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SINGLE-LOCUS COMPLEMENTARY SEX DETERMINATION IN THE ICHNEUMONID *VENTURIA CANESCENS* (GRAVENHORST) (HYMENOPTERA)

by

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ABSTRACT

Some Hymenoptera have single locus Complementary Sex Determination (sl-CSD); heterozygous individuals are female (diploid fertilised eggs) and hemizygous individuals are male (haploid unfertilised eggs). Through inbreeding homozygous diploids can arise which develop into males that are often sterile and sometimes inviable. The phylogenetic distribution of sl-CSD is unclear. In this study, we used inbred crosses to demonstrate that the parasitoid wasp *Venturia canescens* (Gravenhorst) has sl-CSD. Diploid males were detected through heterozygosity at a marker locus (Virus Like Particle protein). They were fully viable at 20°, 25° and 30°C as the sex ratios of inbred crosses did not deviate from controls after adjustment for diploid male frequency. These results further confirm the existence of sl-CSD in the family Ichneumonidae.

KEY WORDS: diploid males, sex determination, single locus, thelytoky, *Venturia canescens*, *Wolbachia*.

INTRODUCTION

All Hymenoptera have haplodiploid sex determination. The most common mode of reproduction is arrhenotoky in which males develop from unfertilised eggs and are haploid, whereas females develop from fertilised eggs and are diploid. The other mode of reproduction is thelytoky, where females develop from unfertilised diploid eggs and there are no males. Thelytoky has apparently arisen multiple times from arrhenotoky, given its patchy taxonomic distribution (WHITE, 1973; BULL, 1983; COOK, 1993).

Sex determination under haplodiploidy takes place without heteromorphic sex chromosomes. The only difference between males and females is the copy number of each chromosome, *i.e.* males have one and females two copies. WHITING (1939, 1943) discovered that sex determination

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in the parasitoid wasp *Bracon hebetor* (Braconidae) depends on the allelic composition at a single locus. Hemizygous haploid eggs develop into males and diploid eggs develop into females when heterozygous, but into diploid males when homozygous at the sex locus. Such diploid males are of biparental origin and can result from inbreeding. They are typically sterile (STOUTHAMER *et al.*, 1992; AGOZE *et al.*, 1994) and sometimes have reduced viability (WHITING, 1943; CROZIER, 1977; PETTERS & METTUS, 1980; COOK & CROZIER, 1995).

Single locus complementary sex determination (sl-CSD) has now been demonstrated in over 40 species of Hymenoptera, including sawflies (Symphyta), parasitoid wasps (Apocrita; Parasitica), ants, bees and wasps (Aprocrita; Aculeata) (STOUTHAMER *et al.*, 1992; COOK, 1993a; PERIQUET *et al.*, 1993; COOK & CROZIER, 1995; BUTCHER *et al.*, 2000a). The widespread occurrence of sl-CSD in species belonging to each major subgroup of Hymenoptera suggests that CSD is ancestral in the Hymenoptera (SCHMIEDER & WHITING, 1947; CROZIER, 1977; COOK, 1993a). However, a number of hymenopteran species clearly have a different mode of sex determination. Many species can be (in)bred in the laboratory for many generations without any problems of diploid males. Controlled experiments of prolonged inbreeding in *Nasonia vitripennis* (Chalcidoidea) (SKINNER & WERREN, 1980) and *Goniozus nephanditis* (Chrysidoidea) (COOK, 1993b) does not lead to diploid males. Moreover, BEUKEBOOM *et al.* (2000) refuted sl-CSD for two Alysiinae species (Braconidae). STOUTHAMER & KAZMER (1994) provided ultimate proof for the absence of any form of complementary sex determination in *Trichogramma*. In this thelytokous wasp gamete duplication leads to complete homozygosity which would lead to all-male production under CSD rather than to all-female. The phylogenetic distribution of CSD is currently unclear and more species need to be tested for a better understanding of the evolution of sex determining mechanisms in Hymenoptera.

