Diploid male production correlates with genetic diversity in the parasitoid wasp Venturia canescens: a genetic approach with new microsatellite markers

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Abstract

Sex determination is ruled by haplodiploidy in Hymenoptera, with haploid males arising from unfertilized eggs and diploid females from fertilized eggs. However, diploid males with null fitness are produced under complementary sex determination (CSD), when individuals are homozygous for this locus. Diploid males are expected to be more frequent in genetically eroded populations (such as islands and captive populations), as genetic diversity at the csd locus should be low. However, only a few studies have focused on the relation between population size, genetic diversity, and the proportion of diploid males in the field. Here, we developed new microsatellite markers in order to assess and compare genetic diversity and diploid male proportion (DMP) in populations from three distinct habitat types – mainland, island, or captive – in the parasitoid wasp Venturia canescens. Eroded genetic diversity and higher DMP were found in island and captive populations, and habitat type had large effect on genetic diversity. Therefore, DMP reflects the decreasing genetic diversity in small and isolated populations. Thus, Hymenopteran populations can be at high extinction risk due to habitat destruction or fragmentation.

Keywords
Diploid males, microsatellite markers, sl-CSD, Venturia canescens.

Introduction

Sex determination takes on many different forms in animals (Beukeboom and Perrin 2014). In insects of the order Hymenoptera, haplodiploidy is the rule: Males develop from unfertilized eggs and are haploid, whereas females develop from fertilized eggs and are diploid. In numerous species including sawflies, pollinating bees, social and solitary wasps, ants, and parasitoids, the underpinning genetic mechanism of sex determination relies on the complementarity of alleles at a single gene (Whiting 1943; van Wilgenburg et al. 2006; Heimpel and de Boer 2008). This gene – csd, for complementary sex determiner – has been described in the honeybee and comes from the duplication of the transformer gene (Geuverink and Beukeboom 2014). The csd gene is probably responsible for arrhenotoky in all species of the order Hymenoptera (Beye et al. 2003; Asplen et al. 2009; Schmieder et al. 2012). Under single-locus complementary sex determination (sl-CSD), unfertilized eggs are hemizygous at the csd locus and develop normally into haploid males (Whiting 1943). Among the fertilized eggs, only heterozygous individuals develop into females; homozygosity at the csd locus yields diploid males that are generally, but not always unviable or sterile (Cowen and Stahlhut 2004; Heimpel and de Boer 2008; Harpur et al. 2013). sl-CSD is the mechanism of sex determination for which empirical evidence prevails in Hymenoptera (Cook 1993; van Wilgenburg et al. 2006; Heimpel and de Boer 2008), but other mechanisms such as multilocus CSD or genomic imprinting also occur (Verhulst et al. 2010).
At the population level, sl-CSD causes higher sensitivity to losses of genetic diversity. A decrease in allelic richness at the csd locus and a subsequent increase in homozygosity are expected to result in more diploid males and, in turn, lower population growth rate. Theoretical models predict that sl-CSD is at the core of a “diploid male vortex,” a feedback between demographic and genetic processes that potentially drives bottlenecked populations toward extinction (Zayed and Packer 2005; Hein et al. 2009; Fauvergue et al. 2015; Bompad et al. submitted). However, diploid males represent such fitness cost that various individual behaviors have evolved to limit their production. Dispersal limits inbreeding and the consequent diploid male production (Ruf et al. 2011), and also allows the recruitment of new csd alleles, so that well-connected populations are expected to produce fewer diploid males (Hein et al. 2009). Some Hymenoptera with sl-CSD have undifferentiated populations beyond a 100-km scale (Estoup et al. 1996; Zimmermann et al. 2011), suggesting high dispersal abilities. Mate choice can also influence the production of diploid males with a reduction expected in case of inbreeding avoidance (Whitehorn et al. 2009; Metzger et al. 2010a; Thiel et al. 2013). Such behaviors may mitigate the diploid male vortex (Hein et al. 2009).

The central assumption of CSD population models is that small, bottlenecked, or isolated populations have a low genetic diversity, including diversity at the csd locus, and consequently a high proportion of diploid males. To date, however, empirical support to this assumption is scarce. Only rare field data on fire ants (Ross et al. 1993), bumblebees (Darvill et al. 2006, 2012), or paper wasps (Tsichida et al. 2014) suggest a relationship between population size, genetic diversity, and the proportion of diploid males. These studies have all relied on genetic markers to estimate within-population genetic diversity, gene flow between distinct populations, mating structure, and, in some cases, the proportion of diploid males.

Evidence for diploid males in natural populations mainly concerns social Hymenoptera. In these species, the dramatic bias in sex ratio toward females renders estimation of the proportion of diploid males very problematic. In parasitoid wasps, the proportion of diploid males was assessed in only three related species: Cotesia glomerata (Ruf et al. 2013), Cotesia rubecula (de Boer et al. 2012), and Cotesia vestalis (de Boer et al. 2015). Parasitoid populations often experience small population size, for example as a consequence of cyclic dynamics (Hassell 2000) or biological control introductions (Hopper and Roush 1993). Given the key role that parasitoid wasps play by controlling the populations of herbivorous insects in natural ecosystems and agrosystems (Shaw and Hochberg 2001), the scarcity of field estimations on the occurrence of diploid males in the field is surprising.

