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Sex determination mechanism in the hymenopteran parasitoid *Aphidius rhopalosiphi* De Stefani-Peres (Braconidae : Aphidiinae)

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ABSTRACT. Three main sex-determining mechanisms have been proposed for Hymenoptera : genetic balance, single locus (sl-CSD) and multilocus (ml-CSD) complementary sex determination. In the last two cases, sex is not determined by the number of chromosome sets but by heterozygosity at one or several loci. Individuals are male when hemizygous (haploid) or homozygous (diploid) at all sex-determining loci. Usually, this results in haploids developing as males and diploids as females, although diploid males can also appear, particularly under conditions of inbreeding. *Aphidius rhopalosiphi* (Aphidiineae : Hymenoptera Braconidae) is a cereal aphid parasitoid that can potentially be used as a biological control agent. Phylogenetic studies suggested that, within parasitoid wasps, the sl-CSD is present in both the Ichneumonoidea superfamily and the Braconidae family. Here, we directly test the sl-CSD model in *A. rhopalosiphi* by inducing diploid male production by brother-sister mating in laboratory-selected isofemale lines. Ploidy levels were analyzed with two complementary methods : DNA flow cytometry and DNA microsatellite markers. We observed a significantly male-biased sex ratio after sib mating, but no diploid males were detected by DNA analysis. The difference between the observed and expected sex ratio suggests that a sl-CSD model with two alleles may be applicable, which would imply that most diploid males are unviable in *A. rhopalosiphi*. Consequences of diploid male production are discussed in terms of the evolutionary biology of Hymenoptera and aphid biological control.

KEY WORDS : Hymenoptera, Aphidiinae, Haplodiploidy, Complementary sex determination, Diploid male, Microsatellite markers, Flow cytometry.

INTRODUCTION

During the past decades, the study of sex-determining mechanisms in sexually reproducing organisms has played a key role in our understanding of evolutionary biology. Testimony of this is HARDY's (2002) recent book "Sex-ratio : concepts and research methods", which presents an exhaustive review of the subject. In haplodiploids such as Hymenoptera, reproduction is based on arrhenotoky. Males develop parthenogenetically from unfertilized haploid eggs while females develop from fertilized diploid eggs. This mechanism gives a high degree of maternal control over offspring sex-ratio (GODFRAY, 1994). Haplodiploidy also has important consequences for the evolution of sex allocation, mating systems, population ecology and social evolution (CHARNOV, 1982; COOK & CROZIER, 1995; PEN & WEISSING, 2002; WEST et al., 2002).

Mechanistically, four genetic models have been proposed to explain the linkage between ploidy and sex in the Hymenoptera : complementary sex determination (CSD; WHITING, 1943), genic balance (CUNHA & KERR, 1957), nucleo-cytoplasmic balance (CROZIER, 1971) and genomic imprinting sex determination (POIRIÉ et al., 1992). Of these, the CSD model seems to be the most widely distributed one, being reported for about fifty species of Parasitica and Aculeata (STOUTHAMER et al, 1992; COOK, 1993a; COOK & CROZIER, 1995). Under the CSD model, sex is determined by multiple codominant alleles at a single locus (sl-CSD) or at multiple loci (ml-CSD). If individuals are heterozygous at any one of the loci, they develop as females, but if they are homozygous (diploid) or hemizygous (haploid) at all loci, they develop as males. The existence of CSD is detected with inbreeding experiments by mother-son and brother-sister matings. Diploid male production can represent a significant load since diploid males are usually sterile (COOK & CROZIER, 1995; HENSHAW et. al., 2002) and often non-viable (PETTERS & METTUS, 1980). Presence of diploid males is strongly correlated to inbreeding and constitutes a genetic load for the population as it results in a biased sex-ratio (females mated to diploid males, like unmated females, produce only male offspring, COOK, 1993a).