Venturia canescens, is a solitary endoparasitoid of pyralid moths (BELING, 1932; VOINOVSKAYA-KRIGER, 1927; SALT, 1976). It belongs to the subfamily Campopleginae of the family Ichneumonidea which contains a very large number of species. Thusfar, all species investigated in this family were shown to have sl-CSD; *Diadromus pulchellus* (PERIQUET *et al.*, 1993) and 7 species of *Diadegma* (BUTCHER *et al.*, 2000a, b). *V. canescens* has been used for many years in various laboratory studies (e.g. AHMAD, 1936; SIMMONDS, 1943; CORBET & ROTHERAM, 1965; ROGERS, 1972; SALT, 1975; HARVEY *et al.*, 1993; DRIESSEN *et al.*, 1995; HELLERS *et al.*, 1996; MARRIS *et al.*, 1996). It is of particular interest to test for sl-CSD because both arrhenotokous and thelytokous females occur in nature (BEUKEBOOM *et al.*, 1999). Certain forms of thelytoky lead to a rapid increase of homozygosity (SUOMALAINEN *et al.*,

1987) and are therefore believed to be incompatible with sl-CSD because homozygosity at the sex locus will result in diploid males rather than females.

The objective of this study was to test whether arrhenotokous *V. canescens* have sl-CSD. Data are presented of inbred crosses in two arrhenotokous strains carrying different alleles of a genetic marker. Experiments were performed at three different temperatures, because the occurrence of diploid males might be temperature dependent. Survival of diploid males may be affected by temperature (WHITING & ANDERSON, 1932) and the expression of sl-CSD may be temperature sensitive (BUTCHER *et al.*, 1998; STOUTHAMER, pers. comm.).

MATERIAL AND METHODS

Strains

Two arrhenotokous strains, Valbonne and Antibes, were used that had been collected in southern France in 1999 (approximately 20 km apart). Populations of adult wasps were kept in plexiglass population cages at 25°C, 16L:8D and provided with water and honey. They were bred on *Ephesia kuehniella* larvae that were cultured on a medium of semolina, flour and some yeast.

Detection of diploid males

Sl-CSD can easily be demonstrated by mother-son crosses because all crosses are matched for the sex locus (*i.e.* two-allelic) and yield 50% diploid males among fertilised eggs (BEUKEBOOM *et al.*, 2000). Another method is brother-sister crosses, but only 50% of those will be matched and yield diploid males. The other 50% will be unmatched (*i.e.* three-allelic) and yield no diploid males (fig. 1). In this study, brother-sister rather than mother-son crosses were used because the latter are not easy to accomplish in *V. canescens*. Thus, in the inbred crosses a total of three sex alleles were present and on average 50% of these were expected to involve a shared allele (F_1 cross nos. 1 and 2 in figure 1) and 50% no shared alleles (F_1 cross nos. 3 and 4 in figure 1). Outbred control crosses between unrelated individuals also involved three sex alleles but these would never contain a shared allele. Further details of the inbred and control crosses are described below.

Two allelic variants of a Virus Like Particle (VLP p40) gene differing in absence ("minus") or presence ("plus") of a 54 bp repeated sequence (HELLERS *et al.*, 1996) were used to detect diploid males. DNA was extracted from wasp abdomen using the Nucleon BACC1 kit (Amersham)

Fig. 1. Inbred crosses to detect single-locus complementary sex determination (sl-CSD). Diploid males develop from homozygous diploid eggs and are detected with a genetic marker (VLP = Virus Like Particle p40 gene). Sex locus alleles are given by lower case letters and the two VLP alleles by "+" and "-". Crosses between homozygous "++" females and "-" males yield heterozygous F₁ females that are mated with one of their brothers. Brother-sister matings are either matched (two-allelic) or unmatched (three-allelic). Matched crosses (nos. 1 and 2) result in 50% diploid males among fertilised eggs, whereas unmatched crosses (nos. 3 and 4) do not result in diploid males. Overall, 50% of F₂ progenies contain diploid males. If the VLP and sex locus are linked, no diploid males become heterozygous "+-" and the overall proportion of diploid heterozygous males among fertilised eggs will be 1/8. Only F₂ progenies of the first and third possible brother-sister crosses are shown.

according to manufacturer's protocol. PCR reactions with p40 specific primers were performed according to the protocol described in HELLERS (1996) and products were separated on 1.5% agarose gels (see MALMBERG *et al.*, 2000).

