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Inbreeding, Male Diploidy, and Complementary Sex Determination in the Solitary Wasp Euodynerus Foraminatus

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INBREEDING, MALE DIPLOIDY, AND COMPLEMENTARY SEX DETERMINATION IN THE SOLITARY WASP *EUODYNERUS FORAMINATUS*

by

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In the insect order Hymenoptera, which includes wasps, bees, and ants, females normally develop from fertilized eggs and are diploid, while males develop from unfertilized eggs and are haploid. Underlying mechanisms of sex determination are more complex and vary among different hymenopteran taxa. One such method is single-locus complementary sex determination, or sl-CSD, in which sex is determined by a single gene locus with many alleles. In species with sl-CSD, diploid individuals that are heterozygous at the sex locus develop as normal females and hemizygous (haploid) individuals as normal males, but diploids homozygous at the sex locus will also develop as males. In many hymenopteran species, such diploid males are inviable or sterile, imparting a high cost to the parents that produce them.

In the solitary vespid wasp *Euodynerus foraminatus*, brothers and sisters frequently mate at the entrance of their natal nest. However, sl-CSD is present in species closely related to *E. foraminatus*. Single-locus CSD and inbreeding are considered incompatible, because inbreeding increases the chances of homozygosity at all loci, including the sex locus, and therefore increases the production of abnormal diploid males.

This study had four parts. First, microsatellite DNA markers were developed for genetic studies of *E. foraminatus*. Second, a controlled breeding experiment was carried out and followed by microsatellite genotyping to confirm the presence of

sl-CSD in this species. Third, individuals sampled from a natural population of *E. foraminatus* from southwest Michigan were genotyped to determine the actual incidence of inbreeding in nature by comparison with Hardy-Weinberg expectations. Fourth and finally, a multi-generation controlled breeding experiment was carried out to determine whether diploid *E. foraminatus* males had normal fertility, as assessed by their ability to father viable, fertile daughters.

E. foraminatus was found to have sl-CSD, and the southwestern Michigan population was found to have inbreeding levels consistent with more than 60% of all matings occurring between siblings. This paradox may be partially resolved by the additional finding that diploid *E. foraminatus* males have normal viability and near-normal fertility, and are capable of fathering viable and fertile diploid daughters.

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CHAPTER I

SEX DETERMINATION IN HYMENOPTERA

The insect order Hymenoptera, which includes wasps, ants, and bees, has a haplodiploid genetic system in which females normally develop from fertilized eggs and are diploid, while males develop from unfertilized eggs and are haploid, carrying only a single set of maternal chromosomes. However, various underlying sex determination systems, rather than ploidy level alone, determine sex in hymenopterans. The best known mechanism is single-locus complementary sex determination, or sl-CSD (Whiting, 1943), in which heterozygotes at a single sex locus develop as females, while hemizygotes (normal haploids) and homozygous diploids develop as males. Other proposed mechanisms include multi-locus complementary sex determination or ml-CSD (Crozier, 1971), in which several loci control sex determination and only a homozygote at all sex loci will develop as a diploid male; genomic imprinting (Beukeboom, 1995), in which a paternal locus carries an active female-development factor that is inactive or absent in the maternal genome; and genic balance (Bridges, 1925; Kerr & Nielsen, 1967) in which a dose-independent "maleness" gene causes male development in haploids while a separate, dose-dependent "femaleness" gene causes individuals of higher ploidy to develop as females. Under the genic-balance model, a haploid has a single copy of each of the male- and female-determining genes, and the effect of the single male-determining gene overrides that of the female-determining gene, resulting in male development. In individuals of diploid or higher ploidy, the presence of a copy of the female-determining

gene in each chromosome set is cumulative; the effects of multiple female-determining genes thus override those of the non-cumulative male-determining genes and result in female development. This relationship can be stated as 2F > M > F (Kerr & Nielsen, 1967). Under genic balance systems, triploids have been predicted to develop as "superfemales" because of the increased dosages of the F gene (Bridges, 1925), although this conclusion has been disputed (Bull, 1983).

These sex-determination hypotheses can be distinguished based on the predictions they generate regarding the offspring of consanguineous matings. Complementary sex determination, whether controlled by a single locus or more than one, can be distinguished from other sex determination systems by an increase in males among the offspring of sibling matings. This is due to the presence of diploid males, which are produced in predictable proportions and can be shown to be heterozygous (and therefore confirmed diploid) via genetic markers. Under non-complementary systems such as genic balance or genomic imprinting, diploid males should be absent (Cook, 1993b), with rare exceptions that are not associated with inbreeding (Whiting, 1960).

As an example of a confirmed non-complementary system, Dobson and Tanouye (1998) have found evidence for genomic imprinting sex determination in the chalcidoid wasp *Nasonia vitripennis*. In *N. vitripennis*, unfertilized eggs develop as males, fertilized eggs with normal paternal chromosomes develop as females, and a supernumerary chromosome that induces paternal genome loss causes fertilized eggs to develop as haploid males.

Unlike genomic imprinting, genic balance in Hymenoptera has not been supported by experimental evidence (Cook, 1993b; MacDougall *et al.*, 1995). If it does

exist in any hymenopteran species, diploid males should not occur in those species, because in any non-haploid individual, the multiple copies of the female-determining genes would override the non-cumulative effects of all copies of the male-determining gene, and result in female development (Cook, 1993b; MacDougall *et al.*, 1995)

It is important to reiterate that the mere presence of diploid males does not confirm that sex determination is complementary. Although no diploid males should be produced in a species with genic-balance sex determination, they may exist in rare cases under genomic imprinting. A mutant polyploid strain of *N. vitripennis* is known to produce diploid males, but, like normal haploids, these diploid males develop only from unfertilized eggs; in this case, the eggs producing diploid males are themselves diploid (Whiting, 1960; Dobson & Tanouye, 1998). Other possible mechanisms of producing diploids without fertilization include parthenogenetic inheritance of the mother's entire diploid genome, which should produce only diploid females identical to the mother (Bull, 1983); fusion of maternal meiotic products (Beukeboom & Pijnacker, 2000), which might, under CSD, produce an occasional sex-allele homozygote (a diploid male) lacking any paternal alleles; or duplication of the haploid egg genome (Beukeboom & Pijnacker, 2000) , which would produce an individual homozygous at all loci (and which would become a diploid male if CSD is also present).

There are two important distinctions between diploid male production under CSD and diploid male production under a non-complementary system such as genomic imprinting. First, non-complementary models predict no association between inbreeding and diploid male production, so that males should account for the same proportion of diploids among inbred and outbred sibships. Second, any diploid males that may occur

under non-complementary systems are predicted to carry only maternal genetic material, since they can develop only from unfertilized diploid eggs, or from fertilized diploid eggs which have lost their paternally-derived chromosomes. In order to support the presence of a CSD system, diploid males must (a) occur in inbred broods in proportions predicted by an sl-CSD or ml-CSD model, (b) be absent from outbred broods except in the rare case of a mating between non-relatives with a shared sex allele (described below), and (c) inherit segregating genetic material from both parents in a Mendelian pattern.

Under sl-CSD, a mating between a male and female which share a sex allele is called a matched mating (Adams *et al.*, 1977) whether or not the male and female are related. Under sl-CSD, the sex locus is expected to be highly polymorphic within populations, and the equilibrium number of sex alleles is expected to increase with population size. Under random mating, the equilibrium frequency of each of the k sex alleles should be equal to $1/k$ as a result of frequency-dependent selection (Yokoyama & Nei, 1979). For random matings within a large population, the probability of a female with sex-locus genotype A_iA_j mating with a male of genotype A_i is $1/k$, her probability of mating with a male of genotype A_j is also $1/k$, and therefore the overall chance of any mating in the population being matched is $2/k$ (Adams *et al.*, 1977; Cook & Crozier, 1995). In a matched mating, 50% of the diploid offspring are predicted to develop as males instead of females, so under random mating the proportion of diploids that are male will be equal to 50% of $2/k$, or $1/k$.

Diploid male production under sibling mating is expected to be much higher. A brother and sister have a 50% chance of sharing an allele at the sex locus, regardless of the number of sex alleles in the population, and therefore half of all sibling matings will

be matched. Because half of the diploid offspring of a matched mating will develop as males, there is an overall 25% chance (50% of 50%) that any diploid offspring of any sibling mating will develop as a male.

Under ml-CSD, expected proportions of diploid males will be smaller than those expected under sl-CSD. The expected proportion of diploid males will vary as a function of the number of loci involved. The simplest case of ml-CSD is the two-locus type, in which a diploid must be homozygous at two separate sex loci to develop as a male, and therefore only a mating between individuals matched at both of these loci can produce diploid sons. In this case, one-fourth of sibling matings are predicted to be matched at both loci. Within these matings, only one-fourth of diploid offspring will be homozygous at both loci and develop as males, so that among all offspring of sibling matings, only one-sixteenth (6.25%) of diploids should be male under two-locus CSD, and these predicted proportions become even smaller under hypotheses of more than two sex loci. If the observed proportion of diploid males produced under inbreeding is consistent with sl-CSD, but too high to support the presence of two-locus CSD, then all forms of CSD involving more than two loci can be ruled out as well.

Single-locus CSD has been documented in more than 40 species distributed widely over the major taxonomic subgroups of the order Hymenoptera (Smith & Wallace, 1971; Cook, 1993b; Beukeboom, 1995), and is the prevalent system in the clade that includes the Ichneumonoidea (Whiting, 1943; Butcher *et al.*, 2000b) and the aculeate (stinging) Hymenoptera (Mackensen, 1950; Ross & Fletcher, 1985; Duchateau *et al.*, 1994). The wide distribution of sl-CSD across multiple clades suggests that it may be an ancestral character state in Hymenoptera (Cook, 1993b), and it is often present in closely

related species; for example, sl-CSD is known in six species in the ichneumonid genus *Diadegma* (Butcher *et al.*, 2000a). However, sl-CSD cannot be assumed for an entire higher taxon merely by its presence in one or more species in that taxon. Exceptions are known both within the aculeate-ichneumonoid clade and in other hymenopteran taxa. Cook (1993a) demonstrated that an aculeate, the bethylid *Goniozus nephantidis*, lacks sl-CSD, and Beukeboom *et. al.* (2000) showed that sl-CSD is not the sex determination system of either of the braconids *Asobara tabida* and *Alysia manducator* (superfamily Ichneumonoidea), despite its presence in another braconid, *Bracon hebetor* (Whiting, 1943).

Diploid males in species with CSD are generally considered a genetic dead end. They may have low viability (Whiting, 1943; Petters & Mettus, 1980), may survive but be unable to mate (Smith & Wallace, 1971), or may be sterile (Cook, 1993b; Krieger *et al.*, 1999). Spermatogenesis in male hymenopterans generally does not include a reductional division (Hogge & King, 1975); consequently, diploid males in some species are known to produce abnormal diploid sperm (Woyke & Skowronek, 1974; Yamauchi *et al.*, 2001), which, if capable of fertilization, results in sterile triploid offspring (Inaba, 1939; Smith & Wallace, 1971; Krieger *et al.*, 1999). The fitness of diploid males is therefore expected to be zero.

Because inbreeding increases the frequency of diploid males under any type of CSD, and because single-locus CSD imparts the highest risk of diploid male production under inbreeding, we would expect selection for inbreeding avoidance in sl-CSD species, and, conversely, against sl-CSD in species whose life histories include high levels of inbreeding (Cook & Crozier, 1995). This is supported by the apparent absence of sl-CSD

in Hymenoptera with frequent close inbreeding, such as the bethylid parasitoid *G. nephantidis* (Cook, 1993a), which is an aculeate, and all species studied to date in the superfamily Chalcidoidea (Cook, 1993b), which is distinct from the aculeate-ichneumonoid clade.

Inbreeding has been observed (Cowan, 1979) in natural populations of the aculeate wasp *Euodynerus foraminatus* (Vespidae: Eumeninae). The experiments in this study were designed to determine (a) whether *E. foraminatus*, like many other aculeates, has sl-CSD, (b) the true levels of inbreeding occurring in a natural population of *E. foraminatus*, and (c) if any previously unknown factors permit both sl-CSD and inbreeding to coexist in this species.

CHAPTER II

DNA ISOLATION AND ANALYSIS METHODS

Introduction

This study required the identification of heterozygous individuals in captive-bred broods and natural populations of the solitary wasp *Euodynerus foraminatus,* and the assessment of allele frequencies and inbreeding levels in the natural population. These efforts required the use of neutral, highly variable, codominant genetic markers, in order to determine parentage and to distinguish heterozygotes from homozygotes. The markers chosen for this study were DNA microsatellites (Tautz *et al.*, 1986; Litt & Luty, 1989; Tautz, 1989), which are variable-length repeats of short (1-6 base pair) DNA motifs. Five variable microsatellite loci from *E. foraminatus* were amplified using a partial genomic library constructed from randomly amplified polymorphic DNA (RAPD) fragments (Williams *et al.*, 1990; Ender *et al.*, 1996; Stahlhut *et al.*, 2002). Four of these microsatellite markers consisted of dinucleotide repeats (repeats of a two-base-pair sequence) and the fifth was a trinucleotide repeat (repeats of a three-base-pair sequence). These protocols describe the isolation of DNA from individual wasps, the procedure used to identify microsatellite markers, and the preparation of amplified wasp DNA for automated fragment analysis.

