < 0.001$, Fam. 5–8: $\Phi(3,26) = 0.463$, $P < 0.001$; Suppl. Table 4, Fig. 4), as well as epigenetic differences (AMOVA; Fam. 1–4: $\Phi(3,26) = 0.511$, $P < 0.001$, Fam. 5–8: $\Phi(3,26) = 0.513$, $P < 0.001$; Suppl. Table 4, Fig. 4). Between families, MSL were significantly correlated with non-ML (Mantel test; Fam. 1–4: $r = 0.631$, $P = 0.001$, Fam. 5–8: $r = 0.587$, $P = 0.001$; Suppl. Table 4). Examining all families revealed that 73% of MSL was non-polymorphic, i.e., detected CCGG-sites were always methylated across all families. For the remaining 27%, DNA methylation occurred in both states (methylated and unmethylated) at least once. In calculating the inheritance of DNA methylation states, i.e., the percentage DNA methylation states at CCGG-sites that remained unchanged from mother to daughter glands, we found that 87.9 ± 0.6% (in Fam. 4) to 96.3 ± 0.4% (in Fam. 6) of DNA methylation states were inherited from mother to daughter, while 12.1 ± 0.6 (in Fam. 4) to 3.7 ± 0.4% (in Fam. 6) of DNA methylation states changed from mother to daughter (Fig. 5). Thus, inheritance of DNA methylation states was not the same for all families (ANOVA; Fam. 1–8: $F(7,52) = 13.75$, $P < 0.001$; Suppl. Table 5). Furthermore, for the 27% polymorphic MSL, methylation states at CCGG-sites were significantly more similar between daughters and their own mother (79 ± 1.7%) than between daughters and mothers from different families (60 ± 1.2%, $t_{(122.7)} = 9.6$, $P < 0.001$). In summary, DNA methylation at CCGG-sites was correlated with genetic variation and mostly consistent across individuals, families, and generations.

To assess the inheritance patterns of DNA methylation, we compared segregation patterns of MSL with AFLP loci and non-ML in 18 daughters of a backcross family [following Verhoeven et al. (2010)]. In comparing Mendelian segregation patterns of MSL with AFLP loci, we found that 93.6% of in total 330 segregating AFLP loci showed Mendelian segregation patterns (Fig. 6, Table 2). Of the 52 systemic MSL and 102 gland MSL, 77.5%, and 75.0% showed Mendelian segregation patterns, respectively. Thus, although most MSL were heritable and followed Mendelian segregation patterns, there was a significant