Aphidius rhopalosiphi De Stefani-Peres (Braconidae : Aphidiinae) is a cereal aphid parasitoid that has attracted considerable interest for biocontrol purposes (LEVIE et al.,

2001). In mass rearing experiments, required for biocontrol via mass-release, we have observed a significantly male-biased sex-ratio (LEVIE & HANCE, unpublished data). This could indicate the production of diploid males, possibly as a result of the occurrence of inbreeding in the rearing experiments. However, as yet, no formal investigation has been conduced on the sex-determining mechanism in *A. rhopalosiphi*.

Phylogenetic studies (COOK, 1993a; COOK & CROZIER, 1995) have suggested that the CSD model is present in the parasitic superfamily of the Ichneumonoidea and in the Braconidae family, to which the Aphidiinae belong, and that single-locus CSD (sl-CSD) is probably the ancestral model in the Aculeate-Ichneumonoid clade (COOK & CROZIER, 1995). However, single-locus CSD has not been formally demonstrated as the sex-determining mechanism for any member of the Aphidiinae.

The aim of the present study was to test whether sex-determination in *A. rhopalosiphi* can be explained by the single-locus CSD model. In order to increase homozygosity at the sex-determining locus and induce the production of diploid males, brother-sister crosses were produced. Presence of diploid males was tested using three different approaches, sex-ratio analysis, microsatellite genotyping and flow cytometry DNA analysis.

MATERIAL AND METHODS

Biological material

A. rhopalosiphi were collected in Belgium from parasitized *Sitobion avenae* Fabricius aphid larvae in September 2000. Laboratory cultures were established on aphids reared on *Triticum aestivum* L wheat. All rearing cultures were maintained at 20˚C, 60% relative humidity and 16-h light : 8-h dark cycles. Wheat was removed once per week and new aphid larvae (reared independently under the same laboratory conditions) were presented to adult parasitoids after each adult emergence.

Establishment of brother-sister crosses

Five isofemale lines were established in September 2002 by crossings between newly emerged virgin females with their brothers over five consecutive generations. For each line and generation, between three to five sibling matings were realized. As control, we also performed twenty random matings using freshly emerged adults from mummies isolated from mass rearing stocks. Matings were performed by placing each male and female pair in a capsule together with 100 stage 2 or 3 aphid larvae on wheat. To prevent superparasitism, adult parasitoids were removed after one to two days. Mummies appeared after 12 days of development and were isolated until emergence. Sex of newly emerged adults was determined as parent fecundity. Adults were fed for two days with a honey solution (5%). All parasitoids (parents and offspring) were stored at -80˚C for future DNA analysis.

Sex-ratio analysis

Because it is difficult to determine the sex-ratio at the egg stage, only the secondary sex-ratio could be recorded. Sex-ratio was estimated as number of males divided by total number of wasps. All-male progenies were excluded from the analysis to prevent confusion of all-male progenies of virgin females with diploid males (BEUKEBOOM et al., 2000). As diploid males can be unviable, brood size and percentage of emerged mummies were compared between control and inbred crosses. Brood size was estimated by absolute number of mummies produced since we always presented 100 aphid larvae to wasps and analyzed results using a non parametric Mann-Whitney test. Sex-ratio and percentage of emerged mummies were analyzed using a general linear model (WILSON & HARDY, 2002). Data were presented in the form of binary responses (0 or 1) and a logistic regression was applied. Statistical analyses were performed using Minitab (version 12.2).

Expected sex-ratios under two different scenarios of sl-CSD (two and three alleles at the sex-determining locus) were calculated from the observed sex-ratio in the control crosses and compared to the observed sex-ratio in the first generation of inbred crosses (for a description of this method, see BEUKEBOOM et al., 2000). Viability parameters of diploid males were also incorporated into the expected sex-ratio calculation (see Table 2). For example, the observed sex-ratio in the control crosses was 0.35 (n=210) and we obtained, in the first inbred generation, a total of 214 wasps (158 males and 56 females). If we considered sl-CSD model with two alleles at the sex-determining locus, 50% of the fertilized eggs are expected to become diploid males (i.e. 0.325) and expected sex-ratio (noted as SR, males proportion) is calculated as :

Case 1 SR1=(0.35 + 0.325) / (0.35 + 0.325 + 0.325); SR1=0.67 if diploid males are viable and

Case 2 SR2=0.35 / (0.35 + .0325); SR2=0.52 if diploid males are unviable.