Isolation and Amplification Protocols

DNA Isolation for Microsatellite Genotyping

Wasps were stored individually in 1.5 µl plastic microcentrifuge tubes at -80°C until ready for DNA extraction. Specimens were removed from the freezer as needed and held on ice, typically for 30 minutes or less, while awaiting handling. One antenna or leg was removed from each specimen and placed in a fresh, UV-sterilized 1.5 µl plastic microcentrifuge tube containing 50 µl of lysis buffer (Gentra Systems, catalog number D-5002) and 1 µl of 10 mg/ml proteinase K. (A cocktail consisting of multiple volumes of this solution may be mixed shortly before use, dispensed in aliquots into the individual tubes, and kept on ice until insect parts are added.) Insect parts were homogenized in lysis buffer with a plastic pestle in a 1.5 ml Eppendorf tube, until the chitin was visibly crushed or broken into pieces (fine pulverization is not necessary). When multiple samples (up to 24) were prepared at one time, crushed material in buffer was returned to ice until all preparations were complete. Tubes were then incubated for 1 to 24 hours at 55°C in a water bath.

After incubation, tubes were removed from the water bath and 17.5 µl of protein precipitation solution (Gentra Systems, catalog number D-5503) was added to each reaction. Reaction tubes were vortexed at high speed for 20 seconds, placed on ice for 5 minutes, and centrifuged at high speed (16.1 X g) for 5 minutes. Supernatant from each tube was removed and added to 70 µl isopropanol in a fresh, UV-sterilized 1.5 µl plastic

microcentrifuge tube, then kept at -20°C for 15 minutes to 24 hours. Pellets of insect tissue and precipitated protein from the previous centrifugation were discarded.

Tubes containing DNA-isopropanol solution were then centrifuged at room temperature at high speed for 5 minutes. Supernatant was carefully discarded and the pellet washed with 250 µl of 70% ethanol. (When DNA is isolated from a sample the size of a wasp leg or antenna, the pellet is usually too small to be visible. Therefore, uniform orientation of the tubes in the microcentrifuge and careful handling during supernatant removal are important for avoiding sample loss.) Tubes were then centrifuged at 13200 rpm for 5 minutes. Supernatant was discarded and the samples dried in a Centrivap concentrator (Labconco) at 37°C for at least 15 minutes or until all traces of liquid had evaporated. Dried DNA was reconstituted with 100 µl of rehydration buffer (Gentra Systems, catalog number D-5004) and stored at -20 °C until needed for fragment analysis.

Modifications to Isolation Protocol for DNA Cloning

In this study, a protocol different from the one described above was used to prepare the whole genomic DNA used in RAPD amplifications. After removing the head from a wasp to prevent contamination of the DNA by eye pigments, genomic DNA was obtained from the whole thorax and abdomen by the protocol above, except that all reagent volumes were doubled. Each dried pellet was rehydrated in 25 µl of rehydration buffer. One µl of each resulting solution was then diluted in 100 µl of the same rehydration buffer, and these dilutions were used in RAPD amplifications as described in

the following section. DNA fragments (such as the RAPD products used here) from multiple individuals may be pooled for use in the competent-cell transformation procedure (Strassmann *et al.*, 1996).

Microsatellite Identification

DNA was isolated from the whole thorax and abdomen of two virgin *E. foraminatus* females, and then reconstituted and diluted, using the protocol for whole insect body segments described above. An aliquot of each stock DNA solution was amplified via polymerase chain reaction, or PCR (Mullis & Faloona, 1987), using 26 RAPD primers (Operon Technologies OPB-3, OPC-11, OPC-13, OPC-17, OPD-1 through 20, and University of British Columbia UBC-406 and UBC-414). PCR amplifications were carried out in 50 µl reactions. Each reaction included 0.35 µl Platinum *Taq* polymerase with 5 µl of 10X PCR buffer (Invitrogen, catalog number 10966018), and 3 µl of the DNA solution described above. Final concentrations of other reagents were 2 mM $MgCl_2$, 0.1 mM dNTPs, and 0.2 µM of the appropriate RAPD primer. PCR reagent cocktails, consisting of all necessary reagents except for template DNA, were prepared in multiple volumes as needed immediately before use. Amplifications were performed using an MJ Research PTC-100 with an initial denaturation step at 94 °C for 1 minute, followed by 40 cycles of 94°C for 30 seconds, 35°C for 1 minute, and 72 °C for 2 minutes, and a final extension at 72°C for 30 minutes. For each product producing bands on a 1.5% agarose gel stained with ethidium bromide, Southern blots were prepared (Southern, 1975; Ausubel *et al.*, 1992). Single-stranded 24-

base DNA probes for 12 di- and tri-nucleotide sequences (Glenn, 1996) were labeled using a Gene Images 3' Oligolabelling and ECF Amplification System kit (Amersham Pharmacia, catalog numbers RPN 5750 and 5770) and hybridized to blots. Blots were viewed on a Molecular Dynamics Storm 860 fluorescent imager. For each RAPD product yielding a positive fluorescent signal on a blot, the corresponding RAPD PCR was repeated. These RAPD products were then used to transform bacteria using a TOPO-TA cloning kit (Invitrogen, catalog number K4500-01). Colony lifts (Glenn, 1996) were prepared from plates containing transformed bacteria, and probed as described above to identify positive bacterial colonies. Inserts from each of 50 positive colonies were amplified in a 50 µl reaction. Each reaction included 5 µl of 10X PCR buffer, 0.18 µl Platinum *Taq* polymerase, and a DNA sample obtained by touching a sterile wooden pick to a single bacterial colony. Final concentrations of other reagents were 1.5 mM $MgCl_2$, 0.1 mM dNTPs, 0.5 µM T7 primer, and 0.5 µM M13 primer. Amplifications were performed with an initial denaturation at 94°C for 5 minutes, followed by 35 cycles of 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 1 minute, with a final extension at 72°C for 5 minutes.

PCR products were then visualized by electrophoresis using 3% agarose gels prepared with 1X TBE buffer (Ausubel *et al.*, 1992) with 100V applied for 2 hours. After ethidium bromide staining, PCR products from twenty-four inserts with distinct sizes were identified by visual inspection. These products were sequenced with T7 and M13R primers, using the ABI PRISM BigDye Terminator kit (Applied Biosystems, catalog number 4390236) and an ABI PRISM 310 genetic analyzer. Sequences were screened for repeats, and to rule out the possibility that any two different inserts

contained identical or overlapping sequences, using ABI PRISM AutoAssembler 2.1. Four AT or GC repeat sequences, for which no probes were used, were also found during sequencing. Primers were designed for eight sequences, using Oligo 6.3 (Molecular Biology Insights). Primers were tested using touchdown PCR with initial denaturing step of 94°C for 1 minute, followed by 10 cycles of 94°C for 30 seconds, 60°C for 30 seconds decremented by 1°C per cycle, and 72°C for 45 seconds, and an additional 25 cycles of 94°C for 15 seconds, 50°C for 15 seconds, and 72°C for 30 seconds, with a final extension at 72°C for 5 minutes. Seven of the eight primer pairs yielded single products and were selected for fragment analysis. For each locus, fragment length was associated with a number of repeat units based on the length of the microsatellite as first identified by sequencing (for example, if a dinucleotide microsatellite known via sequencing to contain seven repeats was 115 base pairs long including amplified flanking regions, a 117-base-pair fragment amplified at this locus in a different individual was assumed to contain eight repeats).

DNA was isolated from one antenna of each of 30 females collected from separate nests in southwest Michigan, and each sample was rehydrated in 100 µl DNA rehydration solution. Each putative microsatellite locus was then amplified from each individual in a 15 µl reaction. Each reaction included 1.5 µl of 10X PCR buffer, 0.1 µl Platinum *Taq* polymerase, and 1 µl of the DNA solution described above. Final concentrations of other reagents were 2.5 mM $MgCl_2$, 0.13 mM dNTPs, 0.13 µM forward primer labeled with amine-reactive BCI dye (ResGen), and 0.13 µM unlabeled reverse primer. Amplifications were performed using the above touchdown PCR protocol.

Fragment analysis was performed on a Beckman Coulter CEQ 2000 XL DNA analyzer following the procedure described below.

Five of the seven loci were polymorphic in *E. foraminatus*. GenBank accession numbers, repeat motifs, forward and reverse primers, numbers of distinct alleles identified, and allele size ranges for this initial sample are shown in Table 1. This preliminary sample of 30 individuals was designed to assess microsatellite variability rather than for drawing conclusions about male diploidy, sex determination, or natural inbreeding levels, but provided sufficient information about microsatellite polymorphisms to identify the markers best suited to studying these phenomena in natural and laboratory-reared broods of *E. foraminatus*.

Table 1

Characteristics of Five *Euodynerus foraminatus* Microsatellite Loci From a Preliminary Sample of 30 Individuals

Locus	GenBank accession number	Repeat motif	Primer sequences (5'-3')	Alleles	Size range (bp)
Efo01	AF485776	$(CTT)_9$	Forward: GGAGAATCT GTCGAGTGTGAGAG Reverse: GCTCTTTCCTTTTCT TACGAATATG	3	194-200
Efo02	AF485777	$(AT)_7$	Forward: GAATTTATGAAATT ACGAATGAAACG Reverse: GTTACCACGATGTA TAGATATTAGG	6	200-210
Efo03	AF485778	$(GC)_5(GT)_{10}$	Forward: CATTTCAGAATAGT ATATGAATGTG Reverse: TTAATTTGTATATG CGTTGACACG	13	111-139
Efo04	AF485779	$(AG)_4N_4(AG)_3$ $N_2(AG)_4$	Forward: ATAAGCGATCAAAG ATAAGCGTC Reverse: TTCTACTTGACAGA ACTTGGCTC	12	211-245
Efo07	AF485782	$(AT)_5$	Forward: AAAATTCTGATCCA GATTCTATGG Reverse: GACTAAACGAAAG CGATTAGAG	4	182-188

DNA Fragment Analysis

After identification of microsatellites, fragment analyses were performed on wasps using DNA extracted from a single leg or antenna of each subject. Amplification of microsatellites from each sample of wasp DNA was performed using the same reagent cocktail (including dye-labeled forward primers) and PCR conditions described in the Microsatellite Identification section above. Dye labels for each forward primer were selected so that labeled fragments from each of the five loci would produce peaks with unique combinations of size range and display color when analyzed using the Beckman Coulter CEQ 200 XL. When amplifying DNA for fragment analysis, the final extension time at 72°C was increased from 5 minutes to 45 minutes to minimize the chances of producing spurious peaks. A pre-labeled 400 bp size standard (Beckman Coulter, catalog number 608098) was included as a reference in all analyses.

After PCR amplification with labeled forward primers, PCR products were loaded into wells of a 96-well sequencing plate. PCR products amplified with primer Efo01 were pre-diluted with sterile water (100-140 µl per 15 µl reaction) before loading, because the dye (D4) used to label the Efo01 forward primer typically produces much stronger peaks than do other labeling dyes. Multiple PCR products (each amplifying a different locus) per sample may be combined in the same well if desired, a procedure called pool-plexing. However, combining too many products in a single well may reduce fragment-analysis performance due to competition between products during injection into the capillary-electrophoresis apparatus. Therefore, whenever genotyping at more than three of the five loci was necessary, two different wells with different combinations of

PCR products were loaded per sample. The best-performing combinations of labeled fragments were empirically determined; combinations of PCR products from loci Efo01, Efo03, and/or Efo04 usually produced clear, highly readable spectra, as did combinations of Efo02 with Efo07. Dyes, screen-display peak colors, pre-loading dilutions and pool-plex compatibilities associated with each labeled DNA fragment are shown in Table 2.

After loading the appropriate volume of each PCR product into sequencing-plate wells, 40 µl of deionized formamide and 0.5 µl of pre-labeled 400 bp size standard were added to each well. (It is convenient to mix multiple volumes of the formamide-standard solution before loading it into sample wells, and leftover solution can be frozen at -20°C and then thawed on ice for later use.) Corresponding wells in a separate buffer plate were filled with separation buffer (Beckman Coulter, catalog number 608053) and both plates were then loaded into the instrument for fragment analysis, using the pre-programmed Frag-3 protocol with automatic fragment-length measurement.

The above protocols were used in all procedures, throughout this study, that required fragment analyses for microsatellite genotyping.