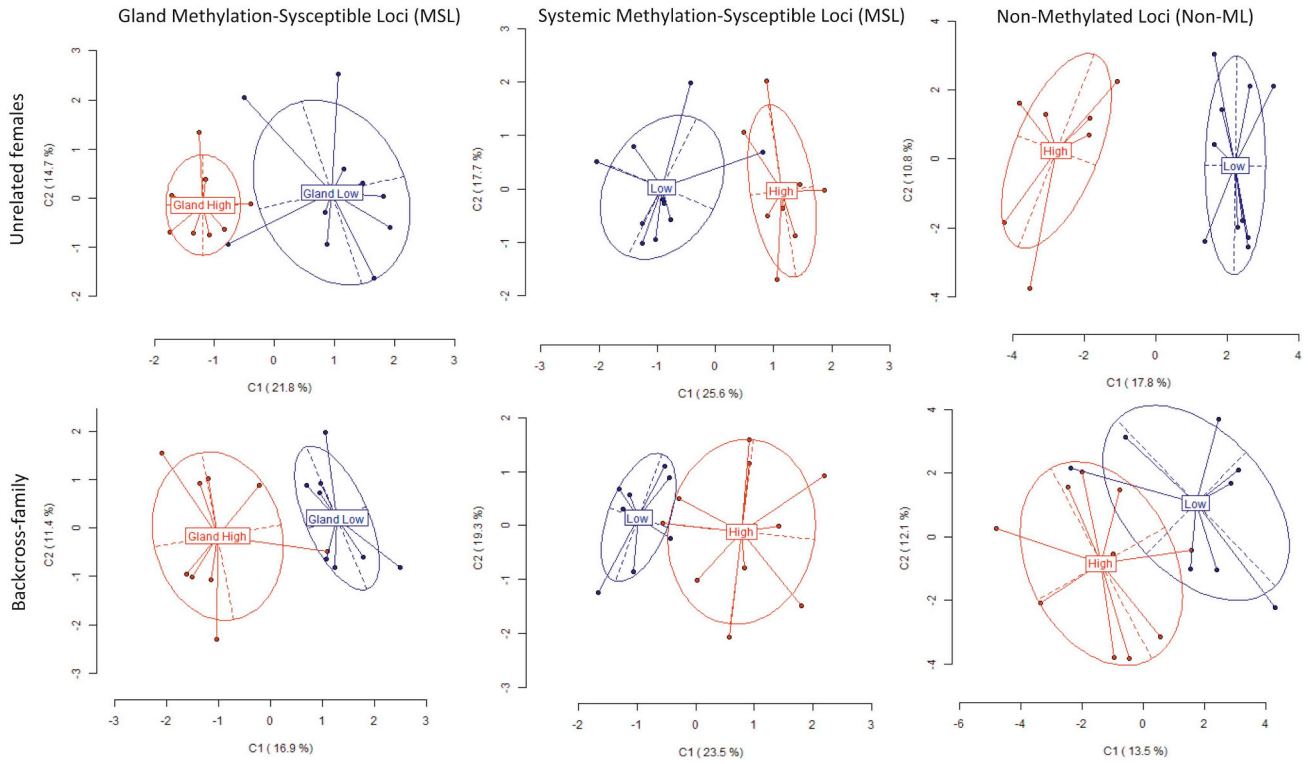

Fig. 3. Principle Coordinate Analysis of epigenetic (using Methylation-Susceptible Loci) and genetic (using Non-Methylated Loci) differences between High pheromone phenotype (red) and Low pheromone phenotype (blue) *Chloridea virescens* females in unrelated females and the backcross family (n = 10 High and 8 Low. The first two coordinates (C1 and C2) are displayed with the indication of the percentage of variance explained in brackets. Dots represent individual females. Labels indicate the centroids of each pheromone phenotype. Ellipses represent the dispersion associated to each value. Systemic MSL are only those gland MSL that are reproducible in leg tissue (for the unrelated females) and thorax tissue (for the backcross-family). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

difference compared to the AFLP segregation patterns (Fig. 6, Table 2). Of the 162 systemic non-ML and 163 gland non-ML, 92.0% and 92.6% of showed Mendelian segregation, respectively, which was similar to the AFLP segregation patterns (Table 2), showing that non-ML are a good estimate of genetic variation. The comparison of MSL with non-ML segregation patterns yielded the same results as the comparison between MSL and AFLP segregating patterns (Table 2).

Deviations from Mendelian segregation patterns indicate *de novo* DNA methylation. When dividing all loci over 4 classes, based on fragment presence or absence in the parents, MSL in the M1P0/M0P1 class (M1: present in mother, P0: absent in father) showed significantly more deviations from Mendelian segregation than AFLP loci, while MSL in the M1P1 class (present in both parents) showed similar segregation patterns as AFLP loci (Fig. 6, Table 2). Loci of the M0P0 class (homozygous absent in both parents) should not segregate, however some gland MSL showed significant deviations from Mendelian segregation (Table 2). Thus, *de novo* gland and systemic DNA methylation in the M1P0/M0P1 class, and gland-specific *de novo* DNA methylation in the M0P0 class appear to occur in *C. virescens*.