In order to detect diploid males among progeny of inbred crosses, females that were heterozygous for the VLP marker were used. They were mated to either a VLP "minus" or "plus" brother. There are a number of possible outcomes. In the absence of sl-CSD, no diploid males will be present and brood sizes and sex ratios will not differ from non-inbred crosses. In the presence of sl-CSD, occurrence of diploid males will result

in broods with elevated proportions of males. Reduced survival of diploid males will be visible as smaller brood sizes of inbred crosses. Detection of diploid males with VLP alleles depends on whether the VLP locus is linked with the sex locus. Under complete linkage there will be no heterozygous diploid males (fig. 1). If there is no linkage, 50% of diploid males will be heterozygous and 50% homozygous for the VLP allele in matched crosses. In that case, the overall proportion of fertilised eggs that will become VLP heterozygous diploid males is 1/8 (fig. 1).

Crosses

Both experimental strains had been recently collected and maintained in large numbers (>50 individuals) prior to the experiment. A homozygous VLP “minus” line from Antibes and a VLP “plus” line from Valbonne were generated by VLP typing 30 stock females, which had mated in the population cage with an unknown male. Of those females that were found to be homozygous, two daughters were also analysed to check for the father’s genotype. A homozygous stock culture was subsequently founded from those homozygous females that had mated a male with the similar allele. Next, heterozygous females were obtained by group mating of homozygous Antibes “minus” females with Valbonne “plus” males and *vice versa* (fig. 1). These interstrain matings provided the individuals that were used in the inbred and control crosses (F_1 generation, see figure 1). They served to prevent accidental matching of sex alleles prior to the experiment. Again, the reason for mating females in groups in population cages was that single pairs often do not mate. All culturing was done at 25°C.

Females were individually provided with approximately 20 hosts in 100 ml glass bottles. For control crosses, virgin F_1 females were obtained from a subset of progenies by isolating all *Ephestia* pupae approximately one week prior to emergence of the wasps. They were placed in a 25 ml tube with a drop of honey on the foam plug. After emergence a number (usually 4-6) of males and female wasps from different progenies were put together in a cage for two days to mate. The other progenies were allowed to emerge within the bottles and subsequently transferred to 32 × 17 × 19 cm plexiglass cages for another two days during which mating between brothers and sisters could occur (= inbred crosses).

A possible drawback of this (group) design could have been that females mated more than once with different males. At the onset of the experiment, it was unknown whether multiple mating occurs in *V. canescens* cultures. However, the results of this study will be informative, at least if sl-CSD occurs; single matings will yield diploid males among 50% of fertilised eggs in 50% of all females, whereas multiple mating will

blur this distinction and result in <50% diploid males among fertilised eggs in >50% of all females (see figure 1).

A total of 96 inbred and 29 control females were given 30-40 hosts overnight in 100 ml glass bottles and a drop of honey on the lid. The next day they were removed from the bottles and kept at 20°C in 25 ml glass tubes closed with a foam plug containing a drop of honey. The following day they were given hosts again. The purpose of the one-day pauses without hosts was to re-adjust the wasps to room temperature (approximately 22°C) and to stimulate egg production. Each female was allowed to oviposit at three different temperatures, 30, 20 and 25°C respectively and this regime was repeated until death of the female with a maximum of nine oviposition bouts (*i.e.* three progenies at each of three temperatures). Females were divided into three cohorts that started the experiment at different moments in order to randomise oviposition temperatures in time. Cohort 2 started two days after cohort 1 at 20°C and cohort 3 four days later at 25°C.

