

Single-locus sex determination in the parasitoid wasp *Cotesia glomerata* (Hymenoptera: Braconidae)

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The parasitoid *Cotesia glomerata* usually produces female-biased sex ratios in the field, which are presumably caused by inbreeding and local mate competition (LMC); yet, sibling mating increases the production of males, leading to the male-biased sex ratio of broods in the laboratory. Previous studies have suggested that the sex allocation strategy of *C. glomerata* is based on both partial LMC in males and inbreeding avoidance in females. The current study investigated the presence of single-locus complementary sex determination (sl-CSD) as a sex-determining mechanism in this species through inbreeding experiment, cytological examination and microsatellite analysis. Cytological examination detected diploid males in nine of 17 single pairs of sibling mating, thus in agreement with the proportion of

matched matings predicted by the sl-CSD model. Sex ratio shifts in these matched sibling matings were consistent with the sl-CSD model with less viable diploid males. The haploid males have a single set of maternal chromosomes ($n=10$), whereas diploid males possess a double set of chromosomes ($2n=20$). Microsatellite analyses confirmed that diploid males produced from the matched matings inherited segregating genetic materials from both parents. Thus, this study provides the first solid evidence for the presence of sl-CSD as a sex-determining mechanism in the braconid genus *Cotesia*.

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Introduction

The basic system of sex determination in Hymenoptera is arrhenotokous haplodiploidy, in which females are diploid, developed from fertilised eggs, whereas males are normally haploid, developed from unfertilized eggs (Crozier, 1977). Increasing evidence suggests that hymenopteran insects have evolved different sex determination mechanisms owing to the diverse mating systems and complex life history patterns in different species (Cook and Crozier, 1995; Butcher *et al.*, 2000b). There are four different models that have been proposed to interpret the mechanisms of sex determination in this order, which are complementary sex determination (CSD) including single-locus CSD (sl-CSD) (Whiting, 1943) and multi-locus CSD (ml-CSD) (Crozier, 1971), genomic imprinting (Beukeboom, 1995) and genic balance (Bridges, 1925; Kerr and Nielsen, 1967).

The sl-CSD is the best known and empirically supported model under which sex is determined by multiple alleles at a single locus; diploid individuals that are heterozygous at the sex locus develop into females, whereas individuals that are hemizygous (haploid) or homozygous (diploid) at the sex locus develop into

males (Whiting, 1943). In Hymenoptera, sl-CSD has been found in at least 20 nonsocial and two social species (Cook, 1993; Butcher *et al.*, 2000a; Beukeboom, 2001; Salin *et al.*, 2004; Stahlhut and Cowan, 2004). The molecular basis of sl-CSD has been revealed in the honeybee, *Apis mellifera*, in which heterozygotes at the sex-determination locus develop into females and homozygotes or hemizygotes into males (Beye *et al.*, 2003). Although genomic imprinting has been found to be a genetic system of sex determination in *Nasonia vitripennis* (Dobson and Tanouye, 1998), ample evidence suggests that sl-CSD is the most plausible and widespread genetic system of sex determination in Hymenoptera (Crozier, 1971; Cook and Crozier, 1995; Paxton *et al.*, 2000), hence leading to the suggestion that CSD might be an ancestral sex-determining mode in this insect order (Bull, 1981).

In the species with sl-CSD, inbreeding will greatly increase the frequency of matched matings (ie the parents share an allele at the sex locus) (Adams *et al.*, 1977) and hence, the production of diploid males. However, the presence of diploid males is also explained genetically by ml-CSD, under which diploid males develop only from those individuals homozygous at all sex-determining loci (Crozier, 1971). Moreover, uniparental diploid males can occasionally occur in some species, such as *N. vitripennis*, through noncomplementary mechanisms (Whiting, 1960; Stille and Dävring, 1980; Dobson and Tanouye, 1998). Indeed, the number of both social and nonsocial species of Hymenoptera in which diploid males have been detected is much

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larger than the cases where sl-CSD has been considered to be the sex-determining mechanism (van Wilgenburg et al, 2006). Therefore, sl-CSD will not be unequivocally confirmed in any species until sex ratio shifts are quantified in inbreeding experiments, as well as the male diploidy is verified through cytological and/or molecular methods.