We further observed that some AFLP loci deviated significantly from Mendelian expectations, a phenomenon that was also found by Verhoeven et al. (2010) and Jiang et al. (2016). Such deviations could perhaps be explained by duplicated loci in the genome or by systematic biases in scoring faint bands as either present or absent (Verhoeven et al., 2010). Some deviations could also be due to DNA methylation changes, as *Eco*RI, which is used for AFLP analysis, is not completely insensitive to DNA methylation. However, this should not have affected the comparison between segregation patterns of AFLP loci and MSL, as possible DNA methylation in *Eco*RI sites are most likely evenly distributed across loci.

4. Discussion

To assess the role of epigenetics in moth pheromone evolution, we used MS-AFLP analysis to examine DNA methylation patterns in the noctuid moth *C. virescens*. We found that DNA methylation patterns are reproducible, and mostly consistent across tissues. However, some gland-specific DNA methylation also occurred. Furthermore, we found differences in DNA methylation patterns between High and Low pheromone phenotype selection lines. DNA methylation patterns were also family-specific, associated with genetic variation and heritable, although some *de novo* DNA methylation also occurred.

4.1. Reproducibility of DNA methylation detection by MS-AFLP

The MS-AFLP technique has been applied in thousands of other studies since its introduction in 1997 (Reyna-López et al., 1997), and its reliability and reproducibility have been demonstrated many times (Richards, C. L. et al., 2017; Schrey et al., 2013). The major shortcoming of MS-AFLP is that it only screens anonymous loci at CCGG-sites which may have no direct connection to the phenotype (Richards, C. L. et al., 2017; Schrey et al., 2013). Thus, we cannot link DNA methylation patterns directly to the High or the Low pheromone phenotype. Despite this shortcoming, MS-AFLP is a powerful method to detect epigenetic differentiation between populations (Herrera and Bazaga, 2010; Liu et al., 2015), tissues (Sun et al., 2014), and to detect heritable and *de novo* DNA methylation patterns (Verhoeven et al., 2010). Furthermore, non-ML are consistently similar to conventional AFLP loci as shown by us (Tables 1 and 2, Fig. 6; Suppl. Figure 2), and in another study (Ardura et al., 2017). Therefore, non-ML provide a good estimate of genetic variation allowing within-dataset comparisons between genetic and epigenetic variation (Pérez-Figueroa, 2013).