Venturia canescens G. (Hymenoptera: Ichneumonidae) is a parasitoid wasp with sl-CSD (Beukeboom 2001). This species is a model organism commonly used in laboratory studies on behavior, physiology, and life history traits (Harvey et al. 2001; Desouhant et al. 2005; Pelosse et al. 2007). Two reproduction modes are known in this species (Schneider et al. 2002): thelytoky (asexual, parthenogenesis) and arrhenotoky (sexual reproduction under haplodiploidy). In arrhenotokous V. canescens, diploid males are fully viable and able to mate. However, they are sterile, and females with whom they mate produce only sons, similar to virgin females (Fauvergue et al. 2015). The prevalence of diploid males in natural or captive V. canescens populations has not been estimated yet, and little is known about the genetic structure of natural populations. So far, the rare population genetic studies on V. canescens showed the absence of genetic structure at the geographic scale of the French Riviera (Schneider et al. 2002; Mateo Leach et al. 2012).

We developed this study with a threefold objective: (1) develop microsatellite markers in order to assess male ploidy and conduct population genetic studies on arrhenotokous V. canescens; (2) estimate the proportion of diploid males in several populations; and (3) estimate the genetic diversity of V. canescens populations under various conditions of population size and isolation. For this, we compared mainland, island, and captive populations, with the expectation of a lower genetic diversity and a higher proportion of diploid males in captive populations compared to insular populations and in insular populations compared to mainland populations.

Materials and Methods

Sampling

Female V. canescens were collected during summer 2010 in two natural populations located in southern France near Nice and Valence (Table 1). To attract parasitoid females, open cups containing a mixture of host-rearing medium (semolina) and larvae of the host Ephestia kuehniella (Lepidoptera: Pyralidae) were hung in trees. Infested semolina is impregnated with host kairomones and is very attractive for V. canescens females (Corbet 1971; Metzger et al. 2008). Females visiting the traps to oviposit were collected, brought into the laboratory, allowed to lay eggs for 2 days on a host patch, and then killed and preserved in 96% ethanol. The reproductive mode of captured females was tested by checking the presence of males among offspring. Only arrhenotokous (sexual) females were used in the following analysis.

In addition, males were searched in eighteen locations of the Mediterranean Basin between 2011 and 2013 during late summer and autumn. Five sites were located on
mainland in Nice (France, 2011 and 2013), Solliès (France, 2013), and Vinyols and Vila-Seca (Spain, 2013). In 2012, we searched *V. canescens* males in five sites located on islands: Mallorca (Spain), Gozo (Malta), Crete (Greece), Sicily (Italy), and Cyprus. Several sites per island were sampled. In 2013, the same protocol was followed to capture males on French islands (Porquerolles and Corsica), in Mallorca (Spain), Cyprus, and in Crete (Greece) (Table 1 and Fig. 1). We selected field sites with at least 10 individual host plants (i.e., carob, fig, pomegranate, citrus, palm, or walnut trees; Salt 1976). On each site, ten male traps were evenly distributed. They were hung in host trees at 50–150 cm from the ground. Traps were checked after 48 h. If one *V. canescens* individual was caught, 20–40 traps were added on the site. *V. canescens* males are attracted by the synergy of pheromones produced by females and

### Table 1. Studied populations: locality, habitat type (mainland, island, and captive populations), geographic coordinates, host plant (Car, Fig, Wal, Pom, Che, Pea, Haz, Cit, and Oli being respectively carob, fig, walnut, pomegranate, cherry, peach, hazelnut, citrus, and olive trees), year of sampling, and year of foundation between parentheses for captive populations, with the corresponding number of males or females sampled. See Figure 1 for a map of localities.

| Populations | Habitat type | Country | Locality | Name | Geographic coordinates | Host plant | Date | Number of males | Number of females |
|-------------|-------------|---------|----------|------|------------------------|------------|------|----------------|-----------------|
|             | Mainland    | France  | Valence  | Val10 | 44°58'21"N 4°55'39"E  | Che/Pea/Haz| 2010 | 0              | 37              |
|             | Nice        | France  | Nice10   | Car   | 43°41'23"N 7°18'6"E  | Car        | 2010 | 0              | 44              |
|             | Nice        | France  | Nice11   | Car   | 43°41'23"N 7°18'6"E  | Car        | 2011 | 190            | 0               |
|             | Nice        | France  | Nice13   | Car   | 43°41'23"N 7°18'6"E  | Car        | 2013 | 90             | 21              |
|             | Solliès     | France  | Sol13    | Fig/Wal/Pom/Che | 43°10'58"N 6°2'55"E  | 2013 | 16             | 2               |
|             | Spain       | Vila-Seca| VS13     | Car/Oli | 41°7'34"N 1°8'07"E  | Car        | 2013 | 58             | 2               |
|             | Vinyols     | Spain   | Vy13     | Car   | 41°6'12"N 1°2'26"E  | Car        | 2013 | 33             | 2               |
|             | Island      | France  | Corsica  | NA    | Not Available  | Not Available | 2013 | 0              | 0               |
|             |             | Porquerolles | NA | Not Available  | Not Available | 2013 | 0              | 0               |
|             | Spain       | Mallorca | Mic12    | Car   | 39°47'58"N 2°57'53"E  | Car        | 2012 | 21             | 0               |
|             |             | Mallorca | Mic13    | Car   | 39°47'58"N 2°57'53"E  | Car        | 2013 | 38             | 3               |
|             | Italy       | Sicily  | NA       | Not Available  | Not Available  | 2012 | 0              | 0               |
|             | Greek       | Crete   | Cre12    | Pom/Fig/Cit/Oli | 35°11'39"N 25°2'09"E  | 2012 | 0              | 1               |
|             |             | Crete   | Cre13    | Pom/Fig/Cit/Oli | 35°11'39"N 25°2'09"E  | 2013 | 0              | 1               |
|             | Malta       | Gozo    | Goz12    | Car   | 36°3'34"N 14°16'34"E  | Car        | 2012 | 0              | 2               |
|             | Cyprus      | Cyprus  | Cyp12    | Car   | 34°39'5"N 33°0'17"E  | Car        | 2012 | 0              | 1               |
|             |             | Cyprus  | NA       | Car   | 34°39'5"N 33°0'17"E  | Car        | 2013 | 0              | 0               |
|             | Captive     | France  | CapNiceA | Car   | 43°41'23"N 7°18'6"E  | Car        | 2013 | 50             | 0               |
|             |             | Nice    | CapNiceB | Car   | 43°41'23"N 7°18'6"E  | Car        | 2013 | 31             | 0               |
|             |             | Valence | Val10    | Car   | 44°58'21"N 4°55'39"E  | Che/Pea/Haz| 2013 | 50             | 0               |
|             | Israel      | Tel-Aviv| Cptsr    | Not Available  | Not Available  | 2013 | 50             | 0               |