Table 2

Loading Volumes and Display Characteristics of Labeled Standards and PCR Products

Labeled fragment	Dye label	Peak display color	Pre-loading dilution (per 15 µl PCR reaction)	Loading volume per well (µl)	Pool-plex compatibility with other loci
400 bp size standard	D1	Red	None	0.5	Required in all analyses
Efo01	D4	Blue	Add 100-140 µl H_2O	1	Efo03, Efo04
Efo02	D3	Green	None	1 to 2	Efo07
Efo03	D2	Black	None	1 to 3	Efo01, Efo04
Efo04	D2	Black	None	1 to 3	Efo01, Efo03
Efo07	D2	Black	None	1 to 2	Efo02

CHAPTER III

SEX DETERMINATION IN *EUODYNERUS FORAMINATUS*

Introduction

The behavior of the solitary hunting wasp *Euodynerus foraminatus* presents a paradox; facultative inbreeding in a species suspected of having sl-CSD. Field observations of this species suggest that brother-sister matings are common. Females nest in cavities such as hollow twigs, in which they provision offspring sequentially in a linear series of cells. Within a nest cavity, mothers usually first provision an uninterrupted series of daughters in the innermost cells, followed by a series of sons in the outer cells. Males receive fewer provisions and develop more quickly than females, and emerge from the nest cavity before their sisters. Males wait at the nest entrance and copulate with emerging sisters; approximately 40% of females have been observed to mate with a brother before dispersal from the natal nest site (Cowan, 1979). Females become sexually unreceptive immediately after mating and will reject further mating attempts by any male (Cowan, 1986). Females not inseminated as they emerge from the natal nest mate at flowers with males that are unlikely to be related. Therefore, significant inbreeding occurs even though opportunities for outbreeding are available. We might therefore predict that *E. foraminatus* lacks sl-CSD.

Inside an *E. foraminatus* nest, the sequence of females in the innermost cells of the cavity is normally uninterrupted, but we have occasionally observed males positioned

between two sisters. These "out-of-order" males receive the allotment of food that is normal for a developing daughter, which is 50% greater than that normally allocated to a developing son in one of the outermost cells. Out-of-order males may be diploids that developed from fertilized eggs, an indication of CSD. Alternatively, they may be haploids which represent mistakes in the mother's oviposition sequence because the mother accidentally withheld sperm, or else they may be cases where the mother released stored sperm to fertilize the egg but fertilization failed, producing a haploid. In order to support the presence of CSD and differentiate between a single-locus and multiple-locus system, we must be able to (a) detect sex-ratio differences between inbred and outbred broods that are consistent with proportions of diploid male production expected under either sl-CSD or ml-CSD, (b) identify diploid males using genetic markers to detect heterozygosity in these individuals, (c) compare diploid male genotypes to those of their parents to demonstrate the presence of both maternal and paternal alleles, and (d) differentiate between sl-CSD and ml-CSD by determining the proportion of diploid offspring of sibling matings developing as males.

To test the hypothesis that *E. foraminatus* has sl-CSD, we performed breeding experiments to compare sex ratios between experimental (sib-mated) and control (outbred) sibships. We then genotyped parents and offspring using microsatellite markers, and made sex ratio comparisons between the broods produced from matched and unmatched sibling matings. We also compared copulation durations, tendency for mated females to nest, and the numbers of nests, provisioned cells and surviving offspring produced per nesting female. These comparisons were made in order to

determine whether either sibling mating itself or matched sex-allele status within sibling matings affected these variables.

Materials and Methods

Collection, Breeding, and Rearing of Wasps

We obtained breeding stock (parental generation wasps) from nature by constructing artificial cavities (trap nests) from sticks of wood with holes drilled in them (Krombein, 1967), and placing these sticks in trees and shrubs where wild females could locate them and use them as nest sites. *E. foraminatus* broods were collected from trap nests collected from six sites in southwest Michigan (Allegan, Barry, and Kalamazoo Counties) in the summer of 1995. Trap nests containing overwintering wasp larvae were kept outdoors through the spring of 1996 so that brood could develop to the pupal stage under natural conditions. Pupae were sexed and placed in separate vials to complete development to adulthood. At maturation, virgin females were caged for mating with either a nestmate (brother) or a male collected from a different site. All matings took place between 28 May 1996 and 1 June 1996. In this parental (F_0) generation, 16 sibling (experimental group) matings and 19 outbred (control group) matings were observed. Copulation durations were timed, beginning when the male mounted the female and ending when the pair unlinked their genitalia. Males were frozen at -80°C after mating. Females were maintained in 30 cm. by 30 cm. by 30 cm. screened rearing cages in a greenhouse with ambient temperature and natural photoperiod. Each female was

provided with three nesting sticks with dimensions of 1.9 cm. by 1.9 cm. by 9.0 cm and with 0.7 cm. diameter holes drilled to a depth of 7.5 cm.; females were also supplied with water, honey, clay soil for making cell partitions, and caterpillars for provisioning nest cells (Chilcutt & Cowan, 1993). Prey caterpillars were eastern spruce budworm larvae (*Choristoneura fumiferana*) that had been reared on artificial diet (Bio-Serv Inc., Frenchtown, NJ). As females nested, we removed the completed nests and replaced them with empty nest sticks. Nesting females were maintained until mid-August, when the experiment was terminated and all nesting females were frozen at -80°C.

Completed nests were first opened several weeks after they were closed by the mother, and inspected regularly afterward to monitor brood development. Developing wasps were sexed at the pupal stage and placed in individual vials until they reached adulthood. Each nest cell containing either unconsumed prey (indicating an egg that did not hatch) or a dead larva was recorded as a dead offspring of undetermined sex. Any male with a nest position further from the entrance than that of a sister was identified as being out of normal nest order. At maturity, each of these F_1 offspring was weighed and then frozen at -80°C.

Microsatellite Genotyping

DNA isolations and microsatellite development were carried out as described in Chapter II. Fragment analysis was performed on a Beckman Coulter CEQ 2000. All parents were genotyped at seven microsatellite loci, of which five were variable and four were informative for this study. Within each family, offspring were genotyped at one to

four of the loci Efo01 (GenBank accession number AF485776), Efo02 (AF485777), Efo03 (AF485778), and Efo04 (AF485779) based on differences between maternal and paternal genotypes. All sons in each sibship were genotyped. Sons were classified as diploid if they were heterozygous at one or more microsatellite loci. Also, at least one daughter per sibship (when available) was genotyped in order to confirm that heterozygotes could be detected via microsatellite polymorphisms and that microsatellite alleles were inherited biparentally, following Mendelian patterns. In one inbred sibship, the mother escaped midway through the season and was unavailable for genotyping; her genotype was deduced from the genotypes of her mate, one son, and one daughter.

Sibling matings in the parental (F_0) generation were classified as matched if they produced diploid sons, and unmatched otherwise. We then compared copulation duration, number of nests produced, number of offspring provisioned, and number of offspring surviving to maturity between inbred and outbred sibships, and between matched (diploid-male producing) and unmatched (non-diploid-male-producing) sibling matings to determine whether either nestmate recognition or sex-allele matching affected reproductive behavior or success.

Statistical Tests

All statistical tests were performed following methods described in Zar (1999) unless otherwise noted. All 2 X 2 contingency tables were analyzed using a two-tailed Fisher exact test. All chi-square tests with one degree of freedom were performed using the Yates correction. Before data from multiple sibships was pooled for contingency

testing, a heterogeneity chi-square was performed to determine whether pooling this data was statistically valid. For t-tests with unequal variances, degrees of freedom were estimated according to Zar (1999).

Results

Sib-mating Effects on Reproductive Behavior and Success

Fourteen of 16 sib-mated mothers and 13 of 19 outbred mothers established nests, showing no significant difference between sib-mated and outbred mothers in tendency to nest (Fisher exact test, $p = 0.24$). Copulation durations, number of nests completed, number of nest cells provisioned, number of offspring surviving to be sexed at the pupal stage, relative proportions of surviving and dead offspring, and sex ratio were then compared between nesting sib-mated ($n = 14$) and nesting outbred ($n = 13$) females. There were no significant differences between sib-mated and outbred groups in copulation time, number of nests completed, number of nest cells provisioned, number of offspring surviving to be sexed at the pupal stage, or mortality (Table 3). These results show that neither reproductive behavior nor overall reproductive success is affected by sibling mating as compared to outbreeding. The relative proportions of sons and daughters were significantly different between the inbred and outbred groups, because of an excess of males in the inbred group when compared to the outbred control.

Table 3

Comparisons Between Sib-mated and Outbred Nesting Mothers

Category	Sib-mated (n = 14)		Outbred (n = 13)		Test	p
	Mean	SD	Mean	SD		
Copulation time (seconds)	68.1	20.6	82.2	39.3	t	0.27
Nests completed (per female)	10.4	4.9	10.5	6.5	t	0.96
Offspring (nest cells) provisioned (per female)	38.1	17.2	38.3	20.3	t	0.98
Offspring surviving to be sexed (per female)	30.4	15.8	31.8	22.4	t	0.86
Total living offspring	426		413		Fisher exact test	0.20
Total dead offspring	108		85			
Total sons	212		129		Fisher exact test	< 0.0001
Total daughters	214		284			

Sex Ratio Comparisons

We performed statistical tests to determine whether offspring sex ratios of the sib-mated and outbred mothers were consistent with sl-CSD, two-locus ml-CSD, or neither. Sib-mated mothers produced a total of 426 offspring, of which 212 were male and 214 female, while the control mothers produced a total of 413 offspring, of which 129 were male and 284 female. Heterogeneity chi-square tests on the control group ($p = 0.99$) and on the experimental group ($p = 0.97$) showed that sex ratio data, when compared among sibships within each group, were sufficiently homogeneous to be pooled for subsequent analyses.

To determine expected sex ratios under sl-CSD, we assumed that sib-mated mothers produced the same proportion of fertilized eggs as did outbred mothers, but that in the inbred group 25% of these fertilized eggs would develop as diploid males instead of as females. Similarly, to determine expected sex ratios under two-locus ml-CSD, we assumed that in the inbred group 6.25% of fertilized eggs would develop as diploid males instead of as females. Observed numbers of males and females (Table 4) were consistent with a null hypothesis of sl-CSD ($p = 0.61$) but not with that of two-locus ml-CSD ($p < 0.0001$). As the observed proportion of males is too large to be accounted for by two-locus CSD, and because ml-CSD models involving more than two loci predict even fewer males, the overall sex-ratio difference between inbred and outbred broods supports sl-CSD but does not support the presence of any form of CSD involving two or more loci.

Table 4

Goodness of Fit Tests for Single-locus and Two-locus CSD Models (Expected Values Based on Outbred Sibship Sex Ratios)

Category		Inbred broods
Experimental results (inbred wasps)	Males	212
	Females	214
Expected with single-locus CSD	Males	206
	Females	220
	p (χ^2)	0.61
Expected with two-locus CSD	Males	151
	Females	275
	p (χ^2)	< 0.0001

Microsatellite DNA Analyses

Outbred Broods

Microsatellite fragment analyses showed that none of the sons in outbred sibships were diploid. In all twelve outbred sibships that produced sons, parental genotypes were sufficiently distinct so that all biparental diploids would have been detected during genotyping. Eleven of these twelve sibships also produced daughters; genotyping of one daughter from each of these sibships identified each of these eleven females as a biparental diploid, confirming that microsatellite fragment analysis could unambiguously identify biparental heterozygotes. No out-of-order males were found within nests in outbred sibships.

Inbred Broods

In a brother-sister mating, the two members of the mated pair are more genetically similar to each other than are the members of an outcrossed pair, and on average, offspring of a sibling mating will be homozygous at more loci than will the offspring of an outcrossing. Based on the presence of at least one diploid son, six of the 14 inbred sibships were identified as products of parents matched at the sex alleles, and in four out of these six matched inbred sibships, it was possible for a diploid offspring to be homozygous at each of the loci scored. If the mother is heterozygous at a locus and the father shares one of her alleles, then, on average, only one half of the offspring will be

heterozygous at that locus and there will be a 50% chance of missing a diploid male by scoring that locus alone. If the mother is heterozygous at two loci and the father shares one allele at each, there will be an 25% chance of missing a diploid male, and, for three loci fitting this description, there will be a 12.5% chance of missing a diploid male. The chances of failing to identify true diploids within these sibships ranged from 0 (in the two sibships where parents shared no alleles at at least one locus) to 0.25 (in one sibship where the parents shared an allele at each locus and the mother was heterozygous at only two loci).

Among the 14 inbred sibships, genotyping of one or more daughters per sibship also clearly identified them as biparental diploids whenever parental genotypes were sufficiently distinct to do so. Among the six sibships produced by matched matings, 25 males developed out of normal nest order, and a total of 51 males were confirmed diploid by genotyping. The other eight inbred sibships produced no out-of-order or diploid males, and were therefore determined to be unmatched. This is consistent with the hypothesis that 50% of sibling matings are matched at a single sex allele (χ^2, $p = 0.78$) as would be expected under sl-CSD, although this 6:8 ratio of matched to unmatched sibling matings is not sufficient to rule out the alternative of two-locus CSD in which 25% of sibling matings are matched at both sex alleles and could therefore produce diploid males (χ^2, $p = 0.21$).

Of the 25 males from matched sib-mated sibships that developed out of normal nest order, 20 were among the 51 males confirmed diploid, four could not be confirmed either haploid or diploid because maternal and paternal genotypes were sufficiently similar to produce a homozygote at all loci tested, and one was an apparent true haploid.

In addition, two males from inbred sibships, in nests that contained no sisters, were out of order with respect to other males that were confirmed diploid. Neither of these males could be confirmed either haploid or diploid based on parental genotypes.

The six males which occurred out of normal nest order, but could not be confirmed either haploid or diploid, were classified as suspected diploid males. Confirmed and suspected occurrences of diploid males among the six matched inbred sibships, and the probabilities that genotyping would fail to identify diploid males in each sibship, are summarized in Table 5.