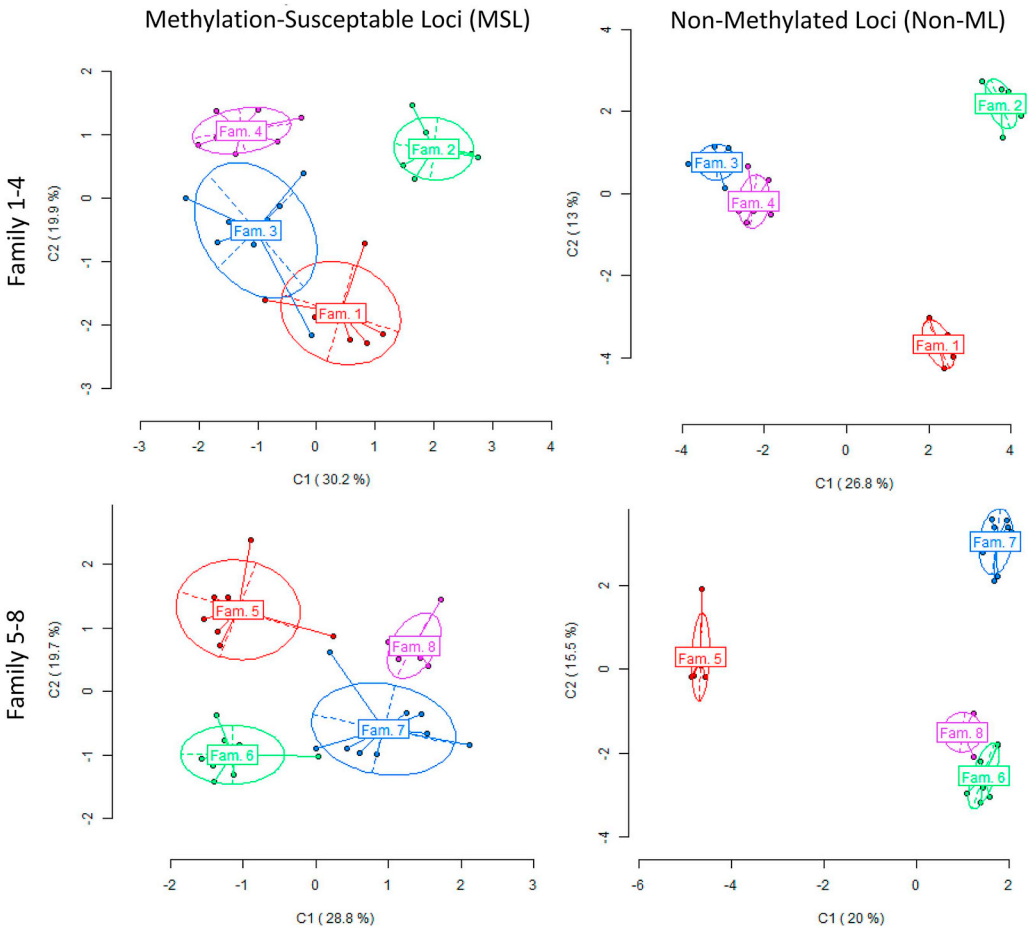

Fig. 4. Principle Coordinate Analysis of epigenetic (using Methylation-Susceptible Loci) and genetic (using Non-Methylated Loci) differences between *Chloridea virescens* families for 2 independent sets of data each containing 4 families with 6–9 daughters per family (total n = 30 in Fam. 1–4, and 30 in Fam. 5–8). The first two coordinates (C1 and C2) are displayed with the indication of the percentage of variance explained in brackets. Dots represent individual females. Labels indicate the centroids of each family. Ellipses represent the dispersion associated with each value.

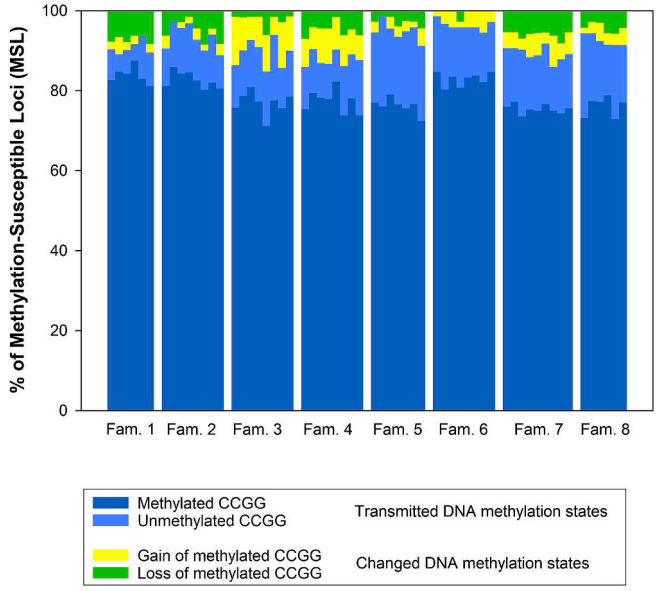

Fig. 5. Inheritance of DNA methylation states at available CCGG-sites from mother to daughter pheromone glands in 8 families (Fam. 1–8, with 6–9 daughters each) of *Chloridea virescens*. Blocks represent families, bars represent daughters, and colors represent DNA methylation events from mother to daughters. Blue colors represent inherited DNA methylation states, yellow represents a gain of DNA methylation, and green represents a loss of DNA methylation. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