1Several sites were searched in these locations, but no wasps were found.
2Sampled in July 2013.
3Sampled in October 2013.
kairomones from their hosts (Metzger et al. 2010b). Traps baited with extracts from these semiochemicals were designed to attract and capture males. A trap consisted of a 125 mm × 200 mm yellow sticky sheet, in the center of which was hung a vial containing the extract. To prepare the extract, 100 g of host-rearing medium containing larvae was immersed during 1 h in 200 mL of hexane. The solution was filtered, and one female was soaked in 100 µL for 3 h. The female was then removed, and the extract was stored at −20°C. A few hours before being used in the field, the extract was evaporated by heating the vial on a hot plate (this step was skipped for the 2013 campaign, after it was shown that evaporation occurred within a few hours in the field). As for females, traps were hung within host trees, and all V. canescens males that stuck on the traps were collected and preserved individually in 96% ethanol.

In parallel, we collected males in three captive (laboratory mass-reared) populations differing in their history and geographic origin (Table 1): (1) a 25–30-generation-old population founded with about 120 females from Nice, (2) a 10–15-generation-old population founded with about 100 females from Valence, and (3) a 15–20-generation-old population founded with 11 females from Israel. Hence, these captive populations experienced different intensities of founding effects, genetic drift, and inbreeding. The three populations were maintained with a standard protocol on larvae of the host E. kuehniella obtained by rearing 50 mg of E. kuehniella eggs (about 2000 eggs) provided by Biotop (Livron-sur-Drôme, France) in plastic boxes (8 × 12 × 25 cm) set with 250 g of wheat semolina. Each week, three boxes containing second to fifth instar host larvae were inoculated with 50 males and 50 females of V. canescens emerging from all available (about six) rearing boxes.

Development of microsatellite genetic markers

Using a DNeasy Tissue Kit from Qiagen (QIAGEN, Hilden, Germany), DNA was extracted from a single 1.5-mL tube containing a pool of 20 adult females of V. canescens recently collected from the Nice population and kept in the laboratory for a few generations. The obtained DNA solution was enriched in microsatellites and pyrosequenced by the company Genoscreen (Lille, France), following the protocol described in Malausa et al. (2011). Using the iQDD program (Meglacz et al. 2010), primer pairs were designed for 675 microsatellite loci (Malausa et al. 2011), among which 124 were screened in monoplex PCR on DNA of one thelytokous and six arrhenotokous V. canescens females from three populations: Valence, Nice, and Antibes (geographic coordinates: 43°33’51”N, 7°0’28”E). Six primer pairs designed by Mateo Leach et al. (2012) were also tested following the same protocol.

The DNA extracts used for these monoplex PCR were obtained using the DNeasy Tissue Kit of QIAGEN. Each monoplex PCR contained 2 µL of DNA, 5 µL of 2X QIAGEN Multiplex PCR Master Mix, and 0.2 µmol/L of each primer. The total volume was adjusted to 10 µL with ultrapure water. PCR was performed as follows: a step of denaturation at 95°C during 15 min, followed by 25 cycles of 30 sec of denaturation at 94°C, 90 sec of annealing at 58°C and 60 sec of extension at 72°C, and a final extension step of 30 min at 60°C. PCR products were separated on a 2% agarose gel stained with ethidium bromide to detect amplification of DNA fragments. For 37 successfully amplified microsatellite loci, forward primers labeled with one of four fluorochromes (Applied Biosystems, Carlsbad, CA) were used in a monoplex PCR with the DNA samples and conditions previously described. Two microliters of PCR product was added to 8.75 µL of Hi-Di formamide and 0.25 µL of GeneScan500 Liz size standard (Applied Biosystems). The mix was loaded on an ABI 3130XL genetic analyzer (Applied Biosystems), and alleles were scored using Gene Marker® version 1.75 (SoftGenetics, State College, PA). Monomorphic markers were discarded, and the remaining markers were combined according to their size and fluorochrome in several multiplex PCRs that were tested on the same seven individuals and in the same PCR conditions as previously.

Figure 1. Location of field sampling. Cre, Cyp, Goz, Mlc, Nice, Sol, Val, VS, and Vy are respectively acronyms for Crete, Cyprus, Gozo, Mallorca, Nice, Sollies, Valence, Vila-Seca, and Vinyols. As no wasps were found in Porquerolles, Corsica, and Sicily, these locations are not presented here.
described. Finally, we selected 19 microsatellite loci amplified using two multiplex PCRs (Table 2).

**Assessment of male ploidy**

We used the genetic markers from the multiplex PCR I (Table 2) to detect diploid males: If a male is found heterozygous for at least one locus, it is considered diploid (Zhou et al. 2006; Armitage et al. 2010; Souza et al. 2010). However, if a diploid male is homozygous for all loci, it will be falsely scored haploid. To estimate the power of the developed microsatellite markers to correctly assess male ploidy, we calculated the probability that a diploid male is homozygous at all the 10 loci of the multiplex and, hence, falsely considered haploid. For this, we first calculated the probability that a diploid male produced by a brother–sister pair is homozygous for all 10 loci of the multiplex PCR I (see Annex). In parallel, we compared ploidy assessed on the same individuals via microsatellite genotyping and flow cytometry. Measure of male ploidy by flow cytometry is based on DNA quantity in cell nuclei (haploid nuclei being expected to have twice less DNA than diploid nuclei). To produce enough diploid males for analysis, brother–sister pairs were formed with individuals from a captive population. From the offspring, 39 males were collected and killed: The thorax was used for genetic analyses and the head for flow cytometry.