Table 5

Total Diploid Offspring (Daughters and Diploid Sons) of Matched Sibling Matings

Sibship	Daughters	Confirmed diploid sons	Confirmed + suspected diploid sons	Proportion of diploid sons expected to be missed
1	12	10	13	0.125
2	15	16	16	0
3	16	13	13	0
4	1	2	2	0.125
5	16	6	8	0.125
6	7	4	5	0.25

When matched sibling matings were compared to unmatched sibling matings, there were no significant differences between these subgroups in copulation duration, number of nests completed or cells provisioned, numbers of offspring surviving to be sexed, or mortality rate (Table 6). Proportions of sons and daughters were significantly different between the two groups ($p < 0.0001$) due to a higher proportion of sons in the matched group. The proportions of sons and daughters (69 sons, 145 daughters) in the

unmatched sibling-mated group was identical to the proportions of sons and daughters (129 sons, 284 daughters) in the outbred group (Fisher exact test; $p = 0.86$).

Table 6

Comparisons Between Matched Sib-mated and Unmatched Sib-mated Nesting Mothers

Category	Matched (n = 6)		Unmatched (n = 8)		Test	p
	Mean	SD	Mean	SD		
Copulation time (seconds)	72.8	33.9	65.1	6.7	t	0.64
Nests completed (per female)	12.0	4.6	9.2	5.0	t	0.32
Offspring (nest cells) provisioned (per female)	45.2	17.2	32.9	6.2	t	0.20
Offspring surviving to be sexed (per female)	35.7	13.8	26.5	16.9	t	0.30
Total living offspring	214		212		Fisher exact test	0.67
Total dead offspring	57		51			
Total sons	145		69		Fisher exact test	< 0.0001
Total daughters	67		145			

To further rule out non-complementary sex determination systems, it is necessary to show that diploid males are produced from fertilized eggs. In order to determine

without ambiguity that a diploid is biparental, the father must possess at least one allele not present in the mother, and this paternal allele must be detectable in the diploid offspring. For example: If a male carrying the $(CTT)_8$ allele at Efo01 mates with a female (homozygous or heterozygous) which does not carry $(CTT)_8$, an offspring carrying both $(CTT)_8$ and one maternal allele is an unambiguously biparental diploid.

When siblings mate, it is often the case that the members of the pair are genetically so similar that the male and female share an allele at each scorable locus. Unambiguous identification of biparentality was thus possible for some but not all of the diploid offspring of sibling matings. Thirty-two of the 51 diploid males were confirmed biparental by the presence of at least one paternal allele that was not present in the mother. The other 19 heterozygous (and therefore diploid) males could not be confirmed biparental in this manner because the mother and father shared an allele at each locus heterozygous in the offspring. However, under the assumption that sex determination is uniform throughout the species, other possible explanations of how these diploid males arose seem unlikely. For example, if diploidy resulted from duplication of the haploid genome of an unfertilized egg, these males would have been homozygous at all loci (Beukeboom & Pijnacker, 2000). If they were produced from unfertilized diploid eggs that carried the entire maternal genome, they would all be clones of the mother, and thus develop as females instead of as males (Bull, 1983). Finally, although fusion of maternal meiotic products (Beukeboom & Pijnacker, 2000) cannot always be ruled out when mother and offspring have the same (heterozygous or homozygous) genotype at one or more loci, each of the 19 males that could not be directly confirmed biparental had a genotype that could be explained by fertilization of a normal haploid egg by normal

haploid sperm from the mother's mate. Therefore, it is likely that fertilization produced these 19 diploid males as well as the 32 that were unambiguously confirmed biparental.

Proportion of Diploid Males in Matched Inbred Broods

In order to determine whether matched inbred broods exhibited the 50% diploid male to 50% female sex ratio expected under sl-CSD, goodness-of fit comparisons for each CSD model of interest (sl-CSD and two-locus ml-CSD) were performed twice. For the first set of comparisons, we counted as diploid only those males confirmed diploid by heterozygosity at at least one microsatellite locus. For the second set of comparisons, we counted as diploid both genetically confirmed diploid males and the six additional males whose genotypes were inconclusive but whose out-of-order nest positions strongly suggested that they were diploid. Heterogeneity chi-square tests showed that sex ratio data was homogeneous across sibships, whether considering only genetically confirmed diploid males ($p = 0.55$) or both confirmed and suspected diploid males ($p = 0.72$). Data from all six sibships was therefore pooled. The results (Table 7) were consistent with the 50% male/50% female ratio expected under sl-CSD for confirmed diploid males ($p = 0.20$) and confirmed plus suspected diploid males ($p = 0.29$), but not consistent with the 25% diploid male to 75% female ratio expected under two-locus ml-CSD for confirmed diploid males ($p < 0.0001$) or for confirmed plus suspected diploid males ($p < 0.0001$). Therefore, sl-CSD is supported, while two-locus CSD is rejected. Since two-locus CSD was rejected because of an excess of diploid males, ml-CSD is also rejected for numbers of loci greater than two.

Table 7

Goodness of Fit Tests for Single-locus and Two-locus CSD Models Based on Matched and Unmatched Inbred Sibship Sex Ratios

Category		Confirmed only	Confirmed plus suspected
Experimental results (matched matings)	Diploid males	51	57
	Females	67	67
Expected with single-locus CSD	Diploid males	59	62
	Females	59	62
	p (χ^2)	0.16	0.26
Expected with two-locus CSD	Diploid males	29.5	31
	Females	88.5	93
	p (χ^2)	< 0.0001	< 0.0001

Diploid Male Viability

The identification of diploid males made it possible to pool diploid offspring of both sexes across maternal mating categories in order to determine whether overall diploid survival to maturity differed, either between sib-mated and outbred mothers or between matched and unmatched sib-mated mothers. This permits assessment of whether diploid males have normal or reduced viability. Outbred mothers produced a total of 284 surviving diploid offspring, all of them daughters. Among sib-mated mothers, those in unmatched matings produced 145 surviving diploid offspring, all of them daughters. Sib-mated mothers whose matings were matched produced 67 daughters and 51 confirmed diploid sons, a total of 118 surviving diploids (or 57 diploid sons and 124 surviving diploids if the six suspected diploid males were included). Therefore, the overall diploid production by sib-mated mothers was 145 + 67 + 51 = 263 diploids if only confirmed diploid males were included, and 145 + 67 + 57 = 269 diploids if suspected diploid males were included.

For each test, the expected number of diploids was calculated using a null hypothesis of no difference between the inbred and outbred groups, thus, each mother would account for the same number of diploid offspring. Because 14 mothers were sib-mated and 13 were outbred, the expected number of diploids produced by sib-mated mothers was equal to 14/27 (52%) of the total diploids produced, while the expected value of diploids produced by control mothers was equal to 13/27 (48%) of the overall total. Similarly, because six sib-matings were matched and eight were unmatched, the expected number of diploid offspring produced by matched sib-mated mothers was equal

to 6/14 (43%) of all diploids produced by sib-mated mothers, while the expected number of diploids produced by unmatched sib-mated mothers was 8/14 (57%) of all diploids produced by sib-mated mothers (Table 8). No difference was found between the numbers of diploids surviving to maturity in inbred and outbred sibships. Also, no difference was found when diploid survival was compared between matched and unmatched matings. Diploid males therefore have viability equal to that of diploid females.

Table 8

Comparisons of Diploid Offspring Survival by Maternal Mating Type

	All sibling matings	All non-sibling matings	Matched sibling matings	Unmatched sibling matings
Observed confirmed diploids	263	284	118	145
Expected total confirmed diploids	284	263	113	150
p (Fisher exact test)	0.23		0.66	
Observed confirmed + suspected diploids	269	284	124	145
Expected total confirmed + suspected diploids	288	265	116	153
p (Fisher exact test)	0.28		0.54	

Size of Diploid Males

Identification of diploid males also made it possible to compare their weights to those of diploid females and haploid males. After all males were genotyped, average live weights were calculated for each of three categories (females, haploid males, and confirmed diploid males). The mean weight of diploid males (N = 51; mean weight = 52.0 ± 16.9 mg.) was intermediate between those of diploid females (N = 479; mean weight = 70.1 ± 12.2 mg) and haploid males (N = 275; mean weight = 38.1 ± 10.3 mg.). Weights were compared via single-factor analysis of variance, which showed a significant difference ($F = 594.7$, $p < 0.0001$). Means differed significantly between each pair of groups according to the Tukey test with unequal sample sizes (Zar, 1999).

Discussion

These results support the hypothesis that sl-CSD is the sex determination system of *Euodynerus foraminatus*, while ruling out alternative hypotheses of ml-CSD and non-complementary systems. The presence of biparental diploid males and the difference in sex ratio between inbred and outbred families together falsify alternative hypotheses of non-complementary sex determination systems, such as genomic imprinting or genic balance. The proportions of diploid males produced by sibling-mated mothers support the presence of sl-CSD rather than ml-CSD. Although diploid males have been observed in other vespid wasps (Strassmann *et al.*, 1994; Chapman & Stewart, 1996), this is the

first detailed study of a vespid to confirm sl-CSD by quantifying both a sex-ratio shift and the production of diploid males.

These results also indicate that sib-mated females suffer no decrease in nesting tendency, fecundity, or offspring viability as compared to outbred females. Also, when considering only sibling matings, nesting tendency, fecundity, and offspring viability do not differ between matched and unmatched matings. Therefore, neither sibling mating itself nor the presence of matched sex alleles in a sibling pair affects reproductive behavior or overall fecundity. Copulation durations, while highly variable, do not differ significantly between sibling and non-sibling matings, nor between matched and unmatched sibling matings, indicating that neither nestmate recognition nor matched sex-allele recognition affects the duration of mating behavior.

Overall numbers of diploid offspring do not differ between sib-mated and outbred mothers, nor between the offspring of matched and unmatched sibling matings. This is consistent with a model in which diploid males arise from fertilized eggs which were "intended" to be daughters but developed as males due to sex-allele matching, and also shows that diploid male viability is not reduced relative to that of normal diploid females.

When weights of females, diploid males, and haploid males were compared, the mean weight of diploid males was intermediate between the mean weights of the smaller haploid males and the larger females. The adult size of diploid males therefore differs from that of both diploid females and haploid males. This may be related to either provisioning, sex-specific developmental rates, or both; in *E. foraminatus*, nesting females provide more prey items to cells containing fertilized eggs than to those

containing unfertilized eggs, while normal haploid males mature more quickly and at a smaller size than do normal diploid daughters (Cowan, 1981, 1983).

These results present an apparent paradox, because the combination of inbreeding with sl-CSD will increase the number of presumably non-functional diploid males. Beukeboom (2000) has proposed that loss of sl-CSD may result from selection imposed by inbreeding, because inbreeding has potentially fewer deleterious effects under non-complementary sex determination systems. For example, chalcidoid wasps in the genera *Melittobia* (Schmieder & Whiting, 1947; Werren, 1993) and *Nasonia* (Werren, 1980, 1983; Dobson & Tanouye, 1998) are known to lack any form of CSD; these species experience inbreeding due to local mate competition (Hamilton, 1967), in which the offspring of only one or a few mothers mate among themselves in a patchy environment. Within the clade consisting of the Ichneumonoidea and the aculeate hymenopteran superfamilies, several species have also been shown to have non-complementary sex determination. These species include two ichneumonoids in the family Braconidae, *Asobara tabida* and *Alysia manducator*, which may experience inbreeding (Beukeboom *et al.*, 2000). An example of a non-CSD species is also known within the aculeate clade. The bethylid *Goniozus nephantidis* lacks CSD; this species is a gregarious parasitoid which, like many other species with this life history, is known to inbreed (Cook, 1993a; Luft, 1996). All of these examples show that non-CSD species can be distributed among taxonomic groups in which sl-CSD species also occur. If sl-CSD is indeed an ancestral condition in the Hymenoptera, these examples indicate that sl-CSD can be secondarily lost.

Conversely, the presence of sl-CSD should select against inbreeding. Some braconids have sl-CSD (Speicher & Speicher, 1940; Whiting, 1943), and one such species, *Bracon hebetor*, has known behavioral mechanisms for sib-mating avoidance despite being a gregarious parasitoid with ample sib-mating opportunities (Antolin & Strand, 1992; Ode *et al.*, 1995). Other behaviors and life history traits are believed to reduce the incidence and/or deleterious effects of sib-mating in other sl-CSD species. For example, ichneumonids in the genus *Diadegma* have sl-CSD but are solitary parasitoids (Butcher *et al.*, 2000a). Because individuals in a solitary parasitoid species are scattered throughout their environment instead of developing on the same host as do their siblings, they presumably have a lower chance of mating with siblings than do gregarious parasitoids. Honey bees (*Apis mellifera)*, which also have sl-CSD, mate far from the natal nest and thereby presumably avoid copulating with siblings (Koeniger, 1986). Also, honey bee queens are polyandrous (Page, 1980; Tarpy & Page, 2002); while polyandry reduces only the variance (and not the overall incidence) of diploid male production, it may minimize the cost of inbreeding to individual queens by making sperm from unrelated males available even if sperm from a close relative is also present in the spermatheca.