4.2. Tissue-specific DNA methylation patterns

Most DNA methylation in insects likely represents germline methylation within gene bodies of ubiquitously expressed housekeeping genes, whereas genes with tissue-specific functions are sparsely methylated (Glastad et al., 2014; Provataris et al., 2018; Sarda et al., 2012). We therefore expected an overall similarity in methylation patterns between the sex pheromone gland and other tissues, a situation that was also found by Jones et al. (2018), who showed that DNA methylation in the noctuid moth *Helicoverpa armigera* was nearly identical across life stages. Even though we did find an overall similarity between tissues, we also found some gland-specific DNA methylation. Gland-specific methylation may have accumulated due to environmental exposure or stochastic variation in DNA methylation maintenance over the adult moths' lifetime. Such age-associated tissue-specific changes in DNA methylation patterns have been frequently observed in mammals (Christensen et al., 2009; Day et al., 2013; Maegawa et al., 2010). Tissue-specificity suggests that DNA methylation is involved in tissue-specific gene expression, as shown in the moth *B. mori* (Xiang et al., 2013; Xu et al., 2018) and *Galleria mellonella* (Mukherjee et al., 2017). Thus, despite overall similarity between tissues, tissue-specific differences in DNA methylation patterns could explain at least part of the epigenetic variation in *C. virescens*.

4.3. Differentiation between High and Low selection lines

We found significant genetic and epigenetic differences between High and Low selection lines of *C. virescens*, and genetic and epigenetic profiles were correlated. In other words, when DNA methylation sites were present, they were likely to be associated with genetic polymorphisms. In general, greater genetic variation also meant greater