After being parasitised by the wasps, hosts were kept at the same temperature until emergence; the resulting developmental times of the wasps were approximately 21, 28 and 35 days at 30, 25 and 20°C respectively. As in most insects (CHAPMAN, 1982), fertilisation in *V. canescens* takes place at oviposition when the egg passes through the oviduct, and the sex of the offspring is determined during early embryogenesis. Therefore, the temperatures at which sex determination occurred were the same as those at which oviposition took place. Numbers of males and females in each progeny were counted and males were frozen for subsequent DNA analysis. An exception formed a subset of 192 males that were individually placed in a cage with a virgin female of one of the stock populations that were homozygous for the VLP alleles. If *V. canescens* was to have sl-CSD and diploid males were surviving, a subset of these males was expected to be diploid. The purpose of this experiment was to determine whether diploid males were functional and fertile, and could produce (triploid) daughters. After two days, the females were hosted as described before and the males frozen for DNA analysis.

RESULTS

Among a total of 96 inbred and 29 control crosses, 67 (70%) respectively 18 (62%) yielded at least one daughter. Ten females did not produce any offspring. Thirty (25 inbred and 5 control) females produced only all-male progenies. They were considered unmated and discarded from the analysis. Among the control group, there was no effect of age on the

progeny sex ratio (Kruskal-Wallis test, $p = 0.538$, $n = 81$ progenies, 18 females, up to 7 oviposition bouts) allowing pooling of data per temperature treatment.

Table 1 lists numbers of males and females among 81 control and 255 inbred progenies respectively. Sex ratios within the control and inbred group did not differ between temperatures (χ^2 -tests, $p > 0.05$). Under sl-CSD, the expected number of diploid males is 1/3 of the number of females, because 25% of diploid eggs are expected to develop into diploid males (see figure 1). The inbred group sex ratios were significantly higher than the control at each temperature (χ^2 -tests, $p < 0.0001$). These results indicate that diploid males occur among the inbred progenies. If the inbred group sex ratios are adjusted for 25% diploid males among fertilised eggs, they do not differ from the control groups at any temperature (table 1b). These results indicate that diploid males are viable and occur at expected frequencies under sl-CSD.

In all crosses the parental females were heterozygous and the males hemizygous for the genetic marker (VLP p40 gene). Table 2 lists the allelic composition of a randomly chosen subset of males in progenies from control and inbred crosses. As expected, no heterozygous (diploid) males were found among 26 offspring of control crosses and the number of (haploid) individuals with the "plus" and "minus" allele were equal (table 2a). In contrast, heterozygous diploid males were found among the progenies of inbred crosses at all three temperatures (table 2b) and demonstrated the presence of sl-CSD.

At each temperature, diploid males occurred in approximately 50% of all progenies (no deviations from 50:50, χ^2 -tests, $p > 0.05$). These results indicated that the VLP locus is unlinked with the sex locus and that multiple mating was rare or absent, because multiple mating would have resulted in diploid males among >50% of progenies (see methods). In some cases, progenies of a single female were VLP-typed from different temperatures. For example V4f progenies were tested at all three temperatures. These did not always give consistent results, *i.e.* they were sometimes classified as having diploid males "absent" and sometimes as "present". This is most likely due to chance, *i.e.* variation in fertilisation proportions (most males were haploid) and small sample sizes.

A subset of 192 males was individually mated to a virgin female prior to VLP analysis in order to test their functionality. Unfortunately, only two males produced daughters among their progeny and both of these males were haploid. All others, including 16 diploid males, produced all-male progenies indicating that they had either not mated or were sterile. Thus, due to the failure of most males to mate in this experiment, it could not be determined whether diploid males were fertile.

TABLE 1

Progenies of control (a) and inbred crosses (b). Given are number of mothers, progenies, females, males and the sex ratio. For the inbred crosses are also given the expected number of diploid males, haploid males, females and the adjusted sex ratio. Adjusted sex ratios are compared to the controls in the last column. Note that one mother may have produced up to 3 progenies at each temperature (see methods).