The genus *Cotesia*, belonging to the family of Braconidae, includes nearly 1000 species worldwide (Michel-Salzat and Whitfield, 2004). Many of these species, including *C. congregata*, *C. kariyai*, *C. rubecula* and *C. glomerata*, are frequently used as biological control agents in agricultural pest management because they are important parasitoids of numerous herbivorous insects (Michel-Salzat and Whitfield, 2004). The empirical studies that have been pursued with three species do not support the presence of sl-CSD as a sex-determining mechanism in this genus (Stouthamer et al, 1992; Niyibigira et al, 2004a, b).

Cotesia glomerata (L.) (Syn. *Cotesia* or *Apanteles glomeratus*) is a gregarious larval endoparasitoid of the small and large white butterflies, *Pieris brassicae* L. and *P. rapae* L. (Liang and Levin, 1982). It has also been employed as a model in basic studies on the physiology (Ockroy et al, 2002), chemical ecology (Mattiacci et al, 2001), behavioural ecology (Gu and Dorn, 2003; Gu et al, 2003) and genetics (Gu and Dorn, 2000; Wang et al, 2003, 2004) of parasitoids. The population of *C. glomerata* wasps generally has female-biased sex ratios in the field, which is often thought to be linked with inbreeding and local mate competition (LMC) (Hardy, 1994). However, previous inbreeding experiments have demonstrated that full-sib mating causes a dramatic increase in the production of males, suggesting the presence of sl-CSD as a sex-determining mechanism in this species (Gu and Dorn, 2003). This hypothesis was supported by the observation of inbreeding avoidance behaviour in *C. glomerata* (Gu and Dorn, 2003), as the species with sl-CSD are expected to avoid inbreeding, thereby to minimize chances of diploid male production (Whiting, 1945; Crozier, 1971; Bull, 1981; Cook and Crozier, 1995). The current study was conducted to investigate the presence of sl-CSD in this braconid species through inbreeding experiment, microsatellite analysis and cytological examination.

Materials and methods

Experimental parasitoids

C. glomerata was collected from a field population in Switzerland in summer 2004, using the same method as described previously (Gu et al, 2003). Potted cabbage plants infested with newly hatched *P. brassicae* larvae were randomly distributed in a cabbage field at Feldi, Canton Zurich (47°34'N, 8°48'E). After 2 days of exposure to parasitoids, these potted plants were retrieved from the field, and the recovered host larvae were reared on cabbage leaves in insect cages (30 × 30 × 30 cm) at 22 ± 1°C, 60% RH, under a 16:8 h light:dark cycle. After pupation, cocoon clusters of *C. glomerata* were individually collected and stored in glass vials (6 × 3 cm) according to their hosts. The female wasp of *C. glomerata* usually deposits 20–60 eggs once into the host (Liang and Levin, 1982). Previous experiments

have also demonstrated that single oviposition of the female wasp on *P. brassicae* larvae do not produce any brood size larger than 62 (Gu et al, 2003). Therefore, the field-collected cocoon clusters sized less than 40 were selected for this study in order to minimize the chance of nonsiblings emerged from a brood. Virgin male and female wasps were obtained by separating individual cocoons from their clusters and allowing them to emerge singly in plastic vials (4 × 2 cm), with the brood noted. Such virgin wasps were used in the following experiment.