Despite the apparent incompatibility between sl-CSD and inbreeding, both sl-CSD and sibling mating persist in *E. foraminatus*. It is possible that sibling mating may have benefits that outweigh its disadvantages even when sl-CSD is present. For example, by mating with her brother, a female increases her own genetic representation in her offspring (and therefore lowers the cost of sex), because many of her own maternally-derived genes are also present in her brother's gametes (Cowan, 1979). It is

also possible that, by mating with her brother immediately after emerging as an adult, a female can produce fertilized eggs as soon as possible while reducing risks associated with prolonged mate-seeking. This behavior would not necessarily prevent the female from mating again at a later time. In some hymenopteran species, multiple mating may reduce overall diploid male production by an already sib-mated female by making sperm from a non-relative available (Tarpy & Page, 2002). However, *E. foraminatus* females seem to become unreceptive immediately after a single mating (Cowan, 1979, 1986). It is possible that mated females become receptive again after an extended period of time; it also remains unknown whether *E. foraminatus* females in nature take advantage of any possible benefits of multiple matings.

Inbreeding levels within natural populations of *Euodynerus foraminatus* have yet to be quantified. Cowan (1979) estimated from field observations that approximately 40% of matings in this species take place between known siblings at their natal nests, while the rest occur among unrelated individuals, following dispersal from the natal nest site. However, it is possible that non-siblings may develop in the same nest, under some conditions that are not easily determined by visual inspection. For example, in some wasps, females may usurp partially completed nests of conspecific females, resulting in broods whose members are not all siblings (Cowan, 1981). Thus, individuals emerging from the same nest cavity might not be siblings, resulting in cryptic outbreeding at nest entrances.

Individual cases of cryptic outbreeding are difficult to identify, but true inbreeding and outbreeding levels in a natural population can be estimated by measuring departures from Hardy-Weinberg equilibrium (Robertson & Hill, 1984). In the next

phase of this study, departures from Hardy-Weinberg equilibrium were measured using microsatellite allele data.

CHAPTER IV

INBREEDING IN A NATURAL POPULATION

Introduction

It has been shown in Chapter III that *Euodynerus foraminatus* has single-locus complementary sex determination, a system normally incompatible with inbreeding because it increases the production of abnormal diploid males (Cook, 1993b). Previous observations of *E. foraminatus* in nature have indicated that the proportion of sibling matings is approximately 40%, with the remainder of matings taking place between unrelated males and females which have dispersed from the natal nest site (Cowan, 1979). Therefore, the presence of sl-CSD in *E. foraminatus* is paradoxical, as other sl-CSD species often exhibit inbreeding-avoidance behavior (Antolin & Strand, 1992; Ode *et al.*, 1995) and other hymenopteran species whose life histories include high levels of inbreeding have been shown to have non-complementary sex determination systems (Dobson & Tanouye, 1998; Beukeboom *et al.*, 2000).

It is unknown whether *E. foraminatus*, in nature, has mechanisms for minimizing the potential costs of inbreeding under sl-CSD. For example, diploid male production by each individual could be minimized if previously sib-mated *E. foraminatus* females were to mate again later, after dispersal, with an unrelated male (polyandry). Under completely random mating (panmixis), polyandry merely reduces the variance, rather than the overall incidence, of diploid male production; polyandry results in occasional

diploid male production by many mothers, while monogamy results in concentrated diploid male production by only those mothers with matched matings (Tarpy & Page, 2002). However, if a female first copulates with her brother and only later mates with an unrelated male, that female reduces the proportion of her diploid offspring that will develop as males, because she now has sperm available from a second male who is much less likely than her brother to share one of her sex alleles. This strategy would be especially effective in a species with last-in, first-out sperm precedence, in which the male that most recently mated with a polyandrous female fathers the majority of her offspring, and which is a common situation in insects (Parker, 1970; Allen *et al.*, 1994). However, there is evidence that single mating is the rule for *E. foraminatus* females. Cowan (1979) observed that *E. foraminatus* females become sexually unreceptive immediately after a single mating. Furthermore, in nests containing multiple males, emerging males compete to be the first to mate with emerging females; one male displaces his brothers, becomes resident at the nest entrance, and mates with as many emerging females as possible. This competition by males to be the first to mate with virgin females also suggests that females mate only once, or, alternatively, that subsequent matings are less important than the first to the production of fertilized eggs.

Cryptic outbreeding may occur if a male-female nestmate pair does not always consist of a brother and sister. This could result from such behaviors as nest usurpation (Cowan, 1981), in which a female takes over a nest which already contains eggs or young of another female, or conspecific brood parasitism ("egg dumping"), in which a female deposits and then abandons one or more eggs in the nest of another female (Tallamy, 1985; Tallamy & Horton, 1990). If nest usurpation or egg dumping takes place among

conspecifics, many pairs that mate at their natal nest entrances could actually be unrelated, so that the presumption that all mating pairs at nest entrances consist of a brother and sister would be incorrect.

Previous field observations of *E. foraminatus* mating behaviors may also have been confounded by the use of artificial trap nests as a study method. The average dimensions of natural cavities used as nests, and their spatial distribution in nature, are unknown, simply because natural nests are difficult to find. Trap nests (Krombein, 1967; Cowan, 1979) are typically used to attract nesting females, observe nesting and emergence behavior, and sample wasp populations; trap nest depth and distribution may not reflect natural conditions. If trap nests tend to contain more or fewer offspring than natural nests, or are more or less likely than natural nests to contain both males and females, then observations of sibling mating at the entrances of artificial trap nests may not accurately reflect the actual levels of sibling mating at natural cavity nests. If the spatial distribution of artificial nests used in previous studies differs from that of natural nests, then the area containing artificial nests may inaccurately reflect the actual density of natural nests, the number of females nesting in the study area, or the average distance between nests produced by the same mother.

It is thus unknown whether either cryptic outbreeding or the use of artificial trap nests has affected previous estimates of sibling mating as observed at natal nests of *E. foraminatus*. Even if these factors are unimportant, complex reproductive behaviors such as polyandry, nest usurpation, egg dumping, and the selection and utilization of natural cavities are difficult to observe and quantify in a population of small, highly mobile flying insects. As an alternative to direct measurement of the relative proportions of

inbreeding and outbreeding, inbreeding levels can be estimated by using genetic data from molecular markers to measure departures from Hardy-Weinberg equilibrium (Robertson & Hill, 1984). Because brothers and sisters share alleles identical by descent from their parents, their offspring will be, on average, homozygous at more loci than the offspring of unrelated mated pairs. Therefore, in a population with high levels of inbreeding, we would expect an excess of homozygotes (heterozygote deficit) relative to Hardy-Weinberg expectations (Rousset & Raymond, 1995; Robertson *et al.*, 1999). DNA microsatellite markers, because of their presumed neutrality and high variability, are especially useful for identifying heterozygotes. Microsatellites tend to be highly polymorphic even in species or populations with low variation at other genetic markers such as allozymes (Hughes & Queller, 1993). As shown in Chapter III, DNA microsatellites can often detect heterozygosity even in diploids whose parents were siblings, and which are therefore likely to be heterozygous at fewer loci than offspring of an outcrossed mating.

In order to estimate natural inbreeding levels in a southwestern Michigan population of *Euodynerus foraminatus*, we collected offspring of free-flying wild wasps and genotyped them using the microsatellite markers described in Chapter II. Parents were free-flying wild wasps which emerged from natural nests and mated under natural conditions. By sampling and genotyping one female (diploid) per nest, it was possible to calculate F-statistics (Wright, 1951; Weir & Cockerham, 1984) and to estimate the percentage of matings taking place among siblings. We also identified which males in these nests were diploid, based on whether they were out of order in the nest, heterozygous at one or more microsatellite loci, or both.

Materials and Methods

Sampling Protocols

Collection of Material from Nature

Trap nests (Krombein, 1967) were placed at three field sites in Allegan and Kalamazoo Counties (Michigan, USA) during the summers of 1996 and 1997. Site 1 was separated from Site 2 by 9.2 km., Site 2 from Site 3 by 29.2 km., and Site 3 from Site 1 by 34.3 km. Wasps which nested in trap nests were free-flying females which had emerged and mated under natural conditions. Completed nests were removed and replaced with fresh empty sticks approximately every ten days throughout the flight season; consequently, no wasps completed development, emerged, or mated at these artificial trap nests. Nests containing immatures were first opened several weeks after closure by the mother, and brood development was monitored until wasps reached the pupal stage. Individuals were then sexed and placed in separate vials for development to adulthood. Fifty-two nests contained males only, 43 nests contained females only, and 59 nests contained both sexes, for a total of 111 nests containing males and 102 nests containing females. At maturation, each adult wasp was frozen at -80°C.

Sampling Females for Inbreeding Coefficient Determination

Because determination of an inbreeding coefficient requires comparisons of homozygous and heterozygous diploids, only females were genotyped for this phase of the study. One female was genotyped from each of the 102 nests that contained female offspring. Twenty-one of these nests were collected from Site 1, 41 nests from Site 2, and 41 nests from Site 3. DNA was isolated from one antenna or leg of each sampled female as described in Chapter II. Each female was genotyped at five loci; Efo01 (GenBank accession number AF485776), Efo02 (AF485777), Efo03 (AF485778), Efo04 (AF485779), and Efo07 (AF485782).

Sampling Males to Determine Diploid Male Count

From each of the 111 nests containing males, we isolated DNA (as described above) from the available male furthest from the nest entrance. Twenty-two of these males were in position 1 (the completed nest cell furthest from the nest entrance), nineteen available males were in position 2, twenty-one were in position 3, and the remainder were in position 4 or higher (closer to the entrance). Males were classified as diploid males if they were either out of order in the nest, heterozygous at one or more microsatellite loci, or both. Whenever a male that was not out of order was shown to be a heterozygote, all available brothers of that diploid male were also genotyped.

Microsatellite Allele Binning

All fragments containing alleles of the trinucleotide microsatellite Efo01 were assigned (binned) to the nearest whole repeat unit (Heath *et al.*, 2002) (see Chapter II for description of repeat units); only two of 102 individuals had allele lengths which did not correspond closely to an integral number of repeats, so their unusual repeat-unit lengths were rounded to the nearest whole number. However, many heterozygotes for the dinucleotide microsatellites Efo02 and Efo07 carried alleles that differed by a fractional number of repeats (for example, two peaks that occurred one, three or five base pairs apart). Repeated PCR amplifications and fragment analyses showed that these fractional-repeat peaks were clearly and repeatably associated with individuals, and not with reagent batches or with DNA amplification or analysis conditions. Binning alleles at these loci to the nearest whole repeat would have misclassified many clearly heterozygous individuals as homozygotes; this would result in artificially high estimates of inbreeding levels. Therefore, two different binning schemes were used and compared for dinucleotide microsatellites:

1. All alleles at Efo02 and Efo07 were binned to the nearest single base (Frydenberg *et al.*, 2002), while all alleles at Efo03 and Efo04 were binned to the nearest dinucleotide repeat unit.

2. All alleles at all four dinucleotide microsatellite loci (Efo02, Efo03, Efo04, and Efo07) were binned to the nearest single base.

The former procedure is more conservative in its definition of what constitutes an allele; that is, it assigns a larger number of alleles to a locus only for those loci at which

heterozygotes with allele lengths 1 bp apart are clearly occurring. The latter procedure is a more consistent method for binning alleles at dinucleotide loci because it treats all dinucleotide loci in the same manner. Under this second method, additional distinct alleles were defined for all four of the dinucleotide microsatellites (Table 9).

Analyses of Population Data

Comparisons of population data to expectations under Hardy-Weinberg equilibrium were performed using GENEPOP (Raymond & Rousset, 1995). Analyses of population F-statistics were performed using FSTAT (Goudet, 1995). All calculations were performed twice; first using single-base allele binning for Efo02 and Efo07 only, and then using nearest-single-base allele binning for all four of the dinucleotide microsatellites Efo02, Efo03, Efo04, and Efo07. Because wasps were collected from three separate sites within the region of interest, the value of F_{ST} (Wright, 1951), estimated as Weir and Cockerham's θ (Weir & Cockerham, 1984), was calculated to determine whether population subdivision was present among the three collection sites. In the absence of significant population subdivision, data from all sites can be combined for calculation of the overall inbreeding coefficient F_{IS} (Wright, 1951), estimated as Weir and Cockerham's f (Weir & Cockerham, 1984). Values of F_{IS} for individual collection sites were also calculated. The value of F_{IT} (Wright, 1951), estimated as Weir and Cockerham's F (Weir & Cockerham, 1984), was also calculated to determine whether the probability of allele identity by descent in the combined population was similar to that of F_{IS} (which takes into account the identification of individuals by collection site). Because

microsatellite markers were used, values of Slatkin's R_{ST} and R_{IS} (Slatkin, 1995; Balloux & Lugon-Moulin, 2002), were also calculated for the same data using GENEPOP, and compared to corresponding values of F-statistics. These R-statistics, developed by Slatkin, are analogues of Wright's F-statistics, but take into account the presumed stepwise mutation that leads to length variation in microsatellite alleles.