In the first inbred generation, the number of observed wasps was 214 and expected male and female numbers (noted as Mnb and Fnb) are :

Case 1 Mnb1=214 x 0.67 and Fnb1=214 – Mnb1 if diploid males are viable

Case 2 Mnb2=214 x 0.52 and Fnb2=214 – Mnb2 if diploid males are unviable

Deviations from the observed and expected male and female numbers of wasps were tested using a chi-squared test with Yates'correction.

The same approach was realized with sl-CSD model with three alleles at the sex-determining locus but, in this case, 25% of the fertilized eggs are expected to become diploid males in the first inbred generation.

Discrimination between haploid and diploid males

Ploidy of male parasitoids was analysed by means of two complementary DNA analysis techniques : microsatellite genotyping of thorax and abdomen tissue and flow cytometry of the head tissue.

Microsatellite DNA genotyping

Diploid males can be identified by genotyping at several loci : if at least one locus is found to be heterozygous they are diploid rather than haploid. Because of their variability, we chose to use microsatellites as genetic markers. Six microsatellite DNA loci (polymorphic codomi-

nant markers) previously developed for *Aphidius ervi* and related species (AF336990, AF336991, AF336992, AF33693, AF336994, AF336998; HUFBAUER et al., 2001) were screened. The applicability on Belgian populations of *Aphidius rhopalosiphi* was previously verified by cloning and sequencing microsatellite loci.

DNA was extracted from thorax and abdomen using a CTAB extraction buffer, chloroform-isoamyl alcohol extraction and isopropanol precipitation. Microsatellite loci were amplified using the polymerase chain reaction. Amplification was performed in a 15 microlitre reaction mixture consisting of 2µl of genomic DNA, 1x PCR Buffer (10x), 2µM $MgCl_2$, 200µM dNTP, 0.5µM of each primer and 0.6 units Ampli Taq polymerase Gold (Applied Biosystems). All PCR reactions were performed using an Applied Biosystem : GeneAmp PCR system 9700, with a thermocycling profile consisting of a 10-min denaturation at 95˚C, 32 cycles of 94˚C for 50 s, 52˚C for 1 min and 72˚C for 1 minute 30s, followed by a final extension at 72˚C for 10 minutes. One primer for the locus was 5'-end-labelled with ABI PRISM® primer (Applied Biosystems). Allele sizes were determined by electrophoresis on polyacrylamide sequencing gels (ABI™ 377 DNA Sequencers), using a 400 HD ROX as a size standard. Gels were analyzed with GeneScan® software.

The efficiency of detecting diploid males among offspring could be determined by genotyping the parents. Initial genotyping of parents of the inbred crosses revealed that, of the six loci screened, only locus AF336990 was polymorphic. Furthermore, the identification of homozygous (hemizygous) and heterozygous genotypes was possible for only one line (line A) since a diploid female (mother, alleles 218-224bp.) presented one allele different to that of her mate (father, allele 218bp.) (see Fig. 2). From their genealogy, it appeared that males presenting a band at 224bp. must have a haploid genotype as this allele was only present in their parent female, but the haplodiploid states of males with a 218bp. band can not be defined as they could have either received this allele from their mother only and be hemizygous (haploid, genotype 218) or they were laid as homozygous diploid individuals receiving the 218 allele from both parents (diploid genotype 218/218). Thus, in the following brother-sister crosses, microsatellite markers allowed us to detect only 50% of all expected diploid males.