We performed flow cytometry analyses as described in de Boer et al. (2007). Only the head of insects was used for flow cytometry because endoreduplication doubles the quantity of DNA in cells of other parts of the body of haploid males, making haploid and diploid nuclei indistinguishable (Aron et al. 2005). To isolate cell nuclei, each insect head was crushed in 0.5 mL of Galbraith buffer by turning the pestle 20 times in a Dounce tissue grinder maintained on ice. The homogenate was filtered through a 40-μm cell strainer cap. Cell nuclei were stained with 20 μL propidium iodide at 1 mg/mL. Nuclei were analyzed on a LSRII Fortessa flow cytometer (BD Biosciences, San Jose, CA) with an excitation wavelength of 561 nm. Using FACSDiva Version 6.1.3 (BD Biosciences), we analyzed 10,000 nuclei per sample in a region that excluded doublets and debris. Flow cytometric DNA histograms of diploid females were used as reference to identify diploid males. The ploidy of each captured male was deduced from its genotype at microsatellite markers from multiplex PCR I. A male was considered diploid if it was heterozygous at least at one locus.

**Population genetics**

**Validation of microsatellites on female wasps**

To be used in population genetic studies, each marker must have several detectable alleles and must not be linked with any other marker or locus under selection. We therefore measured genetic polymorphism, estimated frequencies of null alleles, and test for departures from Hardy–Weinberg (HW) equilibrium and linkage disequilibrium on the two mainland populations from Valence and Nice where enough females were captured in 2010 (Table 1). DNA of each captured female was extracted with the PrepGem Insects kit (ZyGEM, Hamilton, New Zealand). DNA extraction, PCR, and genotyping were performed as previously described. The GENEPOP software version 4.3 (Rouset 2008) was used to calculate the number of alleles, the expected and observed heterozygosity, and to test for HW equilibrium and linkage disequilibrium between loci. We estimated null allele frequencies with the FreeNA program (Chapuis and Estoup 2007).

**Population structure and male ploidy**

For all males captured, DNA was extracted, PCR were performed with multiplex I, and genotypes were analyzed as previously described. Only males with at least eight loci genotyped were used for the analysis. Coexistence of male haploidy and diploidy impedes the estimation of classical population genetic statistics such as observed and expected heterozygosity or Wright’s F statistics. We therefore computed a value of allelic richness per locus and per population with FSTAT version 2.9.3.2 (Goudet 1995) and calculated the number of private alleles in each population (i.e., the number of alleles specific to a population), because allelic richness can be standardized independently of sample size, and both measures are independent of heterozygosity. The allelic richness was computed as in El Mousadik and Petit (1996), that is, a mean number of alleles for each locus in each population, with populations weighted in inverse proportion to their sample sizes in order to give the same weight to all populations. As FSTAT is not able to handle haploid and diploid data in the same analysis, we merged the haploid data from pairs of males to create “false diploids” without changing allelic frequencies. We then analyzed all “diploids” in a single run.

**Statistical analysis**

We used a hierarchical generalized linear model (binomial error and logit link) to assess the effect of population structure on the proportion of diploid males (DMP). Population structure was characterized by different nested explanatory variables: Allelic richness (All_rich) and number of private alleles (Priv_all) nested in the habitat type (Habit), that is, mainland, island, or captive (see Table 1). Data were all analyzed with the statistical software R (version 3.2.2, R Core Team, 2015). The significance of terms in the statistical model was assessed with type II sum-of-
Table 2. Characteristics of two multiplex PCRs amplifying 19 microsatellite loci in *Venturia canescens*.