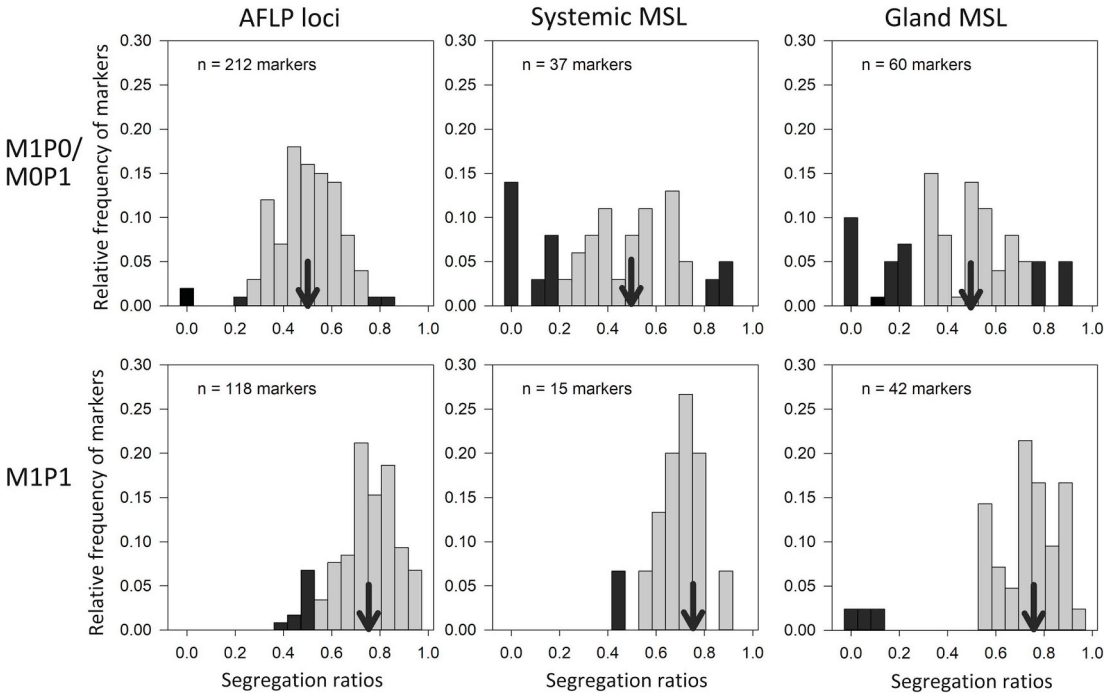

Fig. 6. Mendelian segregation ratios of epigenetic (using Methylation-Susceptible Loci) and genetic (using AFLP loci) loci in marker class M0P1/M1P0 (restriction fragment present in either parent) and marker class M1P1 (restriction fragment present in both parents) in *Chloridea virescens*. Light grey bars are markers that follow Mendelian segregation expectation (indicated by arrows). Black bars deviate from Mendelian segregation. From left to right: AFLP loci, systemic MSL (only those gland MSL that are reproducible in thorax), and gland MSL.

DNA methylation variation. Previously, the genetic basis of the pheromone variation between the High and Low was identified (Groot et al., 2014, 2019), showing that pheromone divergence is at least partly genetically determined. Possibly, DNA methylation differentiation is directly controlled by genetic factors. However, the correlation between genetic and epigenetic variation may be non-causal. For example, DNA methylation variation might affects survival or other fitness parameters and lead to consistent DNA methylation profiles within the High and Low lines over time (Flores et al., 2013). Furthermore, the High and Low selection lines appear to be epigenetically and genetically similarly differentiated. This is in contrast to several studies in wild populations of plants and animals which found greater epigenetic than genetic differences between populations (Lira-Medeiros et al., 2010; Liu et al., 2015; Richards, C. L. et al., 2012). It has been speculated that epigenetic divergence between populations may precede genetic divergence, as DNA methylation is more dynamic than genetic variation and could respond quicker to selection (Lira-Medeiros et al., 2010; Liu et al., 2015; Richards, C. L. et al., 2012). Our experiment was conducted after more than 20 generations of artificial selection, and we do not know whether differences between genetic and epigenetic differentiation occurred in earlier phase of the selection process. Thus, it can be concluded that genetic as well as epigenetic variation may have contributed to the divergence of the High and Low selection lines of *C. virescens*.

4.4. Inheritance of DNA methylation

Our analyses of 8 families and a backcross family showed that DNA methylation patterns were 1) family-specific, 2) correlated with genetic variation, and 3) inherited. The finding that most MSL inherited from mother to daughters and segregated in a Mendelian manner was also found in another invertebrate species, the Pacific oyster *Crassostrea gigas*, where most MSL (90.6%) were stably inherited to offspring in a Mendelian fashion (Jiang et al., 2016). Such inheritance patterns can be due to different processes. For example, methylation could be erased and re-established during development under control of a genetic locus in *cis* or *trans* that segregates in a Mendelian manner. In insects, this has been suggested to occur in honey bees (Remnant et al., 2016; Wedd et al., 2015; Yagound et al., 2019), bumble bees (Lonsdale et al., 2017), and *Nasonia* wasps (Wang et al., 2016). It is also possible that inheritance of DNA methylation occurs through escape from germline erasure. However, Lepidoptera lack *de novo* DNA methyltransferase (DNMT3) to re-establish methylation patterns (Bewick et al., 2017). Glastad et al. (2019) suggested that maintenance DNA methyltransferase (DNMT1) may be sufficient to maintain DNA methylation over generations, and that removal and re-establishment of DNA methylation does not occur in Lepidoptera. This hypothesis is supported by the fact that in F1 hybrids of two species of *Nasonia* wasps (that also lack DNMT3), DNA methylation was faithfully inherited (Wang et al., 2016). Also, DNA methylation has been found in the main embryonic stages of some insects (Drewell et al., 2014; Feliciello et al., 2013). Furthermore, recent studies showed that early embryo reprogramming and genome-wide demethylation may not occur in honey bees (Harris et al., 2019) and other invertebrates (Xu et al., 2019). The persistence of DNA methylation in early embryonic stages could also be mediated by other epigenetic mechanisms (Pavelka et al., 2017). Even though in our study we cannot differentiate between the various scenarios, our results consistently show that most DNA methylation is inherited from mothers to daughters.