Flow cytometry DNA analysis

Flow cytometry was applied after nuclear preparation using a Becton Dickinson Facscan cytometer. We followed the protocol of VINDELOV et al. (1983) for nuclear preparations based on a trypsin solution followed by a trypsin inhibitor-ribonuclease solution and finally by a propidium-spermine tetrahydrochloride solution.

RESULTS

Secondary sex-ratio analyses

Results of first generation inbred (brother-sister crosses) and control crosses are presented in Table 1. The binary logistic regression analyses showed that a significant difference occurred between the mean secondary sex-ratio of control crosses and of the first inbred generation ($z=7.42$; $p < 0.001$). Thus, secondary sex-ratios in the first inbred generation (mean=0.38; SD=0.24) were more male biased than in the control crosses (mean=0.68; SD=0.22). No significant differences were found in the brood sizes (Mann-Whitney test, $W=135$, $p=0.87$) or percentage of emerged parasitoids (binary logistic regression : $p > 0.05$) between control and inbred crosses. No significant differences were observed in sex-ratio of the successive generations of brother-sister crosses (binary logistic regression : $p > 0.05$, Fig. 1). Moreover, we observed extinction of the isofemale line A in the fourth inbred generation.

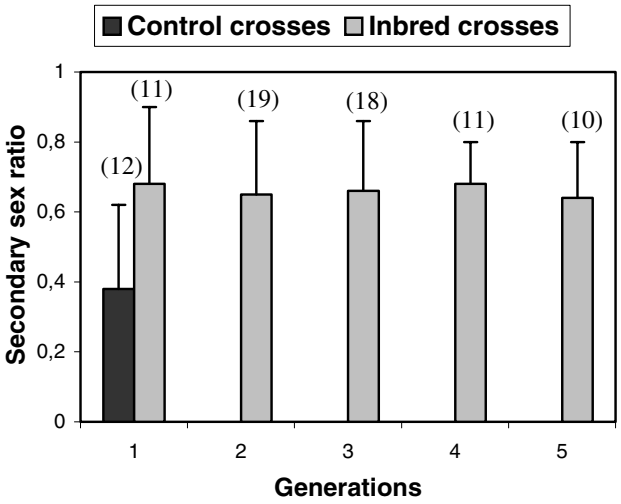

Fig. 1. – Secondary sex-ratio following inbreed crosses (sib mating) through five generations (mean ± SD). Comparison with control crosses. Numbers of crosses are indicated in parentheses.

TABLE 1

Description of the observed results (mean ± SD) in the control crosses and in the inbred crosses of the first generation: brood size (number of mummies), percentage of emerged mummies, mean number of females/pair, mean number of males/pair and secondary sex-ratio (males proportion).

	Control crosses (n=12)	Inbred crosses (n=11)
Brood size	18.00 ± 7.78	21.18 ± 16.38
Percentage of emerged mummies	92.69 ± 8.33	90.54 ± 10.86
Number of females	10.83 ± 7.12	5.09 ± 4.70
Number of males	5.92 ± 3.55	14.36 ± 15.27
Secondary sex ratio	0.38 ± 0.24	0.68 ± 0.22

Expected sex-ratio under sl-CSD assumptions

Expected secondary male sex-ratios, calculated from sex ratios observed in control crosses for different sl-CSD scenarios, are presented in Table 2. There was no significant difference between observed and expected sex-ratio under the assumption of sl-CSD based on two alleles at one single sex-determining locus with viable diploid males. In contrast, the observed sex-ratio was significantly more male-biased than expected if two alleles with non-viable diploid male and three sex alleles were postulated.

TABLE 2

Comparison between observed and expected numbers of males and females and sex ratio (males proportion) in control crosses and in first inbred generation with two and three alleles at the sex-determining locus. Viability of the diploid males were also integrated in the analysis. Significance of χ^2test were indicated as: NS, no significant difference and S, significant difference.