| Multiplex | Locus  | Repeat motif          | F-Primer  | R-Primer  | F 5' label | Concentration (μmol/L) | Size range (bp) | GenBank no. |
|-----------|--------|-----------------------|-----------|-----------|------------|------------------------|----------------|-------------|
| I         | VC068  | (GA)<sup>9</sup>      | TATCCTTCAGCATTCCGTCCTC | CTCGCTCGGTGAACACTAC | FAM | 0.1 | 104–120 | KX258853 |
|           | Vcan071| (CAA)<sub>11</sub>    | TCTCTACCCACCTCCCTTCAC | TGTACGGTGCACCTTGAGC | FAM | 0.1 | 223–254 | GU053679 |
|           | VC092  | (AG)<sup>9</sup>      | TGTTGGCTCTGTGTAAGGT | CTCGTCGAATGTTGACGT | FAM | 0.1 | 284–307 | KX258854 |
|           | VC094  | (G)<sup>118</sup>     | TCTAGTGGCTGAACTCTCTG | CATATTTTCCCTTGGACAC | FAM | 0.2 | 429–436 | KX258855 |
|           | VC009  | (CAA)<sub>16</sub>    | AACAAGCAACAGCAACAGGAG | ACTTGCGAACGTGATTTC | VIC | 0.1 | 313–337 | KX258847 |
|           | VC036  | (TTG)<sub>12</sub>    | GTCCAGGCTACACAGGCACG | GTACGCTCCTTATCTGCAG | VIC | 0.1 | 226–254 | KX258849 |
|           | VC002  | (AG)<sub>11</sub>     | TCTGGTTTCTGCTGATTAATCCA | ATGATTGCTGACCTGCCGC | NED | 0.4 | 324–342 | KX258845 |
|           | VC001  | (AG)<sub>10</sub>     | TTTGGCGCGATTTGCTGAAAG | ACAGAAACGAAATTTGACAATC | NED | 0.4 | 391–447 | KX258844 |
|           | VC066  | (CAA)<sub>7</sub>     | AACAATGCAACTGCAATCGAA | TCCTTGAGCTCACAATTAC | PET | 0.2 | 87–90 | KX258852 |
|           | VC060  | (CT)<sup>12</sup>     | TATCTGGCTCTATTCGAGGA | AGGCGCTGAATGGAAAGTTA | PET | 0.2 | 206–231 | KX258851 |
| II        | VC106  | (AG)<sub>11</sub>     | CAAGCAGTATGATGAGCCGTG | CTGGAATCTTTCGCGGTTGGC | FAM | 0.2 | 89–97 | KX258856 |
|           | Vcan073| (TG)<sub>15</sub>     | GGGTCCACGGTACTTCCGTA | ACTGGCGATGCTACCTTGGA | FAM | 0.2 | 227–267 | GU053681 |
|           | VC047  | (AG)<sub>11</sub>     | ACCATGAGGCGACTATTTGCCTT | CGGAAGTTAATTTCTAGCAGGAC | VIC | 0.2 | 141–157 | KX258850 |
|           | VC031  | (TC)<sub>11</sub>     | TCGATGCAATCTGAGTCCGTTGAAGA | GGGTTGCTAAATAGCGAGGAGG | VIC | 0.2 | 227–261 | KX258848 |
|           | VC006  | (AG)<sub>8</sub>      | GACTAATGGGAGAGGAGGTGTCG | GCCACGTTTATGTTGCAGGAGC | VIC | 0.2 | 317–357 | KX258846 |
|           | VC120  | CCCCTT<sup>(CT)<sub>2</sub></sup> | CAATCGATCACGATACATTCG | GCAAGGTTAGCGCTTAGTG | VIC | 0.2 | 92–128 | KX258858 |
| Vcan106   | (TC)<sup>24</sup> | CTCATCTGAGGGAGGAGGATT | ATC GGCGAGTTGCTGGATTTT | NED | 0.2 | 183–222 | GU053714 |
| VC107     | (CAG)<sub>13</sub>(CAA)<sub>12</sub> | AAACACTGACACAAACACCA | CACTGGCAATATGCTGTGCG | PET | 0.2 | 85–108 | KX258857 |
| Vcan088   | (CAA)<sub>4</sub> | AGTAACCCTCGCTGCCATTTG | CGACTCCACATTTCCACACA | PET | 0.2 | 133–150 | GU053696 |

All the microsatellites were developed in this study, except Vcan071, Vcan073, Vcan106, and Vcan088 that come from Mateo Leach et al. (2012).
squares tested with analyses of deviance with the likelihood ratio test based on the \( \chi^2 \) distribution (\textit{car} package (Fox and Weisberg 2010)). We used nonparametric analysis to test for population differentiation (Kruskal–Wallis test on allelic richness and the number of private alleles). Due to the low sample size (11 when using populations and three when testing the effect of habitat types) and therefore the lack of statistical power, we estimated the effect size by computing the Cohen’s \( d \) (Cohen 1977; Nakagawa and Cuthill 2007) with \textit{effsize} library in R (Torchiano 2015) and used the threshold provided in Cohen (1992) to assess the magnitude of the effect, that is, \(|d| < 0.2\): negligible, \(|d| < 0.5\): small, \(|d| < 0.8\): medium, otherwise large.

In all multiple analyses such as tests for HW equilibrium, \( P \)-values were corrected for multiple testing via the false discovery rate (FDR) procedure (Benjamini and Hochberg 1995). Means are thereafter presented with standard errors unless indicated otherwise.

**Results**

**Insect sampling and microsatellite markers**

A total of 108 females were collected between 2010 and 2013, and 627 males were collected in 11 populations (Table 1 and Fig. 1). No males were found in the French, Italian, Greek, Malta, and Cyprus islands, and as only one to three females were sampled in these locations, they were not used for the microsatellite development. Therefore, we used 75 females from Nice and Valence (2010) for the development of genetic markers.

**Validation of microsatellite markers for population genetic studies**

For all the nineteen microsatellites developed, amplification was obtained for all the arrhenotokous \textit{V. canescens} females trapped in Nice (Nice10 population) and in Valence (Val10 populations). The number of alleles ranged from 2 to 14 (mean number of alleles \( \mu = 7.95 \pm 0.76 \), Table 3). The two multiplex PCRs displayed a similar range (multiplex I: 2–12 alleles, multiplex II: 4–14 alleles) and mean number of alleles (multiplex I: 7.3 \pm 0.92, multiplex II: 8.67 \pm 1.25, \( t \)-test: \( t = -0.88, df = 15, P = 0.39 \)). The markers had a maximum frequency of null alleles equal to 8.5% (Vcan073 locus, Val10 population; mean: 1.7 \pm 0.6% in Nice10 population and 1.8 \pm 0.7% in Val10 population) and a null median frequency for both populations. No departure from the HW equilibrium was found in any of the populations.