(a)						
Treatment	Temperature	No. mothers	No. progenies	No. offspring	No. females	No. males
Control	20	16	29	364	193	171
	25	12	20	365	208	157
	30	18	32	451	215	236
	Total	18	81	1180	616	564
(b)						
Treatment	Temperature	No. mothers	No. progenies	No. offspring	No. females	No. males
Inbred	20	44	92	1220	454	766
	25	42	65	1026	457	579
	30	57	98	1296	477	819
	Total	67	255	3542	1388	2164
Treatment	Temperature	Expected No. diploid males ¹	Expected No. haploid males ²	Total No. fertilised eggs ³	Adjusted sex ratio ⁴	Sex ratio of control vs inbred
Inbred	20	149.8	616.2	603.8	0.505	$\chi^2 = 1.38, P = 0.239$
	25	150.8	428.2	607.8	0.413	$\chi^2 = 0.32, P = 0.571$
	30	157.4	661.6	634.4	0.510	$\chi^2 = 0.21, P = 0.648$
	Total	458	1706	1846	0.482	$\chi^2 = 0.02, P = 0.890$

¹ Number of females $\times$ 1/3 (25% of females are expected to become diploid males);

² number of males – expected number of diploid males;

³ number of females + expected number of diploid males;

⁴ expected number of haploid males / total number of offspring.

In summary, this study proves the presence of single locus Complementary Sex Determination in *Venturia canescens*. Moreover, it demonstrates that the occurrence of diploid males is unaffected by temperature, at least in the range 20-30°C.

DISCUSSION

It was shown that sex determination in *Venturia canescens* is controlled by the allelic state of a single locus. To date, this form of single locus Complementary Sex Determination has been found in 40 species of Hymenoptera including all major superfamilies. *Venturia canescens* belongs to the subfamily Campopleginae of the family Ichneumonidae. Thusfar, all species tested in this family appear to have sl-CSD. BUTCHER *et al.* (2000a, b) recently demonstrated sl-CSD in seven species of *Diadegma* following the work of PERIQUET *et al.* (1993) on *Diadromus pulchellus*. Thus, adding *V. canescens* brings the total number of species with sl-CSD among the Ichneumonidae at nine.

In contrast to previous reports, no evidence was found for an effect of temperature on the occurrence of diploid males in *V. canescens*. WHITING & ANDERSON (1932) found a temperature effect on CSD in *Bracon hebetor*. This effect was apparently due to the temperature at which the parental females were kept prior to the experiment rather than to the temperature at which their progeny was reared. They reared all their wasps at 30°C, but kept one group at 20 and another group at 30°C after emergence. Diploid males were less frequent among progeny of mothers kept at 20°C than among progeny of mothers kept at 30°C. These results could either be due to higher lethality of matched *versus* unmatched embryos, or reduced expression of diploid males in progenies of mothers that had been kept at 20°C. In the latter case, one would expect to find some daughters with matched sex alleles, but this was not investigated. In my experiment I did not vary the temperature at which wasps were kept before oviposition; all wasps were reared at 25°C and kept at room temperature after emergence. I found no differences in proportions of diploid males among progenies reared at 20, 25 and 30°C. Moreover, there appeared to be no mortality of diploid males in contrast to *B. hebetor* in which diploid males have reduced viability compared to haploids. BUTCHER *et al.* (1998) also reported a temperature effect on CSD. They claim that diploid males in *V. canescens* only appeared when broods were reared above 22°C. This observation is not supported by the data of this study.

SI-CSD may have drastic evolutionary consequences. First, many parasitic wasps perform natural inbreeding where mating occurs among siblings. Under such conditions, sl-CSD will cause a large fraction of fertilised eggs to develop into diploid males whose sterility poses a heavy genetic load on the population. In *V. canescens*, natural inbreeding is probably rare, because it is a solitary parasitoid of moth larvae that do not have an aggregated distribution (DRIESSEN & BERNSTEIN, 1999). Moreover, both sexes are fully winged and can disperse over large distances (Desouhant, unpublished results). Unfortunately, sterility of diploid males in *V. canescens* could not be confirmed in this study.