	Number of males and females	Sex-ratio (males proportion)	χ^2test (95%)
Control crosses	71 / 130	0.35 (n = 210)	
Observed results in the inbred crosses	158 / 56	0.74 (n = 214)	
sl-CSD: two alleles at the sex-determining locus			
Expected results with viable diploid male	144.45 / 69.55	0.67	NS
Expected results with non-viable diploid male	110.85 / 103.148	0.52	S
sl-CSD: three alleles at the sex-determining locus			
Expected results with viable diploid male	109.14 / 104.86	0.51	S
Expected results with non-viable diploid male	89.88 / 124.12	0.42	S

Search for diploid male production

Genealogical analysis with Microsatellite DNA markers

As explained previously, only locus AF336990 was polymorphic and the identification of homozygous (hemizygous) and heterozygous genotypes was possible for only one line (line A). Genotypes of parents, of the successive generations and of their male progeny are summarized in Fig. 2. Normally, those diploid males should be sterile, and if females mate with them, they

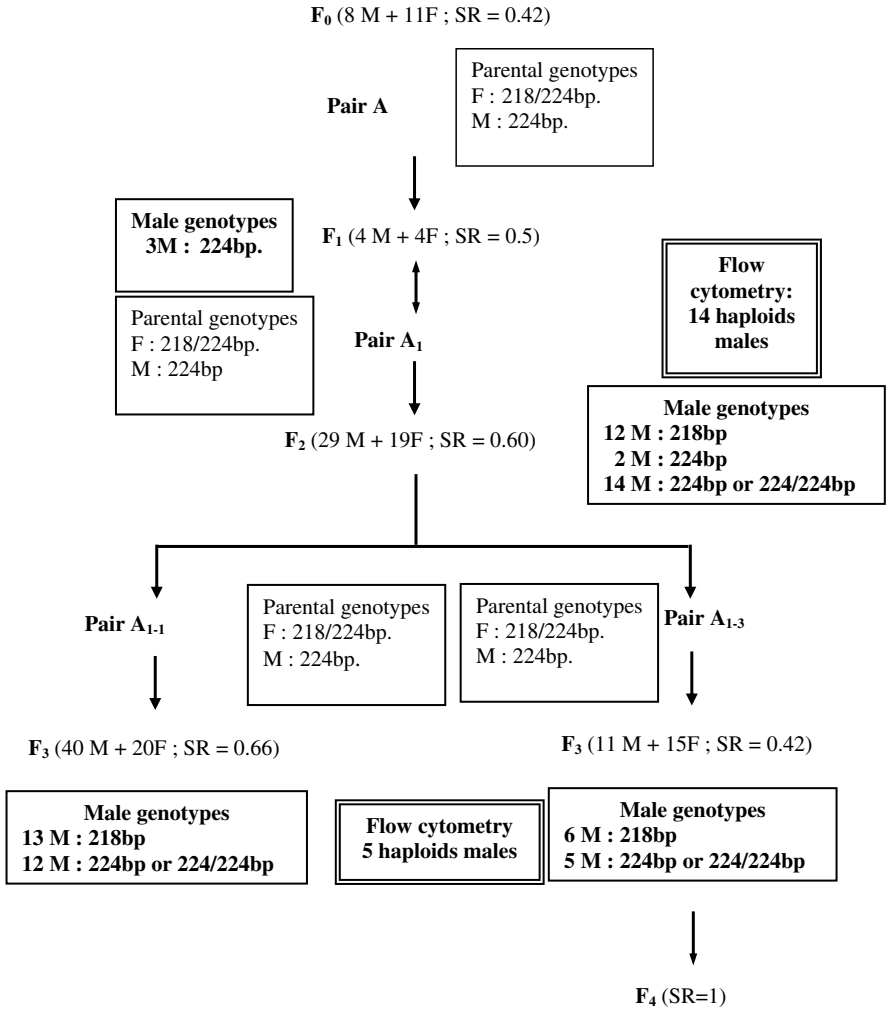

Fig. 2. – Genealogy of the isofemale lines using microsatellite DNA analysis. Secondary sex ratio (noted SR) and observed number of males are reported. Single line boxes represent parents genotypes whereas double line boxes represent genotypes of the male offspring. Results of flow cytometry analysis are also indicated.

should be able to produce haploid males only. In the first generation, three of the males had a haploid genotype as their progeny contained females (sex-ratio < 1). By the same reasoning, the second generation gave, of the 29 males genotyped : (i) 14 haploid males (12 with genotype 218 and two with genotype 224 (offspring tested) and (ii) 14 males that could either be haploid (genotype 224) or diploid (genotype 224/224). For the third generation, of the 11 males obtained, six were haploid and five could have been haploids or diploids.