### Table 3. Genetic diversity at 19 microsatellite loci for natural populations of Venturia canescens sampled in 2010 near Nice and Valence, southeast of France.

| Population | Nice10 (n = 44) | Val10 (n = 37) |
|------------|----------------|---------------|
| **Multiplex** | **Primer name** | Number of alleles | \( H_e \) | \( H_o \) | HW \( P \)-value | Null allele frequency | \( H_e \) | \( H_o \) | HW \( P \)-value | Null allele frequency |
| I | VC068 | 7 | 0.742 | 0.727 | 0.848 | 0.012 | 0.826 | 0.865 | 0.771 | 0.000 |
| | Vcan071 | 11 | 0.809 | 0.750 | 0.493 | 0.036 | 0.798 | 0.838 | 0.728 | 0.000 |
| | VC092 | 7 | 0.636 | 0.705 | 0.390 | 0.000 | 0.600 | 0.676 | 1.000 | 0.000 |
| | VC094 | 4 | 0.592 | 0.545 | 0.771 | 0.034 | 0.511 | 0.541 | 0.813 | 0.000 |
| | VC009 | 8 | 0.697 | 0.864 | 0.135 | 0.000 | 0.649 | 0.703 | 1.000 | 0.000 |
| | VC036 | 7 | 0.540 | 0.568 | 0.771 | 0.000 | 0.450 | 0.432 | 0.771 | 0.000 |
| | VC002 | 7 | 0.577 | 0.591 | 0.813 | 0.026 | 0.716 | 0.757 | 0.771 | 0.000 |
| | VC001 | 12 | 0.600 | 0.477 | 0.370 | 0.090 | 0.655 | 0.595 | 0.176 | 0.046 |
| | VC066 | 2 | 0.487 | 0.386 | 0.509 | 0.069 | 0.491 | 0.486 | 1.000 | 0.003 |
| | VC060 | 8 | 0.549 | 0.591 | 0.927 | 0.000 | 0.558 | 0.568 | 0.135 | 0.011 |
| II | VC106 | 4 | 0.711 | 0.750 | 0.976 | 0.000 | 0.728 | 0.676 | 0.307 | 0.019 |
| | Vcan73 | 10 | 0.672 | 0.818 | 0.746 | 0.000 | 0.706 | 0.541 | 0.135 | 0.092 |
| | Vcan04 | 5 | 0.573 | 0.636 | 0.821 | 0.000 | 0.607 | 0.541 | 0.396 | 0.013 |
| | Vcan01 | 14 | 0.650 | 0.591 | 0.390 | 0.048 | 0.674 | 0.595 | 0.493 | 0.083 |
| | Vcan06 | 11 | 0.673 | 0.773 | 0.813 | 0.000 | 0.593 | 0.595 | 0.771 | 0.000 |
| | VC120 | 7 | 0.515 | 0.500 | 0.771 | 0.000 | 0.593 | 0.459 | 0.135 | 0.059 |
| | Vcan106 | 14 | 0.875 | 0.886 | 0.400 | 0.008 | 0.836 | 0.919 | 0.746 | 0.000 |
| | VC107 | 6 | 0.724 | 0.705 | 0.493 | 0.000 | 0.603 | 0.622 | 0.479 | 0.022 |
| | Vcan088 | 7 | 0.644 | 0.682 | 0.813 | 0.009 | 0.489 | 0.541 | 0.821 | 0.000 |

*\( n \): number of females analyzed; \( H_e \): expected heterozygosity; \( H_o \): observed heterozygosity; HW \( P \)-value: \( P \)-value of the test for Hardy–Weinberg equilibrium after FDR correction.*

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(Table 3), and the observed heterozygosity ($H_o$) was similar in the two multiplex PCRs in both populations (Mean $H_o$ multiplex I: 0.63 ± 0.03, multiplex II: 0.66 ± 0.03; $t$-test: $t = -0.54$, df = 36, $P = 0.59$; Table 3). Two pairs of microsatellites were detected as linked when both populations were considered: Vcan073/Vcan106 ($\chi^2 = \infty$, df = 4, $P < 0.0001$) and VC107/Vcan106 ($\chi^2 = 24.2$, df = 4, $P = 0.0063$). However, when populations were analyzed separately, the pairs of loci were linked only in one population (Vcan073/Vcan106 in Val10 and VC107/Vcan106 in Nice 10), we therefore decided to keep all loci. Therefore, the two multiplex PCR can be used for genetic studies with equivalent performance.

**Validation of markers for ploidy assessment**

The probability that a diploid male produced by sibmating is homozygous for all microsatellite loci of the multiplex I was low enough ($P = 0.0023$) to rely on microsatellites for ploidy assessment. This was confirmed by the congruence between flow cytometry and microsatellite genotyping results. Haploid and diploid males were easily discriminated by flow cytometry, with diploid males presenting a profile similar to that of diploid females (Fig. 2). For the 39 males tested, the ploidy measured by flow cytometry and genotyping analyses matched perfectly.

**Population genetic structure and proportion of diploid males**

After genotyping with the markers of multiplex I, we discarded 28 males for which more than one loci did not amplify. As a result, 599 males (95.5%) were successfully genotyped and used in the analysis.

**Population differentiation**

The population structure was estimated through different parameters such as allelic richness (All_Rich) and the number of private alleles (Priv_all) from male genotyping data (Table 4). The number of private alleles was marginally significantly different between habitat types (mainland, island, and captive, Kruskal–Wallis test, $\chi^2 = 5.68$, df = 2, $P = 0.058$), and allelic richness significantly differed between habitat types (Kruskal–Wallis test, $\chi^2 = 6.72$, df = 2, $P = 0.035$) and populations (Kruskal–Wallis test, $\chi^2 = 18.51$, df = 10, $P = 0.047$). Both measures shared the same trend suggesting that the diversity is the lowest in captive populations and the highest in mainland (Table 4). Effect sizes were medium or large for each comparison for both variables (mainland–island, All_Rich: $d = 2.35$, Priv_all: $d = 1.09$; mainland–captive, All_Rich: $d = 1.56$, Priv_all: $d = 1.52$; island–captive, All_Rich: $d = 0.67$, Priv_all: $d = 1.41$). The sampling year had no impact on the mean allelic richness for populations sampled over 2 years ($t$-test, Nice11–Nice13: $t = -0.247$, df = 15.9, $P = 0.808$ and Mlc12–Mlc13: $t = -0.124$, df = 15.9, $P = 0.903$). Allelic richness of the captive population from Israel (CapIsr) appeared different
from all the other populations except the captive populations from Nice and Valence (CapNiceB and CapVal, pairwise t-tests between populations with FDR correction for multiple testing, data not shown). As the CapIsr population was highly differentiated from the other populations, it could potentially drive the significant trend observed. Indeed, when allelic richness was compared without CapIsr, the difference between populations disappeared (Kruskal–Wallis test, $\chi^2 = 4.745$, $df = 9$, $P = 0.856$), and the difference between habitat types became only marginally significant (Kruskal–Wallis test, $\chi^2 = 5.804$, $df = 2$, $P = 0.055$).