In this study, most (73%) MSL were non-polymorphic, i.e., available CCGG-sites were always methylated across all families and generations. Although MS-AFLP does not provide any information on the DNA methylation state of a site outside the CCGG-context, i.e., at HCGH-sites, absence of a CCGG-site could mean that the site is no longer available for methylation due to the loss of a CG-dinucleotide. Such sites represent genetic and epigenetic polymorphisms simultaneously, and their phenotypic impact may be substantial (Wedd et al., 2015). Alternatively, if the DNA methylation status remains unaffected by DNA polymorphisms at CCGG-sites, then consistent DNA methylation across families and generations may also reflect its potential role in

maintaining expression patterns of housekeeping genes (Provataris et al., 2018; Sarda et al., 2012).

The DNA methylation status at some CCGG-sites did change from mother to daughter gland, but these changes did not affect all families equally. Subtle maternal transgenerational effects, e.g., through unintended environmental variation between mothers, could account for some of these differences, although rearing and handling conditions between individuals were carefully kept constant across the experiments. Mothers might also differ in their ability to transmit DNA methylation to offspring (Herrera et al., 2014). In addition, DNA methylation changes in pheromone glands could also be paternally - instead of maternally - inherited loci, which were not taken into account in the family experiment. The fact that in some cases, some sites were subject to spontaneous *de novo* DNA methylation can be deduced from our observation that MSL deviated more often than AFLP loci from Mendelian inheritance patterns. This suggests that DNMT1 may have (partly) taken over the role of *de novo* DNA methylation from DMNT3. Stochastic changes might also be caused by imperfect re-establishment of DNA methylation by DNMT1, or other random processes at the cellular level (Vogt, 2015). This could be an important source of epigenetic variation and a key mechanism for the appearance of phenotypic diversity in the absence of genetic mutations (Feinberg and Irizarry, 2010; Schmitz et al., 2011; Vogt, 2015).

Since some systemic MSL, i.e., those that were consistently methylated across tissues, also deviated more often from Mendelian inheritance patterns than AFLP loci, we speculate that spontaneous *de novo* DNA methylation may affect the germline as well. *De novo* DNA methylation could accumulate over multiple generations when it affects the germline (Jablonka, 2013) and escapes germline reprogramming. In *C. virescens*, we cannot say to what extent the inherited DNA methylation patterns that we observed originated from past *de novo* DNA methylation. However, the level of methylation changes from parents to daughters that we observed (22.5–25.0% of segregating MSL deviated from Mendelian ratios) suggests that methylation variation may have built up over generations, and this could have contributed to the observed epigenetic divergence between High and Low phenotypes. Our finding that 75–78.5% of the MSL were inherited indicates that inheritance of DNA methylation should be taken into account in evolutionary scenarios of moth sex pheromone divergence.

In summary, we explored the evolutionary potential of DNA methylation variation in the noctuid moth *C. virescens*. We assessed whether DNA methylation is 1) tissue-specific, 2) differentiated between pheromone phenotype selection lines, and 3) heritable, and we provide evidence for all these phenomena. Our study suggests that DNA methylation has a diverse role in *C. virescens*. Some genes are systemically methylated, while other genes are methylated only in the sex pheromone gland. In addition, some methylated genes are associated with the pheromone phenotype selection lines. The fact that some sites were subject to *de novo* DNA methylation, but most DNA methylation was heritable, suggests that DNA methylation could potentially generate heritable phenotypic variation that may become subject to selection and may have a direct impact on evolution of moth sex pheromones. Taken together, our results present an important initial step towards understanding the role of DNA methylation in the evolution of sexual communication signals in moths.

Declaration of competing interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ibmb.2020.103370.

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