Inbreeding experiment

In total, 20 single pairs of full-sibling mating were established using the virgin males and females. We placed one female and one male wasp, both newly emerged from the same brood, into a vial (4 × 2 cm). After 2 days, the male wasp was transferred from the vial into a prelabelled Eppendorf tube and stored at –80°C, whereas the female wasp was transferred into an insect cage, where 20–25 *P. brassicae* larvae were provided for parasitism. To reduce superparasitism (Gu et al, 2003), the female wasp was removed from the cage 24 h later and frozen in a prelabelled Eppendorf tube at –80°C. The parasitized host larvae were reared on cabbage leaves until the parasitoids egressed and pupated. Parasitoid cocoon clusters were collected and individually kept in vials with their motherhood noted. When emergence of adult wasps from a brood was completed, female and male numbers were counted to calculate the sex ratio, and the unemerged cocoons were checked to estimate the brood size and pupa mortality. After counting, some males from each brood were used for cytological examination, whereas the remaining male wasps were stored at –80°C for microsatellite analysis. Among the 20 sibling mating pairs, three produced only male progeny, probably owing to unsuccessful mating; these pairs, as well as their progeny, were not used in microsatellite analysis and cytological examination.

Microsatellite analysis

In all, 17 of the 20 single pairs of parents, which produced both female and male progeny, were genotyped at five microsatellite loci, including Cot1 (GenBank accession number AY804182), Cot2 (AY804183), Cot3 (AY804184), Cot4 (AY804185) and Cot5 (AY804186). These codominant microsatellite markers were also used to detect heterozygous bi-parental diploid males in the F1 progeny derived from these pairs of parents. Microsatellite development, DNA isolation and DNA genotyping were carried out as described by Zhou et al (2005). PCR amplified microsatellite alleles were separated on 6% denaturing polyacrylamide gel using a Sequi-Gen GT sequencing apparatus (Bio-Rad, Reinach, Switzerland), then visualized by silver staining (Creste et al, 2001). Gel patterns were scanned using a UMAX Powerlook 1100 Firewire scanner.

Among the five microsatellites tested, Cot1 was highly polymorphic (eight alleles were detected) and informative in the sib-mated pairs tested. In comparison, polymorphic level was much lower at Cot2, Cot3, Cot4 and Cot5 (one to four alleles were detected). Microsatellite analyses of the 17 pairs of parents showed uninformative results at these loci because an allele was shared by mating partners. Consequently, only Cot 1 was

used to genotype the sons of the informative parents. As controls, two daughters in each of the sibships were randomly selected and genotyped to check their bi-parental heterozygosity at this locus. Sons were classified as bi-parental diploids if they carried both maternal and paternal alleles.

Cytological examination

Preliminary studies using cerebral ganglia and testes as materials were conducted to select a suitable tissue for chromosome preparation in males. The results showed that testes had a much higher amount of spermatocytes in active division, as compared with cerebral ganglia, and colchicines treatment (>1 h) greatly increased the yields of high-quality metaphases. Therefore, testes were used for the metaphase spread.

Testes of newly emerged males were dissected out in colchicines-hypotonic solution (0.05% colchicine in 0.075 M potassium chloride solution) on a cavity slide, then transferred into fresh colchicines-hypotonic solution and incubated at room temperature for at least 1 h. Chromosome preparations were made using the air-drying technique modified from Imai *et al* (1977). After air-drying overnight, the preparations were stained for 10 min in freshly prepared Giemsa solution (Fluka product diluted 1:20 in 1/15 M, pH 6.8 phosphate buffer: KH_2PO_4 4.54 g/l, Na_2HPO_4 4.75 g/l). Chromosomes were observed under a Zeiss Axioplan 2 Imaging microscope, coupled with AxioCam digital camera and AxioVision software (version 2.0.5; Zeiss).

Statistical analysis

To compare sex ratio and brood size between matched and unmatched matings, data were first checked for normality using the Kolmogorov-Smirnov test, and the homogeneity of variances using Levene's test. Accordingly, Student's *t*-test was applied in data analyses. Similar methods were used to compare the mortality of pupae in matched and unmatched matings. Deviations of the observed matched matings from the expected values under sl-CSD and ml-CSD models were analysed using χ^2 goodness of fit test (Zar, 1999). All analyses were performed using SPSS 11 for Macintosh.

Results

Microsatellite analysis

In five of the 17 single pairs of sibling mating, distinct alleles were shown between mother and father at locus Cot1. For the remaining 12 parental pairs, three showed ambiguous genotypes (more than two bands were presented in females), and other pairs carried shared alleles at the locus. Therefore, only sons of the five informative parent pairs were subjected to further genotyping analyses.