Results

Allele Diversity

Allele diversity was calculated across all 102 genotyped individuals, using both allele-binning schemes, which differed only in the numbers of alleles identified at loci Efo03 and Efo04 (Table 9). In one individual, amplification of locus Efo01 failed repeatedly; amplification of Efo04 failed repeatedly in a different individual. In both cases, peaks for these loci were visible in the output of the fragment analyzer, but these peaks were poorly defined and could not be clearly interpreted as representing one allele or more than one.

Table 9

Homozygotes, Heterozygotes, and Allele Diversity at Five Microsatellite Loci

Locus	Observed homo-zygotes	Observed hetero-zygotes	Did not amplify	Number of alleles	
				Single-base binning used for Efo02 and Efo07 only	Single-base binning used for all four dinucleotide loci
Efo01	71	30	1	7	7
Efo02	45	57	0	9	9
Efo03	30	72	0	16	28
Efo04	27	74	1	15	25
Efo07	54	48	0	8	8

Hardy-Weinberg Equilibrium

A one-tailed test for heterozygote deficiency (Louis & Dempster, 1987; Raymond & Rousset, 1995) was performed using GENEPOP, under the null hypothesis of no heterozygote deficiency as compared to Hardy-Weinberg expectations (Table 10). This test was performed on data pooled from all collection sites. Significant heterozygote deficiency was present at all loci under both binning schemes. Observed heterozygote deficiency may be due to either inbreeding or population subdivision (Hartl & Clark, 1997), so tests for population differentiation (Wright, 1951; Weir & Cockerham, 1984) were also performed as described in the next section.

Table 10

P-Values for One-Tailed Test for Heterozygote Deficiency

Locus	Single-base binning used for Efo02 and Efo07 only	Single-base binning used for all four dinucleotide loci
Efo01	< 0.001	0.001
Efo02	< 0.001	< 0.001
Efo03	0.001	< 0.001
Efo04	< 0.001	< 0.001
Efo07	< 0.001	< 0.001

Population Differentiation

When nearest-single-base binning was used for only Efo02 and Efo07, the overall value of population differentiation (Weir and Cockerham's θ, analogous to Wright's F_{ST}), as calculated with FSTAT for the three populations, was 0.003. When nearest-single-base binning was used for all four dinucleotide microsatellites, the overall value of population differentiation value for the three populations was also 0.003. The 95% confidence intervals as determined by bootstrapping included zero in both cases (Table 11). Therefore, all three collection sites can be considered to be parts of the same population, and the observed heterozygote deficiency is likely to be due to consanguinity, not population differentiation among collection sites. Data from all three collection sites (all 102 females) was combined in order to estimate inbreeding coefficients.

Inbreeding Coefficient

When nearest-single-base binning was used for only Efo02 and Efo07, the inbreeding coefficient (Weir and Cockerham's *f*, estimating Wright's F_{IS}) for the population was 0.284 (Table 11). When nearest-single-base binning was used for all four dinucleotide microsatellites, the inbreeding coefficient for the population was 0.298.

Inbreeding coefficients were also calculated separately for each collection site; for all three sites, separately calculated inbreeding coefficients were similar to the overall inbreeding coefficient, and by-site 95% confidence intervals all overlapped with that of the overall inbreeding coefficient for all three sites. Under both binning schemes, values

of Weir and Cockerham's F (estimating Wright's F_{IT}) were similar to values of the inbreeding coefficient (F_{IS}), and 95% confidence intervals overlapped; the calculated probability of alleles being identical by descent was therefore not dependent on whether or not the data were subdivided by collection site, nor was this calculated probability affected by the difference between the two binning methods. F-statistics, including values for each locus and 95% confidence intervals for the overall values, are shown in Table 11. Usage of the estimators F, θ, and f (as estimators of, respectively, F_{IT}, F_{ST}, and F_{IS}) follows that of Weir and Cockerham (1984), This more detailed analysis using F-statistics further supports the conclusion that the observed heterozygote deficiency (as compared to Hardy-Weinberg equilibrium expectations) is due to mating between relatives, not population differentiation due to fragmentation.

Table 11

Population Differentiation and Inbreeding Coefficients

	Single-base binning used for Efo02 and Efo07 only			Single-base binning used for all four dinucleotide loci		
	F (F_{IT})	θ (F_{ST})	f (F_{IS})	F (F_{IT})	θ (F_{ST})	f (F_{IS})
Efo01 (all sites)	0.444	-0.001	0.445	0.444	-0.001	0.445
Efo02 (all sites)	0.312	0.004	0.310	0.312	0.004	0.310
Efo03 (all sites)	0.213	0.020	0.197	0.248	0.011	0.239
Efo04 (all sites)	0.162	-0.002	0.164	0.196	0.006	0.192
Efo07 (all sites)	0.377	-0.007	0.382	0.377	-0.007	0.382
All loci, Site 1	--	--	0.261	--	--	0.274
All loci, Site 2	--	--	0.248	--	--	0.263
All loci, Site 3	--	--	0.327	--	--	0.341
Overall	0.286	0.003	0.284	0.300	0.003	0.298
Lower 95% CI for overall value	0.210	-0.004	0.205	0.238	-0.003	0.233
Upper 95% CI for overall value	0.383	0.012	0.384	0.383	0.008	0.384

R-Statistics

R-statistics were calculated, using GENEPOP (Raymond & Rousset, 1995), for the same data used in the calculation of F-statistics. The overall value of R_{IS} as estimated by Rousset's ρ_{IS} (Rousset, 1996) was 0.344 when only Efo02 and Efo07 were binned to the nearest base pair, and 0.290 when all dinucleotides were binned to the nearest base pair. Both of these estimates fall within the 95% confidence intervals calculated for F_{IS}; therefore, both methods produce consistent results for inbreeding coefficients. Values of R_{ST} (as estimated by Rousset's ρ_{ST}) were 0.029 when only Efo02 and Efo07 were binned to the nearest base pair, and -0.012 when all dinucleotides were binned to the nearest base pair. This disparity is more difficult to interpret but is consistent with the higher variability reported for R-statistics (Balloux & Lugon-Moulin, 2002). Because the range between these two estimates included zero, they are interpreted as being consistent with previous F_{ST} calculations showing no differentiation among collection sites.

Sibling Mating

Based on previous behavioral observations of nestmate mating (Cowan, 1979), calculated inbreeding levels were assumed to be wholly or primarily due to sibling mating. The lower of the two estimates of the inbreeding coefficient ($f = 0.284$) obtained from single-base-pair binning for Efo02 and Efo07 only, was used to estimate α, the level of sibling mating using the equation $\alpha = 4f/(3f+1)$ (Pamilo, 1985; Paxton *et al.*, 2000).

This yields an estimate of $\alpha = 0.613$, equivalent to approximately 61% of all matings in this population taking place between siblings while the remaining 39% are random matings.

This calculation was repeated for the lower and upper bounds of the 95% confidence interval for f (as calculated with single-base-pair binning for Efo02 and Efo07 only). For the lower bound ($f = 0.205$), $\alpha = 0.508$; for the upper bound ($f = 0.384$), $\alpha = 0.71.4$; therefore, sibling matings are estimated with 95% confidence to account for between 50.8% and 71.4% of all matings in this population.

Diploid Male Production

A total of twelve diploid males were identified from seven nests. Five of these males were heterozygous at at least one of the five microsatellite loci used for genotyping. In each of the five cases, this male was the only confirmed diploid male; one diploid male had three non-diploid brothers, two males each had one non-diploid brother, and two diploid males had no brothers. The other seven presumed diploid males were out-of-order males; four in one nest and three in another. None of these out-of-order males could be confirmed heterozygous at any of the five loci used for genotyping.

Under the estimated 61% sibling-mating level, 30.5% of matings are expected to be matched, and therefore we would expect 0.305 X 0.5 = 15.25% of all diploids in this sample to be male. Twelve diploid males and 199 females were observed, for a total of 211 diploids. The expected number of diploid males would therefore be 15.25% of 211, or 32, and the expected number of females would be 211 - 32, or 179. These observed

numbers differ significantly from expectations (χ^2, $p < 0.0001$). This apparent shortage of diploid males could not be interpreted with confidence because in many cases the innermost nest cells, which are those most likely to contain diploids, experienced high mortality.

Discussion

Population Genetics

These results demonstrate significant departures from Hardy-Weinberg equilibrium due to an estimated 61% of all matings in this population taking place between siblings. This is an even larger proportion of sibling matings than the 40% observed at trap nests by Cowan (1979). If cryptic outbreeding had been present, genetic data would have revealed a lower, not higher, proportion of sibling mating than this previous estimate. Because the parents of the sampled broods were free-flying, naturally-mated wasps from natural nests, these results should be unaffected by the use of artificial trap nests used to collect their offspring.

Although the three collection sites were separated by distances ranging between 9.2 and 34.3 km., the negligible value of F_{ST} shows that there is no significant genetic differentiation among the three collection sites; they were therefore considered as a single population. *E. foraminatus* is a strong-flying insect which is expected to have considerable opportunity for dispersal when seeking either mates (in cases where males encounter unmated females at flowers after both have left their natal nests) or nesting

sites. Furthermore, the reproductive lives of mated females under laboratory conditions have exceeded two months (Chapter III); if mothers in nature produce nests over a wide distance during a long lifespan, young adults emerging from nearby nests may have a low probability of being related, thus reducing the chances of close relatives encountering each other and mating away from nest sites. Significant opportunities for dispersal and outbreeding across a wide geographic distance are therefore likely in this population. However, sibling mating persists, as indicated by the combination of a negligible F_{ST} (no population subdivision over the distance sampled) and a high F_{IS} consistent with between 50% and 71% of matings occurring between siblings. In short, both sibling mating and random mating appear to coexist in this population.

Results obtained with different allele binning schemes, and between estimates of F-statistics and R-statistics, were statistically indistinguishable. This demonstrates that the microsatellite genotyping methods used in this study were robust under slightly different interpretations of natural allelic variation and its sources (Slatkin, 1995; Balloux & Lugon-Moulin, 2002)

Diploid Male Production

Based on the calculated sibling-mating level of 61% and the occurrence of 131 females in the nests that contained males, we would expect to find 32 diploid males in those nests (as well as an occasional diploid male that was the product of a rare, sex-allele-matched outbred mating). However, only twelve diploid males were found. Paxton (2000) found a similar diploid male deficiency compared to expectations in a

natural population of a bee species, *Andrena scotica*, in which microsatellite analysis suggested that 44% of matings were among siblings (with expected diploid male production of 11% if sl-CSD is present), while observed diploid male production was only 0.03%. Paxton suggested three possible explanations for this phenomenon: (a) multiple-locus rather than single-locus CSD, (b) "sex-allele signaling" in which siblings could recognize a shared sex allele and therefore be more likely to mate when the mating would be unmatched, and (c) sperm selection, in which unmatched sperm has a higher probability than matched sperm of being used by the mother to fertilize an egg, if both types of sperm are present in the spermatheca. Breeding experiments (Chapter III) have ruled out ml-CSD as a sex-determination mechanism in *E. foraminatus*. In order to determine whether sex-allele signaling influences acceptance of a sibling as a mate, a mate-choice experiment would be required, in which females are presented with a choice between a brother known to be matched at the sex allele and another brother known to be unmatched. Similarly, for a controlled test of the hypothesis of sperm selection, it would be necessary to determine directly whether matched sperm and unmatched sperm had different probabilities of fertilizing an egg. The possibility that either sex-allele signaling or sperm selection occurs in *E. foraminatus* can therefore not be determined from data obtained in this study.

In laboratory breeding experiments (Chapter III), it was also clear that, under controlled conditions, diploid males do not suffer increased mortality with respect to their siblings of normal ploidy. It cannot be determined from this data whether high mortality in innermost cells in this natural population indicates lower survivorship of diploid males in nature. Innermost cells appear to be heavily affected; it is possible that high mortality

due to parasitism primarily impacted diploids of both sexes in this population, although this cannot be quantified from the available material. Therefore, conclusions cannot be drawn from this sample regarding the causes of diploid male deficiency.

Consequences of Inbreeding

Because *E. foraminatus* is a species with sl-CSD, and because half of all sibling matings are expected to be matched at the sex allele, more than 30% of the matings in this population are expected to be matched as the result of sibling mating; since 50% of diploids in a matched mating develop as males, then 15% of all diploids in this population should be male rather than female The loss of female sexual receptivity after a single mating has been previously documented (Cowan, 1986). However, even if re-mating occurred among the mothers of sampled broods in this population, the effective level of sibling mating remains extremely high. Therefore, *Euodynerus foraminatus* should incur a high cost of diploid male production, unless there are other mitigating factors present. The question remains: Why do inbreeding and sl-CSD persist together in this species?

The cost of diploid male production under sl-CSD is one manifestation of the more general phenomenon of inbreeding depression, in which inbreeding leads to homozygosity at deleterious recessive alleles, thereby increasing the frequency of low-fitness homozygous genotypes relative to that of high-fitness heterozygous genotypes. Inbreeding depression is widely recognized as a selective pressure leading to inbreeding avoidance in animals (Foster, 1992; Pusey & Wolf, 1996). In some cases, inbreeding

depression may be underestimated because the most homozygous (and therefore most affected) individuals are inviable and are thus neither observed nor sampled in the course of population studies (Keller & Waller, 2002). We would therefore expect inbreeding avoidance to be a general phenomenon, and one especially pronounced in an sl-CSD species where the average sib-mated female loses one-fourth of her investment in daughters because sterile diploid males are produced instead.