Flow cytometry analysis

Flow cytometry was used to test the ploidy of males coming from generations 2 and 3 (generation 2 : 14 males; generation 3 : 5 males) that could not be resolved using microsatellites genotyping (for details, see Fig. 2). Before males were analyzed, several diploid females were tested as a control, so that the relative fluorescence index could be standardized (Fig. 3). None of the males tested by flow-cytometry, however, were diploid; all were normal haploid males. Hypotheses than can explain this observation are discussed in the following section.

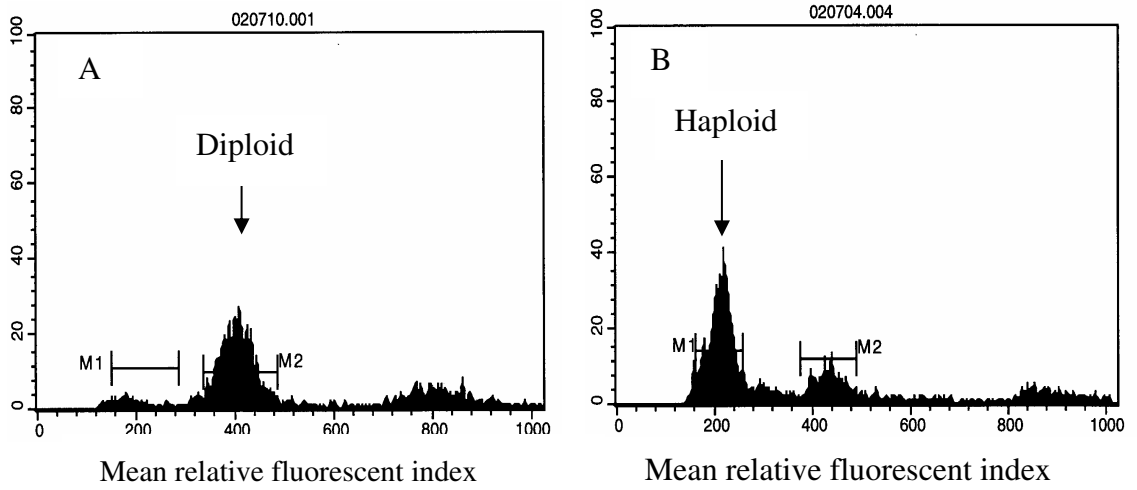

Fig. 3. – Profile of diploid female (A) and haploid male (B) using flow cytometry DNA analysis. M1 and M2 correspond to mean relative fluorescence peak of haploid and diploid cells respectively

DISCUSSION

This study presents the first investigation of the sex determination mechanisms in the braconid subfamily Aphidiinae. Experiments with laboratory cultures of *A. rhopalosiphi* showed that inbreeding results in a more male-biased secondary sex-ratio than that observed either in control crosses or under natural conditions (0.43 ± 0.03, LEVIE, unpubl.). Statistical analysis comparing observations with the expected sex-ratio under a sl-CSD assumption supported the simplest model based on two alleles at a single sex-determining locus with viable diploid males. Nevertheless, DNA microsatellite genotyping and flow cytometry analysis yielded useful complementary information on the ploidy of males since no diploid males were detected in the subsequent inbreeding generations of the analysed isofemale line. Even if these analyses do not allow us to draw clear conclusions on the sex determination mechanisms, our data suggest that sl-CSD with unviable diploid males is the likely sex determination mechanism in *A. rhopalosiphi*.