A total of 159 sons were randomly selected from the five sibships for genotyping at locus Cot1. Among them, five diploid males were detected in sibship 1 ($n = 48$) and three diploid males were found in sibship 2 ($n = 39$); no heterozygous male was detected in the remaining three sibships. Examples of the microsatellite genotypes were shown in Figure 1. One diploid male (lane 2) in sibship 1 inherited one allele (257 bp) from its father (lane 3) and another allele (278 bp) from its mother (lane 4). Similarly,

one diploid male (lane 8) in sibship 2 carried two distinct alleles with 266 bp from its mother (lane 5) and 305 bp from its father (lane 6). The same heterozygous pattern was also presented in its sister (lane 7), showing the bi-parental nature of diploids at this locus. In contrast, the haploid males shown in lanes 1 and 9 only carried one allele from their mothers. Thus, it is clear that the diploid males of *C. glomerata* developed from fertilized eggs and inherited the genetic materials from both parents.

Cytological examination

Metaphase pictures clearly show that haploid males in *C. glomerata* have 10 maternal chromosomes ($n = 10$) (Figure 2a) and the diploid males possess a double set of chromosomes ($2n = 20$) (Figure 2b).

Cytological examination detected diploid males in nine of the 17 examined pairs of sibling mating (Table 2),

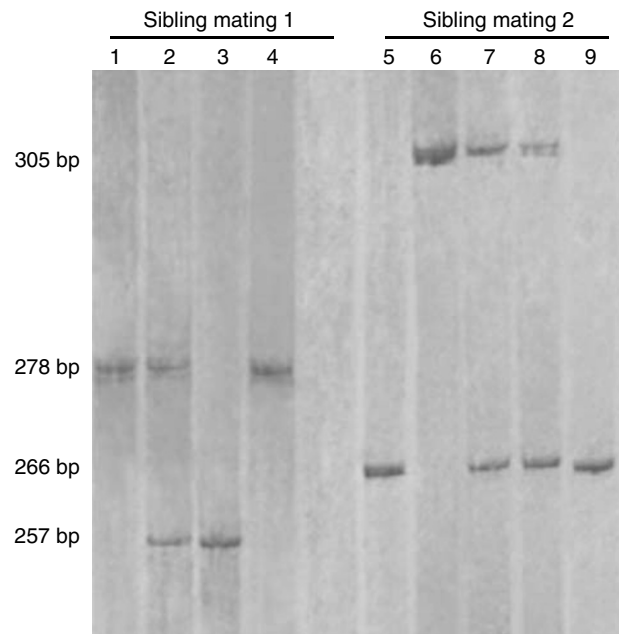


Figure 1 Microsatellite analyses for the existence of bi-parental heterozygous diploid males in *C. glomerata*. Lanes 1–4 show the genotype of haploid son, diploid son, father and mother in sibling mating 1, respectively; Lanes 5–9 show the genotype of mother, father, daughter, diploid son and haploid son in sibling mating 2, respectively.

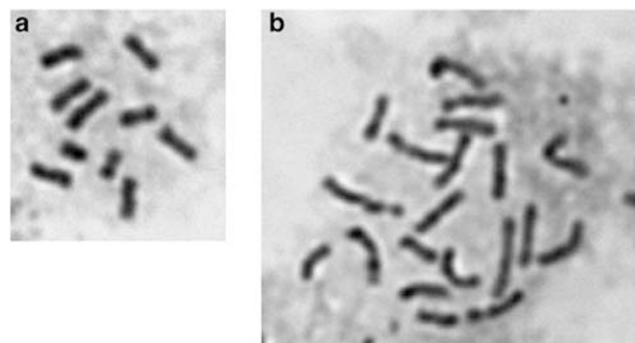


Figure 2 Metaphase spreads of spermatocytes in *C. glomerata*, stained with Giemsa. (a) Haploid male ($n = 10$), (b) diploid male ($2n = 20$).

suggesting that these matings are matched. This result is well in agreement with the prediction of sl-CSD model (goodness of fit test; $\chi^2 = 0.059$, $P > 0.05$). According to the ml-CSD model, the chance that a matched mating occurs in brother–sister crosses is $(0.5)^k$ if there are k sex loci in the species (Cook and Crozier, 1995). Thus, under two-locus ml-CSD, the chance of matched mating should be less than 25%, which is significantly different from the observed data (goodness of fit test; $\chi^2 = 7.078$, $P < 0.05$). In this context, the chance would be even smaller when more sex loci were involved. Therefore, the presence of ml-CSD in *C. glomerata* is ruled out.