### Diploid male proportion and genetic diversity

Diploid males were found in all populations, but in variable proportions (from DMP = 0.02 ± 0.02 in CapVal to DMP = 0.16 ± 0.05 in CapIsr; Table 4 and Fig. 3). The sampling year had no impact on the proportion of diploid males for populations sampled over 2 years (proportion test, Nice11–Nice13: $\chi^2 = 0.760$, $df = 1$, $P = 0.383$ and Mlc12–Mlc13: $\chi^2 = 5.10^{–32}$, $df = 1$, $P = 1$). As expected, the DMP was affected by habitat type (LR $\chi^2 = 7.07$, $df = 2$, $P = 0.029$, Table 5), with a negative relationship between the DMP and the allelic richness of each habitat types (Fig. 4). When removing the CapIsr population, the effect of the habitat type became non-significant ($P = 0.105$), but we still found a negative influence of allelic richness (nested within habitat types) on DMP (LR $\chi^2 = 6.80$, $df = 2$, $P = 0.033$, Table 5).

#### Discussion

We developed highly variable microsatellite markers for the parasitoid wasp *V. canescens*. Polymorphism, HW equilibrium, the absence of linkage disequilibrium, and negligible null allele frequencies make these markers suitable for population genetic studies. In addition, relatively high rates of heterozygosity enable a reliable measure of male ploidy, confirmed by flow cytometry. Using these markers, we estimated within-population genetic diversity and the proportion of diploid males in eleven locations.
around the Mediterranean Sea, as well as in captive populations. We found a tendency for genetic diversity to be reduced in small and isolated populations, and the proportion of diploid males to increase with decreasing genetic diversity. These patterns are expected theoretically in a scenario whereby reduced population size yields decreased genetic diversity (and decreased allelic polymorphism at the sl-CSD locus), increased proportion of diploid males, decreased population growth rate, and, if dramatic enough, population extinction (a scenario referred to as the diploid male vortex; Zayed and Packer 2005). Hence, our results suggest that habitat type and population history are key features that should be considered when studying genetic diversity in parasitoids with sl-CSD.

Table 5. Effect of habitat type and genetic diversity on diploid male proportion.

| Response variable | Diploid male proportion |
|-------------------|-------------------------|
|                   | LR $\chi^2$ | df | $P$ |
| With CapIsr population |            |    |     |
| Habit             | 7.0710       | 2  | 0.029 |
| Habit:All_rich    | 2.4942       | 2  | 0.287 |
| Habit:Priv_all    | 0.5125       | 1  | 0.474 |
| Without CapIsr population |        |    |     |
| Habit             | 4.5108       | 2  | 0.104 |
| Habit:All_rich    | 6.7997       | 2  | 0.033 |
| Habit:Priv_all    | 0.5125       | 1  | 0.474 |

LR, Likelihood ratio; df, degrees of freedom; Habit, habitat type; All_rich, allelic richness; Priv_all, private alleles. $P < 0.05$ highlighted in bold.

The effect of habitat type on genetic diversity was marked by a lower diversity in captive populations. These bottlenecked and completely isolated populations were characterized by a lower allelic richness compared to either island or mainland populations, and a lower number of private alleles compared to mainland populations (Table 3). Because the number of private alleles is highly dependent on sampling effort, the effect of habitat type on the number of private alleles could simply result from the high number of individuals captured in the mainland population of Nice. Besides, the captive population from Israel was highly differentiated from all populations as a result of a very small foundress number (11 females). This population had the lowest allelic richness and mean number of alleles, and hence, it is likely that these extreme values had driven the effect of habitat type. Indeed, the population effect disappeared when discarding the population from Israel. Nonetheless, the effect of habitat type remained marginally significant, suggesting that the trend of higher allelic richness in mainland compared to island and captive populations is indeed real.

The decrease in genetic diversity co-occurring with higher rates of inbreeding in small and isolated populations, such as island populations, has been documented in various species (mammals, birds, fishes, insect or plants, Frankham 1997; Furlan et al. 2012). In V. canescens, the higher genetic diversity in mainland compared to captive populations could be explained by efficient dispersal. In this species, adults are good dispersers (Desouhant et al. 2003), a feature that corroborates the absence of genetic structure at the scale of southeastern France (Schneider et al. 2002). In island and captive populations, founder effect and genetic drift combined with constrained dispersal should lead to genetic erosion, a phenomenon that is also observed in other species of the order Hymenoptera such as bumblebees or orchid bees (Ellis et al. 2006; Schmid-Hempel et al. 2007; Boff et al. 2014).