We propose here two nonexclusive explanations for the disparities between the results of the statistical analysis and the DNA analysis. First, under laboratory conditions, sex-ratio may be influenced by the existence of an inbreeding depression phenomenon. This hypothesis might be corroborated by the high male-biased secondary sex-ratio and the extinction of the isofemale line A in the fourth generation (only all-male progenies). Perhaps inbreeding depression might result in differential mortality of the sexes (high female mortality rate) explaining the absence of brood size differences between control and inbred crosses. However, if inbreeding depression occurs in haplodiploid systems, expression of lethal genes would be higher in haploid than diploid individuals and so would result in a higher mortality rate of haploid males. Inbreeding depression has already been reported within inbred *Diadegma chrysostictos* and *Trichogramma pretiosum* wasps (ANTOLIN, 1999; BUTCHER et al., 2000).

A second explanation may be the absence of CSD sex-determining model in *A. rhopalosiphi* although low or non-viability of diploid males due to egg and larval mortality would also explain this pattern. Low viability of diploid males associated with CSD has been reported for Hymenoptera *Bracon hebetor* (WHITING, 1943; PETTERS & METTUS, 1980). In future, it may perhaps be possible to directly demonstrate differential mortality between haploid and diploid males, e.g. by comparing primary sex-ratio with secondary sex-ratio.

Moreover, STOUTHAMER et al. (1992) and COOK (1993a, 2002) underlined that wasp populations from laboratory cultures may have a lowered genetic diversity resulting from the fixation of several sex loci (homozygous). Populations may then contain only two sex alleles at one non-fixed sex locus. In this case, diploid male production would not increase in subsequent generations as the isofemale line cannot lose another sex allele or it will become extinct (COOK, 1993b) as observed in our laboratory culture (inbred generations 2 to 5, Fig. 1). Although our laboratory culture has been established for

only one year from specimens collected in nature, we cannot exclude the existence of some inbred crosses during that period or an initial reduced genetic diversity in the field samples. Thus we cannot reject definitively the existence of a ml-CSD model even if sex-ratio analyses correspond better to sl-CSD. Further investigations would be needed to exclude ml-CSD as the sex-determining mechanism in *A. rhopalosiphi*, since the number of generations and repetitions we used is probably insufficient to find diploid males.

Taking a phylogenetic overview, sl-CSD is likely to be the ancestral model in the Aculeate-Ichneumonoid clade (COOK, 1993a; COOK & CROZIER, 1995). It has indeed been described in four species of the Braconidae family (three species from the Braconinae subfamily and one species from the Microsgasterinae subfamily, SPEICHER & SPEICHER, 1940; WHITING, 1943; CLARK & RUBIN, 1961; STEINER & TEIG, 1989). However, phylogenetic patterns are still uncertain (see WHARTON et al., 1992) especially since BEUKEBOOM et al. (2000) first reported the existence of non-sl-CSD in a species belonging to the Alysiinae subfamily (Braconidae). Moreover, the Aphidiinae in concert with the Mesostoinae subfamilies form a subgroup in the cyclostomes Braconidae isolated from other subfamilies (BELSHAW et al., 1998). We, therefore, plan further investigations on this subfamily, particularly on *A. rhopalosiphi* and the related *Aphidius ervi* species. These data should contribute to elucidation of the evolution of the CSD in the Braconidae family. DNA flow analysis on males collected from natural populations could bring interesting information on both the complementary sex determination model but also on mating strategy. Production of diploid males, which are sterile, in natural populations will generate a costly genetic load in haplodiploid species, and reduce the reproductive success of parents (COOK & CROZIER, 1995; ZAYED & PACKER, 2001).

Industrial mass rearing is pivotal if cereal aphid parasitoids are ever to be used as biological control agents. However, knowledge of the sex determination mechanisms and sex-ratio biases could greatly reduce the costs of parasitoid production and increase the efficiency of mass-release programs.

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