A second consequence of sl-CSD is that it may prevent the evolution of thelytoky. Most forms of thelytoky lead to partial or complete homozygosity (SUOMALAINEN *et al.*, 1987). For example, thelytoky in some species is caused by infection with *Wolbachia* bacteria (reviewed in WERREN, 1996; COOK & BUTCHER, 1999). STOUTHAMER & KAZMER (1994) showed that in *Trichogramma* these bacteria cause gamete duplication of haploid eggs, leading to complete homozygosity within a single generation. Such a form of thelytoky is clearly incompatible with sl-CSD, because it would result in 100% diploid males rather than females. Nevertheless, both arrhenotokous and obligate forms of thelytoky have been found in *V. canescens* (BEUKEBOOM *et al.*, 1999). BEUKEBOOM & PIJNACKER (2000) showed that thelytoky is not caused by *Wolbachia*, but involves automixis with a restitution metaphase after the reduction division. The genetic consequences are similar to central fusion (SUOMALAINEN *et al.*, 1987), *i.e.* loci distal from a cross-over event have a 50% chance to become homozygous. The long term effect is that loci located distally

TABLE 2

VLP alleles among control (a) and inbred (b) progenies. Control progenies were only analysed from 30°C. Inbred progenies were analysed at all three temperatures and are divided into ones without and with diploid males.

(a)

Treatment	Family	No. males analysed	Hemizygous or homozygous +	Hemizygous or homozygous -	Heterozygous + -
Control 30°C	V2a	2	1	1	0
	V2b	3	1	2	0
	V2c	6	3	3	0
	V2e	4	2	2	0
	V7d	4	2	2	0
	V12b	4	1	3	0
	V16a	4	4	0	0
	Total	27	14	13	0

TABLE 2
(Continued).

(b)

Treatment	Family	No. males analysed	Hemizygous or homozygous +	Hemizygous or homozygous -	Heterozygous + -
Inbred 20°C	V1c	7	1	6	0
	V3c	2	1	1	0
	V16d	8	4	4	0
	V16e	5	5	0	0
	V16i	7	4	3	0
	Total	29	15	14	0
	V2d	2	1	0	1
	V3d	6	1	4	1
	V3f	8	4	3	1
	V3i	8	2	5	1
	V4f	3	1	1	1
	V4g	7	3	1	3
	A7d	4	2	1	1
	A25c	5	1	2	2
	Total	43	15	17	11
Inbred 25°C	V1g	1	1	0	0
	V3e	10	7	3	0
	V4f	5	2	3	0
	Total	16	10	6	0
	V2f	9	2	4	3
	V4g	6	2	1	3
	Total	15	4	5	6
Inbred 30°C	V2d	7	3	4	0
	V3d	10	4	6	0
	V4f	5	3	2	0
	V8d	5	4	1	0
	V12f	6	3	3	0
	V12g	4	2	2	0
	V13e	5	1	4	0
	V16h	6	6	0	0
	Total	48	26	22	0
	V2f	11	4	6	1
	V3i	8	4	3	1
	V8e	5	1	2	2
	V12k	9	3	4	2
	V23c	8	3	4	1
	A25d	6	2	2	2
	A27c	9	3	5	1
	Total	56	20	26	10

on the chromosome arms will become homozygous over time, whereas those close to the centromere (where cross-overs are absent) will retain their heterozygosity. Male production in thelytokous *V. canescens* females has never been observed, except for a single case after irradiation (SPEICHER *et al.*, 1965), indicating permanent retention of heterozygosity at the sex locus. Indeed, the sex locus in *V. canescens* is expected to be physically close to one of the centromeres (SPEICHER *et al.*, 1965; CROZIER, 1971; BEUKEBOOM & PIJNACKER, 2000). Moreover, the VLP locus was found to be unlinked with the sex locus and is therefore likely to be located on one of the other linkage groups (*V. canescens* has 11 chromosomes). MALMBERG *et al.* (2000) did not find any VLP heterozygotes among 19 thelytokous females tested, suggesting that the VLP locus quickly becomes homozygous after the origin of thelytoky due to a more distal location on a chromosome arm than the sex locus.

To summarise, *V. canescens* was shown to have single locus Complementary Sex Determination. Diploid males were fully viable in the temperature range tested (20-30°C), but it could not be determined whether they were fertile. This mode of sex determination has apparently not prevented the evolution of thelytoky, although it may have prohibited infection with thelytoky-inducing *Wolbachia* bacteria. Finally, these results show how knowledge of the sex determining mechanism and the cytological mechanism of thelytoky is needed for a full understanding of the evolution of reproductive modes in Hymenoptera.

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