However, inbreeding may have benefits as well. In a sibling mating, a female acquires her brother's sperm, which contains many alleles identical by descent to her own and therefore increases her genetic representation in her offspring (Cowan, 1979; Greeff, 1996). Also, when brothers and sisters mature in close proximity, sibling mating may be the most efficient way for a female to find a mate while minimizing the hazards and energy expenditure associated with mate-seeking over long distances.

Long-term effects of inbreeding may also be mitigated by haplodiploidy itself. In haplodiploids, even rare deleterious alleles are regularly exposed to selection in the haploid sex (normal males) and presumably eliminated quickly, while heterosis (heterozygote advantage, normally associated with outbreeding) cannot be an important factor in the fitness of any traits important to both sexes (Bruckner, 1978). In fact, haplodiploidy may reduce the costs of inbreeding because haplodiploid populations recover more quickly than full diploids from the transient genetic load imposed by the first few generations of inbreeding, and can more readily take advantages of any benefits of inbreeding (Werren, 1993). Nevertheless, there is ample documentation of inbreeding avoidance by other hymenopteran species with sl-CSD (Antolin & Strand, 1992; Foster,

1992; Ode *et al.*, 1995), and even Werren's 1993 treatment emphasizes sex-determination load as an obstacle to the transition from outbreeding to inbreeding.

Hymenopteran species whose life histories include presumed or confirmed high levels of natural inbreeding tend to have non-complementary sex determination systems (Cook, 1993a; Dobson & Tanouye, 1998). For example, the braconids *Alysia manducator* and *Asobara tabida* lack CSD (Beukeboom *et al.*, 2000), while their relative *Bracon hebetor* has sl-CSD (Whiting, 1943); it has been suggested that *A. manducator* and *A. tabida* are inbreeding species (Beukeboom *et al.*, 2000), while *B. hebetor* is known to have inbreeding avoidance mechanisms (Antolin & Strand, 1992). These observations, combined with the known distribution of sl-CSD across over 40 species in multiple hymenopteran superfamilies, support a model in which sl-CSD was ancestral in the Hymenoptera but has been lost multiple times under selection such as that imposed by inbreeding (Cook, 1993b).

The persistence of both sl-CSD and inbreeding in *Euodynerus foraminatus* suggest that unknown mitigating factors may be present. As shown in Chapter III, neither sibling mating alone nor the presence of a matched sex allele affects nesting behavior, fecundity, fertilization rate, or offspring survival. If other traits exist that mitigate the costs of inbreeding in this sl-CSD species, they may include previously unsuspected mechanisms, such as higher-than-expected diploid male fertility.

CHAPTER V

FERTILITY OF DIPLOID MALES AND THEIR DESCENDANTS

Introduction

It has been shown in Chapters III and IV that *Euodynerus foraminatus* has single-locus complementary sex determination as well as exhibiting high inbreeding levels in natural populations. If diploid males in *E. foraminatus* are the genetic dead ends that they are known to be in other species (Crozier, 1977; Cook & Crozier, 1995; Beukeboom, 2001) then diploid males represent a heavy cost to their parents because diploid sons cannot transmit parental genes. In addition, if diploid males produce abnormal diploid sperm but can still copulate with females, a singly-mated female whose mate is a diploid male will incur a loss in fitness. If the diploid sperm cannot penetrate the egg, the mate of a diploid male will fail to produce daughters (MacBride, 1946); if diploid sperm can fertilize eggs, the resulting daughters will be triploid and therefore sterile due to aneuploidy during meiosis (Inaba, 1939; Smith & Wallace, 1971). The data presented in previous chapters show that diploid *E. foraminatus* males have viability comparable to that of other offspring. If adult diploid males are found in nature and can indeed mate, then fertilization failures and/or triploid female production are expected to contribute to sex determination load.

The observed association of sl-CSD and high levels of inbreeding in *E. foraminatus* raises the possibility that diploid males may not be as costly as previous

work with other species suggests. To test this possibility, we conducted a breeding experiment to compare the fertility of diploid and haploid males, and of any descendants they might produce.

Materials and Methods

Collection, Pairing, and Rearing of Wasps

Wasps were obtained from nature by placing trap nests (Krombein, 1967) at five locations in Allegan and Kalamazoo Counties in southwest Michigan. Trap nests were constructed as described in Chapter III. Each collection site was separated by at least five kilometers from any other collection site. Completed nests (identified by a visible mud plug at the entrance) were collected and opened in the laboratory. Larval development was monitored as described in Chapter III. At pupation, wasps were sexed, removed to individual glass vials, and reared to adulthood. Wasps reared from the same nest were presumed to be siblings, and wasps from separate collection sites were presumed to be unrelated. Sibling pairs selected for mating were confined in screened cages two to three days after adult eclosion, and observed to confirm that copulation took place. Individual females were placed in screened cages and provisioned with food, caterpillars, and nesting materials as described in Chapter III.

Experimental Breeding Protocol

The parental (F_0) generation was defined as the free-flying wasps that produced broods in trap nests in the field; wasps reared from these trap nests constituted the F_1 generation as shown in Figure 1 (Cowan & Stahlhut, in preparation). Thirty-one nestmate (sibling) pairs from the F_1 generation were mated as described above. Under sl-CSD, half of these matings were expected to be matched at the sex allele, and therefore to produce diploid offspring with a 1:1 male:female ratio, as well as normal haploid males, in the F_2 generation. The other half was expected to be unmatched, and therefore to produce only haploid males and diploid females in the F_2 generation. Both control (haploid) and experimental (diploid) F_2 males were offspring of sibling matings, to ensure that any possible inbreeding effects unrelated to paternal diploidy would be uniform in subsequent generations. To avoid introducing any further inbreeding effects, all pairings in subsequent generations were between wasps whose ancestors nested at different field locations.

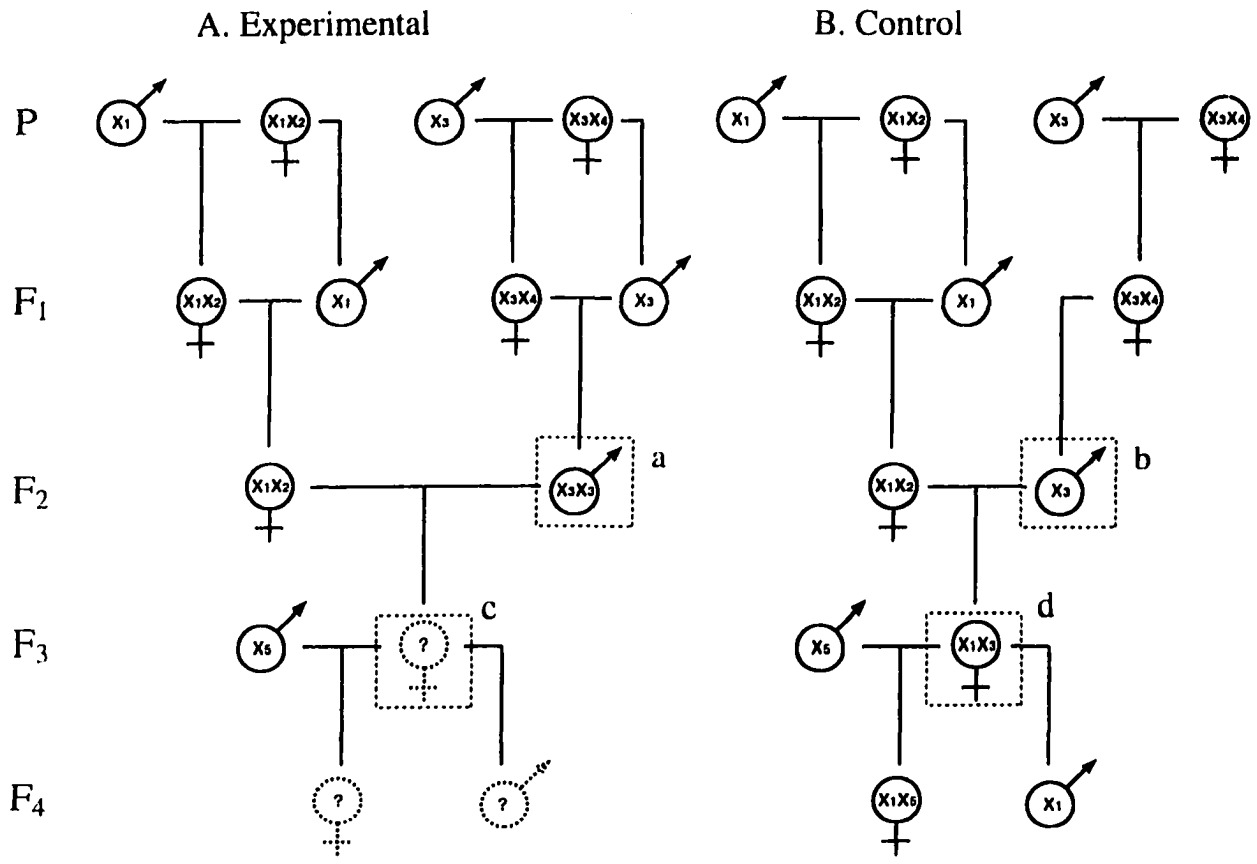

Figure 1. Breeding Protocol for Determining Fertility of Diploid Males and Their Descendants

To determine whether diploid males were fertile, haploid and diploid F_2 males were mated to unrelated females, which produced offspring of the F_3 generation. Because diploid F_2 males fathered F_3 daughters, fertility of these daughters was tested by mating them to unrelated males to produce the F_4 generation. Mating durations and offspring production were recorded for laboratory-mated pairs, and statistical analyses performed according to methods described in Zar (1999) and in Chapter III.

Assignment of Males to Experimental and Control Groups

Experimental and control groups were defined based on whether diploid (experimental) or haploid (control) males from the F_2 generation were the fathers of F_3 females. The ploidy of male F_2 wasps was determined by genotyping at the microsatellite loci Efo01, Efo02, Efo03, Efo04, and Efo07. The F_1 matings that produced F_2 sons were classified as matched if they produced either (a) any out-of-order males or (b) any males determined to be diploid because of heterozygosity at one or more microsatellite loci. F_1 matings were classified as unmatched if they produced (a) no diploid males and (b) nests with an uninterrupted series of daughters followed by an uninterrupted series of sons; the underlying assumptions were that, in matched matings, each diploid had a 50% chance of being male and that diploids would be interior to haploids in the nest. For example, if a nesting F_1 female produced a nest containing three daughters in the innermost positions and two sons in the positions nearest the entrance (FFFMM), a second nest with the sequence FFMM, and a third nest with the sequence FFFFM, the probability, or

p(matched), that this sequence of sexes resulted from a matched mating was $(1/2)^6$ or 1/64, because the three nests together have six females interior to other females.

Results

Identification and Characteristics of Matched and Unmatched Matings

Thirty-one brother-sister pairs from the F_1 generation were mated to produce the F_2 generation. Three of the 31 females did not nest, and two others produced insufficient numbers of offspring to determine whether these matings were matched or unmatched. Of the remaining 26 matings, 14 were classified as matched because they produced sons that were out of order, heterozygous, or both. The other twelve were classified as unmatched because they produced no out-of-order males, and because their probabilities of actually being matched (see Methods section) ranged from $(1/2)^5$ to $(1/2)^{22}$. These observed values of 14 matched and 12 unmatched matings agree with the prediction that in the presence of sl-CSD, half of all sibling matings will be matched at the sex allele and the other half unmatched (χ^2; $p = 0.84$).

Offspring of matched and unmatched matings were significantly different in sex ratio, with an excess of sons found in broods produced by matched matings (Table 12). Sex ratios within individual broods, expressed as the ratio of living sons to living daughters, ranged from 0.47 to 0.65 in matched matings and from 0.07 to 0.33 in unmatched matings. This lack of sex-ratio overlap between broods classified as matched

and those classified as unmatched further supports the method described above of classifying matings by whether or not they produced diploid or out-of-order males.

The mean number of cells provisioned by females with matched (32.3) and unmatched (28.2) matings did not differ significantly (t -test; $p = 0.15$). In matched matings, out of 452 provisioned cells, 386 offspring survived to maturity (85% survivorship), while in unmatched matings, 302 offspring from 338 cells survived (89% survivorship). These numbers of surviving and dead offspring are not significantly different between matched and unmatched matings (Fisher exact test, $p = 0.12$), supporting previous observations (Chapter III) that diploid male survival is comparable to that of male and female offspring of normal ploidy.

Table 12

Sex and Survival of Offspring of Matched and Unmatched Matings

	Matched matings (n = 14)		Unmatched matings (n = 12)	
	Total	Mean (per mother)	Total	Mean (per mother)
Living daughters	167	11.9	255	21.2
Living sons	219	15.6	48	4.0
Total living offspring	386	27.6	302	25.2
Dead offspring	66	4.7	36	3.0
Total cells provisioned	452	32.3	338	28.2

When data from matched matings were pooled, 167 daughters and 219 sons were found; in unmatched matings, 255 daughters and 48 sons (all presumed haploid) were found. Under sl-CSD, the number of daughters and the number of diploid sons in matched matings should be equal. Therefore, in matched matings, the expected number of diploids is 167 X 2, or 334, while the expected number of haploids is 219 - 167, or 52. When these expected numbers of haploids (52) and diploids (334) produced by matched matings are compared to the observed numbers of haploids (48) and diploids (255) produced by unmatched matings, sex ratio is consistent with the expectation under sl-CSD that half of diploids developed as males in the matched-mated broods (Fisher exact test, $p = 0.91$). These observations further support the initial assessment of which matings were matched and which were unmatched.