Consistent with these effects of habitat type on diversity at microsatellite loci, the proportion of diploid males was affected by the habitat type with more frequent diploid males in island and captive populations than in mainland locations. The highest proportion (16%) was observed in the captive population from Israel, which is congruent with the lowest genetic diversity estimated. In other species, diploid male production was also found to be higher in isolated population compared to larger genetically diverse populations (Kukuk and May 1990). Overall, the proportion of diploid males in V. canescens (6.8% overall, CI 95% [3.0–9.2%]) is in the order of magnitude of estimations in other species of parasitoids with sl-CSD: 10% in a native population of C. glomerata (Ruf et al. 2013) and 15% in a population of C. rubecula introduced for biological control (de Boer et al. 2012).
It is however important to notice that despite a high sampling effort in several islands, we caught males only on one island (Mallorca), for two consecutive years. It is therefore possible that the trend of lower genetic diversity and higher proportion of diploid males compared to mainland populations are due to a peculiar composition specific to Mallorca population, and rather than an effect of the habitat type “island” and its isolation. The habitat isolation effect is nonetheless corroborated by the genetic erosion observed in captive populations, and thus likely to be real, but would require new samplings in other islands to be confirmed.

Behaviors such as natal dispersal and mate choice can mitigate the effects of decreased genetic diversity and hence attenuate diploid male production. In C. glomerata, a biased fertilization occurs and limits genetic incompatibility, leading to a DMP lower than expected in field populations (Ruf et al. 2013). On the contrary, the lack of mate discrimination in C. rubecula could explain the relatively high DMP observed in its field populations (de Boer et al. 2012). Laboratory studies in Braccon brevicornis have reported an avoidance of mating with a partner harboring the same allele at the sl-CSD locus (i.e., avoidance of matched matings; Thiel et al. 2013), and sib-mating avoidance was observed in V. canescens (Metzger et al. 2010a). Such behaviors should reduce the DMP and thus lessen the production of unfit offspring (Parker 1983; Chuine et al. 2015).

The higher proportion of diploid males in captive and island populations of V. canescens could result from a lower genetic diversity in isolated populations, or a down-grade in discrimination of females against related males. In a number of species, females adapt future mate choice according to the genotype of their first mate [lizards (Laloi et al. 2011; Breedveld and Fitze 2015); beetles (Dowling et al. 2007); Drosophila (Chapman and Partridge 1998)]. This hypothesis may not hold for V. canescens because females are monoandrous (Metzger et al. 2010b). However, as island or captive populations are genetically eroded, the probability for a female to encounter a male bearing a similar allele at the csd locus is higher; possibly, successive encounters with low-quality males before mating could lead to an adaptive decline in sib-avoidance. This could explain the increase in diploid male production in small or isolated populations. At last, the presence of diploid males even in mainland populations where the genetic diversity is high and where no departure from HW equilibrium was detected could also be due to imperfect sib-mating avoidance, as observed under the laboratory conditions (Metzger et al. 2010a).

Our study suggests that diploid male production in insects of the order Hymenoptera reflects fitness decline resulting from inbreeding in small and isolated habitats. Sex determination via sl-CSD imposes a major genetic constraint and may reflect an “unintelligent design” (sensu van Wilgenburg et al. 2006), which makes some hymenopterans highly sensitive to isolation. Hence, our study raises the question of population conservation (Zayed 2009) and highlights that small organisms such as insects may also suffer from habitat destruction and fragmentation.

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Conflict of Interest

None declared.

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Annex:

Calculation of the probability that a F2 male, produced by sibmating, is homozygous for all 10 markers from multiplex I.

A sample of 30 females was collected in a three- to five-generation-old captive population (founded in autumn 2009 with about 40 females from Nice). These females constitute the F0 generation and had presumably mated randomly in the population. Their offspring (generation F1) were constrained to mate between siblings (Fig. A1). We computed the probability that an F2 male, produced via sibmating, is homozygous for all genetic markers, using allelic frequencies and inbreeding coefficient \( F_{is} \) of F0 females.

We first calculated the probability that an F2 diploid individual is homozygous at one microsatellite locus, given the genotypes of its grandparents (F0 generation) at this locus (Fig. A1). Then, for each locus \( k \), we calculated the probability that an F0 female is heterozygous at this locus as \( P_{k}(\text{HetF0}) = 1 - F_{is} \times H_e \) and the probability that she is homozygous as \( P_{k}(\text{HomF0}) = 1 - P_{k}(\text{HetF0}) \), where \( H_e \) is expected heterozygosity at loci \( k \) under HW equilibrium.

We calculated as follows the conditional probabilities that the F0 male carries at loci \( k \) a different (DA) or common (CA) allele with the F0 female, knowing that the female is heterozygous (HetF0) or homozygous (HomF0):

\[
P_k(\text{CA|HetF0}) = \frac{\sum_{i=1}^{n} \sum_{j=1}^{n} \left( \frac{2p_i p_j}{H_e} \right) (p_i + p_j)}{1 - H_e} \]

\[
P_k(\text{DA|HetF0}) = 1 - P_k(\text{CA|HetF0})
\]

\[
P_k(\text{CA|HomF0}) = \sum_{i=1}^{n} \left( \frac{p_i^2}{1 - H_e} \right) p_i
\]

\[
P_k(\text{DA|HomF0}) = 1 - P_k(\text{CA|HomF0})
\]

\( p_i \) and \( p_j \) are the frequencies of alleles \( i \) and \( j \), respectively. Using the proportions of F2 homozygous from Figure A1, the probability that an F2 diploid is homozygous as locus \( k \) was as follows:

\[
P_k(\text{HomF2}) = P_k(\text{HetF0})(0.5P_k(\text{CA|HetF0}) + 0.25P_k(\text{DA|HetF0})) + P_k(\text{HomF0})(P_k(\text{CA|HomF0}) + 0.5P_k(\text{DA|HomF0}))
\]

The probability that a diploid F2 is homozygous at all 10 loci from multiplex I was as follows:

\[
P(\text{HomF2}) = \prod_{k=1}^{10} P_k(\text{HomF2})
\]

Figure A1. Proportion of heterozygous diploid F2 at one microsatellite locus, according to the genotype of the F0 pair.