Under sl-CSD, 50% of the fertilized (diploid) progeny are expected to develop into diploid males in the nine matched matings, and thus the ratio of diploid males to females in siblings should be approximately equal. However, the calculated ratio of diploid males to females, based on unambiguously determined diploid and haploid males from these matched matings, ranged from 0.083 to 0.667, with an average of 0.260. Indeed, even the ratio of diploid males to females calculated by considering those undetermined males as diploids was lower than the prediction of sl-CSD model in seven of the nine matched matings (Table 1). As compared to the eight unmatched matings, the nine matched matings had significantly higher sex ratios (the proportion of males) (t -test, $P < 0.05$) (Table 2). On the other hand, the mean brood size was significantly smaller in the matched matings than in the unmatched matings (t -test, $P < 0.05$), although no significant differences were shown in pupa mortality between the matched and unmatched matings (t -test, $P = 0.57$) (Table 2). These results indicate that diploid males produced from the matched matings of

C. glomerata have suffered a relatively higher mortality in a pre-pupa stage, relative to diploid females.

Discussion

Cytological examinations have physically identified diploid males from sibling matings, and furthermore, microsatellite analyses have revealed that such diploid males inherit segregating genetic materials from both parents. Thus, empirical evidence is sufficient to rule out the possibility of non-CSD mechanisms. The males used in our microsatellite analyses and cytological examinations were derived from single-pair sibling matings, so that these findings truly reflect the bi-parental nature of the diploid males produced as such. The quantitative analysis of the cytological data obtained from the single-pair sibling matings has shown that sex ratio shifts in the matched matings are consistent with sl-CSD model rather than any ml-CSD model. As our breeding experiments started directly with a large sample of field-collected cocoon clusters, the presence of sl-CSD in *C. glomerata* unlikely result from the collapse of a ml-CSD system owing to a bottleneck effect. Therefore, this study provides solid evidence to support our previous hypothesis that sl-CSD functions as a sex determining mechanism in this braconid species (Gu and Dorn, 2003).

So far, the presence of sl-CSD has not been found in the braconid genus *Cotesia*. Although diploid males were recorded in *C. rubecula*, no experimental data are available to support the presence of sl-CSD in this solitary species (Stouthamer et al, 1992). Modelling analyses for the field populations shows no evidence for the presence of sl-CSD in two other gregarious species, *C. flavipes* and *C. sesamiae* (Niyibigira et al, 2004a), and further laboratory inbreeding experiments have ruled out the presence of sl-CSD in *C. flavipes* (Niyibigira et al, 2004b). Therefore, our study has, for the first time, confirmed the presence of sl-CSD as a sex-determining mechanism in this large genus of theoretical and practical interest (Michel-Salzat and Whitfield, 2004). The presence of species with and without sl-CSD in this genus suggests that sex determination mechanisms are diverse even within the same genus. Furthermore, it raises interesting questions about evolutionary changes in the genetic system of sex determination in closely related species, and the influences of variable mechanisms on the behaviour and population biology of these species (Cook and Crozier, 1995; van Wilgenburg et al, 2006).