Classification of Males by Ploidy

Diploid Males

Males were ruled diploid if they were either out of normal nest order, heterozygous at one or more of the five microsatellite loci available for scoring, or both. A total of 21 F_2 males were classified as diploid. Of these 21 males, seven were ruled diploid by genotyping (heterozygosity) alone, two by nest position alone, and twelve because they were both out of normal nest order and heterozygous at one or more loci.

Haploid Males

A total of 18 males of the F_2 generation were classified as haploid based on either nest position or genotype. Males were ruled haploid (without genotyping) if they were offspring of unmatched matings, identified as those which produced large numbers of females in sequence without any out-of-order males present. For each such mating, a p(matched) was calculated as described above. Eleven of the 18 males were ruled haploid by this method. Two of these eleven came from matings with a p(matched) of $(1/2)^8$, one from a mating with a p(matched) of $(1/2)^{10}$, one from a mating with a p(matched) of $(1/2)^{14}$, two from matings with a p(matched) of $(1/2)^{16}$, one from a mating with a p(matched) of $(1/2)^{21}$, and four from matings with a p(matched) of $(1/2)^{22}$.

The remaining seven of these 18 males were ruled haploid by genotyping, when they were found to have only maternal alleles but came from matings in which paternal alleles could be unambiguously identified.

Reproduction by Diploid Males and Their Mates

Copulation, Nest Provisioning, and Offspring Survival

When presented with virgin females, all 21 diploid males and 16 of 18 haploid males readily mounted females and copulated with them. For males that mated, there was no difference between haploid and diploid males in copulation time; mean copulation time was 81 seconds for haploids and 80 seconds for diploids (t-test; $p = 0.86$). Females

mated to diploid males provisioned on average 37.1 nest cells, compared with an average of 32.1 cells for females mated to haploid males; this difference is not statistically significant (t-test; $p = 0.09$). When offspring survivorship was compared between the two groups, females mated to diploid males produced 466 offspring surviving to maturity from 631 provisioned cells, while females mated to haploid males produced 317 surviving offspring from 417 provisioned cells. These proportions are equivalent (Fisher exact test; $p = 0.47$). Therefore, nest provisioning and offspring survival did not differ between the mates of diploid and haploid males.

Reproductive Success of Diploid Males and Their Mates

Daughters. Diploid males fathered on average 16.4 daughters each, while haploid males fathered on average 21.2 daughters each. This difference is not statistically significant as determined by a two-tailed t-test ($p = 0.10$). However, when individual diploid males were considered, production of daughters ranged from 0 to 36, while the range for haploids was narrower (7 to 32) and did not include zero.

Sons. When females mated to diploids were compared with females mated to haploids, there were significant differences between groups in the number of sons produced. Females mated to diploids produced on average 11.0 sons each, while females mated to haploids produced an average of 3.2 sons each (t-test; $p = 0.01$). However, because these pairings were between non-relatives, it is likely that all of these sons were haploid (carrying only maternal genes) and therefore did not reflect directly on male reproductive success (but see below).

Sperm utilization. When the first half of each female's nesting life is compared to the second half, all females produce fewer daughters and more sons during the latter half of nesting life. Females mated to haploid males produced on average 11.5 daughters during the first half of nesting, and 9.7 daughters during the second half, a slight numerical difference that is not statistically significant (t-test; $p = 0.06$). For females mated to diploid males, the reduced production of daughters was more dramatic and statistically significant; these females produced on average 11.0 daughters during the first half of nesting and 5.3 during the second half (t-test; $p < 0.0001$). This may indicate that diploid males transfer smaller amounts of functional sperm than haploid males; it is possible that females mated to diploid males may run out of sperm over the course of their reproductive lives.

Overall fertility of fathers. In this experiment, the difference in daughter production between the mates of haploid and diploid males is not statistically significant based on the results of the two-tailed test (described above), but son production and sperm utilization differ significantly between the two groups. Therefore, fertility of diploid males is interpreted relative to that of normal haploid males; diploid males have 77% of the fertility of haploid males ($16.4/21.2 = 0.773$).

Daughters of Diploid Males

Ploidy

In eight pairings of diploid males and their mates, we observed combinations of microsatellite alleles that permitted unambiguous identification of their F_3 daughters as diploid or triploid. Microsatellite genotyping showed that all of these daughters were diploid rather than triploid, and that each carried one maternal and one paternal allele. These broods were too small to test for consistency with Mendelian segregation, but these data imply that either diploid males can produce haploid sperm, or else some other mechanism results in the presence of only one paternal chromosome set in the zygote.

Reproductive Capabilities

Daughters in the F_3 generation exhibited similar mating behaviors whether their fathers were haploid (n = 15) or diploid (n = 19); all mated quickly when presented with an unrelated male. Mean copulation times were 92 seconds for the daughters of haploids and 117 seconds for the daughters of diploids (t-test; $p = 0.08$.)

Of the 34 F_3 females used in mating experiments, 12 of the 19 females with diploid fathers nested, compared to 12 of the 15 with haploid fathers (χ^2; $p = 0.49$). Nesting females with diploid fathers provisioned on average 24 cells, while females with haploid fathers provisioned on average 25 cells (t-test; $p = 0.37$). Daughters of diploid males provisioned 286 nest cells that produced 168 surviving offspring, while daughters

of haploid males provisioned 300 nest cells that produced 168 surviving offspring (Fisher exact test, $p = 0.50$). These data show that fertility and offspring viability do not differ when daughters of haploid males are compared to daughters of diploid males.

Discussion and Conclusions

Previous results have demonstrated that *Euodynerus foraminatus* has both single-locus CSD and a high level of inbreeding in nature, and that diploid males of *E. foraminatus* have viability equivalent to that of haploid males and diploid females. The results in this chapter show that diploid *E. foraminatus* males can also mate normally and father diploid daughters, and that these daughters have normal viability and fertility. No triploids have been observed among offspring fathered by diploid *E. foraminatus* males. This contrasts with previous studies of other hymenopterans with sl-CSD, in which diploid males were shown to have low viability (Petters & Mettus, 1980), low fertility (Duchateau & Marien, 1995; Krieger *et al.*, 1999), or to produce diploid sperm (Woyke & Skowronek, 1974; Yamauchi *et al.*, 2001) that cannot penetrate the egg (MacBride, 1946) or else results in sterile triploid offspring (Inaba, 1939; Smith & Wallace, 1971; Krieger *et al.*, 1999).

The average fertility of diploid males in this study was approximately 77% of the average fertility of haploid males. Although all *E. foraminatus* females switch to producing more sons later in their nesting lives, the magnitude of this switch is much larger for the mates of diploid males. Taken together, these observations may indicate

that females mated to diploid males may have less stored sperm available than if they had mated with haploid males.

Previous studies of sperm production in hymenopterans have shown that spermatogenesis begins with a reductional division (meiosis I) which is aborted during metaphase I (Woyke & Skowronek, 1974; Crozier, 1975; Hogge & King, 1975), as only one set of chromosomes is present in a normal haploid male. The diploid sperm observed in diploid males of other species (MacBride, 1946; el Agoze *et al.*, 1994; Yamauchi *et al.*, 2001) is presumably the result of the reductional division aborting even when two sets of chromosomes are present in the spermatogonium. If this is the case, spermatogenesis in these other sl-CSD species follows the same path regardless of whether it occurs in a haploid male or in a diploid male. However, either of the two alleles at a microsatellite locus can be passed by an *E. foraminatus* male to his daughters. This suggests that a complete reductional division occurs during spermatogenesis in *E. foraminatus* diploid males. Alternatively, it is possible that one paternal chromosome set from a diploid sperm is eliminated after fertilization, resulting in a diploid zygote; in contrast, when a zygote is fertilized by haploid sperm, the paternal chromosome set is known to be retained because diploid offspring of haploid males are biparental (Chapter III). Either of these processes implies a mechanism for recognizing ploidy, either of the spermatogonia during meiosis or of the zygote after fertilization. The result is the development of biparental diploids regardless of the father's ploidy. *E. foraminatus* may therefore be polymorphic for either the mode of sperm production or the fate of paternal chromosomes after fertilization. These possible polymorphisms are previously unknown in Hymenoptera.

Some variation in diploid male reproduction is found in other sl-CSD species. One example is the rare production of diploid daughters by diploid males of the ichneumonid *Diadromus pulchellus*, although fertilization by the sperm of *D. pulchellus* diploid males much more commonly produced inviable zygotes (of unknown ploidy) than viable diploids (el Agoze *et al.*, 1994). If a species with these characteristics were to start inbreeding, the frequency of diploid males would increase and the variation in their reproductive abilities would permit selection for increased diploid male fertility.

Several previous studies of natural hymenopteran populations have revealed unexpectedly high diploid male frequencies (Ross & Fletcher, 1985; Kukuk & May, 1990; Chapman & Stewart, 1996; Roubik *et al.*, 1996; Zayed & Packer, 2001), and have attributed diploid male production either to close inbreeding or to loss of sex-allele diversity by drift in small isolated populations. Because either of these circumstances can result in high diploid male production, they can also strengthen selection for an increase in diploid male fertility. Consequently, these species may be of interest as candidates for having fertile diploid males.

Single-locus CSD has been confirmed in hymenopteran species which represent a wide taxonomic distribution, leading to speculation that is is the ancestral form of sex determination in the modern Hymenoptera (Cook, 1993b), secondarily lost by some taxa under selection pressures perhaps most commonly related to inbreeding (Cook, 1993b; Cook, 1993a; Beukeboom *et al.*, 2000). In the model proposed by Bull (1981), haplodiploid arrhenotokous parthenogenesis (the production of haploid males from unfertilized eggs) could plausibly have arisen from a fully diploid, ZZ/ZW (male homogametic) ancestral sex determination system in which fertile males were normally

diploid. If haploid males could be produced parthenogenetically and had a fitness greater than one-half that of diploid males, mothers producing haploid sons could reduce the cost of sex, and haploid male production would be favored. Under this model, only later in evolutionary time would diploid males lose reproductive abilities, possibly because of loss-of-function mutations, or else because of other mutations that are beneficial to haploid males in a single dose but have deleterious effects in diploids. The relatively high fertility of diploid *E. foraminatus* males may therefore be a case in which the ancestral condition of diploid male fertility was retained. Or, it could represent the loss and later re-acquisition of one or more traits controlling fertility, such as a complete reductional division during meiosis, or even the evolution of a novel mechanism such as loss of one paternal chromosome set after fertilization (Reed & Werren, 1995; Dobson & Tanouye, 1998). The evolution of these traits is unlikely to result from any specific advantage of male diploidy under the model in which haploid males are favored if their fitness is greater than one-half that of diploid males (Bull, 1981); diploid males with 77% of the reproductive fitness of haploid males would thus not be favored in *E. foraminatus*. It is more likely that the retention or reacquisition of high levels of fertility in diploid *E. foraminatus* males simply offsets some of the sex-determination load in this facultatively inbreeding species.

Some important questions remain about the reproductive genetics and mating behavior of *E. foraminatus*. For example, if diploid male fertility is an evolutionary reversal, it may result from reactivation of long-dormant genetic mechanisms, but may also have been restored by more recently evolved processes. The actual mechanism of

ploidy reduction, and whether it occurs during spermatogenesis or after fertilization, also remains to be identified and characterized.

The initial expectations of this study were that *Euodynerus foraminatus* would either have a sex-determination mechanism other than sl-CSD or else have extremely low levels of inbreeding in nature, because of the expected high costs of diploid male production when consanguineous matings occur in a species with sl-CSD. Instead, *E. foraminatus* has been shown to have sl-CSD, to exhibit high natural levels of sibling mating, and to produce diploid males which have near-normal fertility and father fully viable and fertile diploid (rather than sterile triploid) daughters. This is the first observation of this combination of characteristics in any species. The long-held assumption that diploid males in any hymenopteran species with sl-CSD must have zero or near-zero reproductive fitness can therefore no longer be taken for granted.

Appendix

Permission to Use Exempt Recombinant DNA Technology

Department of Biological Sciences

Kalamazoo, Michigan 49008-3899

WESTERN MICHIGAN UNIVERSITY

December 3, 2002

TO WHOM IT MAY CONCERN

This is to certify that all experiments involving recombinant DNA included in the Ph.D. thesis entitled "Inbreeding, Male Diploidy, and Complementary Sex Determination in the Solitary Wasp *Euodynerus foraminatus*" involved only wasp and commercially available cloning vector DNA. Neither of these is considered pathogenic. All these experiments are considered exempt for regulatory purposes. None of these experiments have resulted or will result in the release of experimental material into the environment. The recombinant DNA protocol approval number for this laboratory is 01-KEa.

Karim Essani, Ph.D.
Professor

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