Sibling matings lead to the production of diploid males in *C. glomerata*. Our cytological examinations have shown that such diploid males possess a double set of chromosomes ($2n = 20$), whereas haploid males contain a single set of maternal chromosomes ($n = 10$). Based on the stylized figures from Hegner (1915), Gokhman and Quicke (1995) postulated that the haploid and diploid chromosome numbers of *C. glomerata* were 12 and 24, respectively, but in the meantime, they pointed out that Hegner did not provide a definitive statement on chromosome numbers, and hence such data should be considered with extreme caution. The basic number of chromosomes in *C. glomerata* revealed in the current study is the same as in many other braconid parasitoids, including *Habrobracon* (*Bracon*) *hebetor* Say (Torvik-Greb, 1935), *H. pectinophorae* Watanabe (Inaba cited in Mikino,

Table 1 Production of diploid males from nine matched sibling matings in *C. glomerata*

Sibling matings	Females	Males			Proportion of diploid males to females ^a
		Diploid	Haploid	Undetermined	
1	52	13	58	6	0.250–0.365
2	46	9	73	8	0.196–0.370
3	24	4	22	3	0.167–0.292
4	23	11	14	1	0.478–0.522
5	12	8	25	2	0.667–0.833
6	63	7	87	6	0.111–0.206
7	62	8	96	18	0.129–0.419
8	60	5	68	1	0.083–0.100
9	220	52	279	26	0.261–0.355

^aData are given as a range from a minimum (where all undetermined males were considered haploid males) to a maximum (where all undetermined males were considered diploid males).

Table 2 Comparison in sex ratio, brood size and pupa mortality between matched and unmatched sibling matings in *C. glomerata*

	Sex ratio (%)	Brood size	Pupa mortality (%)
Matched sibling matings ($n = 54$)	61.6 ± 2.3	23 ± 3	5.6 ± 1.1
Unmatched sibling matings ($n = 48$)	42.7 ± 7.0	34 ± 4	4.6 ± 1.2
P -value (t -test, $df = 15$)	0.017	0.037	0.568

Means ± SE are presented.

1951), *H. serinopae* Ramakrishna (Rasch *et al*, 1977), *Meteorus gyrator* Thunberg (Gokhman and Quicke, 1995), *M. pallipes* Wesmael (Gokhman and Quicke, 1995) and the related species, *C. congregata* (Belle *et al*, 2002).

Diploid males in some species with sl-CSD have a normal viability, although they can be either sterile (El Agoze *et al*, 1994; Krieger *et al*, 1999) or unable to fertilize females (Smith and Wallace, 1971; Naito and Suzuki, 1991). We have shown that diploid males in *C. glomerata* appear to have low survivorship, as compared to diploid females. As a high survival rate of pupae was found in the broods produced from both matched and unmatched matings, the death of diploid males might have occurred in the early developing stages. Although the survived diploid males of *C. glomerata* resemble haploid males in both size and viability (Y. Zhou, personal observation), we do not know about the fertility of diploid males in *C. glomerata* yet. Certainly, information on this aspect is important to understand the effects of sl-CSD on population biology in this species.

Natural populations of *C. glomerata* normally produce female-biased sex ratio in the field (Tagawa, 2000; Gu and Dorn, 2003), which is presumably the result of LMC (Hamilton, 1967). In this situation, mating is restricted to a local area or natal patch where a mother produces the minimum number of sons sufficient to inseminate all of her daughters in each patch, thus minimizing the level of competition between sons. Therefore, the species that manifest LMC are generally believed to have a high level of inbreeding (Hardy, 1994). Although *C. glomerata* has been reported to show a high inbreeding rate (about 60%) in the field because most of the emergent females are inactive and tend to mate with males from the same brood (Tagawa and Kitano, 1981), the post-emergence behaviour of the wasp determines that the inbreeding level of this species is much lower than we have intuitively assumed. Indeed, 30% of males and over 50% of females left their natal patch before copulating, and hence only 27.5% of females mated with the males emerging from the same brood, on average (Gu and Dorn, 2003). This inbreeding avoidance behaviour and consequently a lower inbreeding level are expected for the species with sl-CSD. Confirmation of sl-CSD in *C. glomerata* sheds a light on our previous suggestion that this parasitoid species adopts a sex allocation strategy based on both partial LMC in males and inbreeding avoidance in females (Gu and Dorn, 2003). However, more laboratory and field data are required to elucidate the sex allocation and mating system of this gregarious parasitoid in relation to its sex determination mechanism.

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