Heredity **73** (1994) 317–327

Received 28 February 1994

Cytogenetics of microbe-associated parthenogenesis and its consequences for gene flow in *Trichogramma* wasps

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Cytogenetics and gene flow were studied in microbe-associated parthenogenetic (thelytokous) forms of three species of the genus *Trichogramma* (*T. pretiosum, T. deion* and *T. nr. deion*). The chromosome behaviour in newly laid eggs indicated that the mechanism allowing restoration of diploidy in unfertilized thelytokous eggs was a segregation failure of the two sets of chromosomes in the first mitotic anaphase. This results in a nucleus containing two sets of identical chromosomes. The mechanism is known as gamete duplication and results in complete homozygosity. This was confirmed by investigation of the segregation pattern of allozymes in the offspring of heterozygous thelytokous females. Contrary to the generally assumed genetic isolation of thelytokous lines, thelytokous females of these species can mate and will use the sperm to fertilize some of their eggs. These fertilized eggs give rise to females whose genome consists of one set of chromosomes from each parent. Egg fertilization and the resulting syngamy of the sperm and egg pronucleus apparently precludes the gamete duplication that would have taken place if the egg had remained unfertilized. Most field populations of *Trichogramma* contain both parthenogenetic (thelytokous) and sexual (arrhenotokous) forms. In the two field populations that we studied there was evidence for high levels of gene flow from the sexual (arrhenotokous) fraction to the parthenogenetic (thelytokous) fraction of the population. The implications of the cytogenetic mechanism of parthenogenesis, i.e. gamete duplication, for the mechanism of sex determination in Hymenoptera are discussed.

Keywords: arrhenotoky, cytogenetics, parthenogenesis, thelytoky, *Trichogramma, Wolbachia.*

Introduction

Most members of the insect order Hymenoptera reproduce by arrhenotoky, a form of parthenogenesis in which unfertilized eggs give rise to haploid males and fertilized eggs give rise to diploid females. However, complete parthenogenesis, i.e. thelytoky, is also found in many hymenopterous lineages (Luck *et al.*, 1992). In thelytoky, unfertilized eggs give rise to daughters and these females can also produce daughters from unfertilized eggs. Consequently, thelytokous lines can persist for multiple generations in the absence of males and sexual reproduction.

It is generally assumed that thelytokous lines are genetically isolated from each other and form apparently conspecific, arrhenotokous forms (White, 1984). Genetic isolation of thelytokous and arrhenotokous forms is probable in cases of hymenopteran thelytoky where the two forms are geographically isolated (Carter, 1937; Benson, 1950; Flanders, 1950; Young, 1990). However, thelytokous and arrhenotokous conspecifics are known to occur sympatrically in the Hymenoptera (Smith, 1941; Flanders, 1945; Mackay, 1955; Rössler & DeBach, 1973a,b; Aeschlimann, 1986; Day & Hedlund, 1988; Stouthamer *et al.*, 1990a,b). A growing body of evidence suggests that sympatry between arrhenotokous and thelytokous forms may be common (Day & Hedlund, 1988; Stouthamer, 1993).

Two mechanisms are known that would allow gene flow between sympatric arrhenotokous and thelytokous forms. First, many thelytokous forms produce rare males and these males are capable of fathering offspring through arrhenotokous females (Flanders, 1965; Orphanides & Gonzalez, 1970; Rössler & DeBach, 1973b; Legner, 1987; Stouthamer, *et al.*, 1990a,b). This mechanism allows gene flow from the

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thelytokous lines to the arrhenotokous population but not vice versa. Secondly, one case is known where males are capable of fathering thelytokous offspring through thelytokous females (Rössler & DeBach, 1973b). This allows gene flow from the arrhenotokous population to the thelytokous lines. Bidirectional gene flow between thelytokous and arrhenotokous forms and gene flow among thelytokous lines are possible if both mechanisms are operational in the same species. This possibility of gene flow to thelytokous lines is most interesting because this would remove or reduce the presumed long-term 'costs' of asexual reproduction, i.e. the accumulation of mutations via Muller's ratchet and the inability to adapt to changing environments (Muller, 1964).

The ability of thelytokous females to utilize the sperm of conspecific males and thereby produce offspring sexually will likely depend on the cytogenetic mechanism and form of thelytoky. For instance, in cases such as the ichneumonid wasp *Venturia canescens* (Speicher, 1937) and the sawfly *Thrinax maculata* (Peacock & Sanderson, 1939), the number of chromosomes is never reduced to the haploid number and fertilization would result in triploid offspring. Such offspring are expected to be sterile (Smith, 1955). However, under those cytogenetic mechanisms that restore diploidy by either a fusion of the pronucleus with a polar body (central fusion or terminal fusion) or by fusion of mitotic products of the pronucleus (gamete duplication or endomitosis), fertilization and the production of fertile offspring may be possible if sperm penetration supersedes subsequent fusion. The majority of cases in which the cytogenetic mechanism of thelytoky has been studied in Hymenoptera involve a fusion of some kind (Smith, 1941; Tucker, 1958; Rössler & DeBach, 1973a; Stille & Dävring, 1980; Verma & Rüttner, 1983; Sanderson, 1988).

At present two forms of thelytoky can be recognized in the Hymenoptera: revertible or microbe-associated thelytoky and nonrevertible thelytoky. In microbe-associated thelytoky, bacteria of the genus *Wolbachia* (Stouthamer *et al.*, 1993; Stouthamer & Werren, 1993) are thought to cause parthenogenesis and the removal of the microbes by antibiotic or high temperature treatment leads to the production of progeny that reproduce sexually (Stouthamer *et al.*, 1990a; Stouthamer, 1991; Stouthamer & Werren, 1993). Microbe-associated thelytoky has been reported for nine species in the genus *Trichogramma* and several other parasitic wasp species (Zchori-Fein *et al.*, 1992; Stouthamer *et al.*, 1993) and more cases of thelytoky in Hymenoptera are likely to be of this type (Stouthamer *et al.*, 1990a; Stouthamer & Werren, 1993). In nonrevertible parthenogenesis microbes are not present and neither temperature nor antibiotic treatments cause a reversion to arrhenotoky (Stouthamer *et al.*, 1990b; Stouthamer & Werren, 1993). Several cases of nonrevertible thelytoky are also reported from *Trichogramma* (Stouthamer *et al.*, 1990b).

In this paper we examine the cytogenetics and potential for gene flow in microbe-associated thelytokous forms of *Trichogramma*. *Trichogramma* wasps are minute parasitic wasps that oviposit into the eggs of moths and butterflies. Their life cycle takes 10 days at 25°C. Approximately 150 species have been described in this genus and thelytokous forms of species have been found (Pinto & Stouthamer, 1994), 10 of which are known to carry the *Wolbachia* causing the parthenogenesis (Stouthamer *et al.*, 1990a,b, 1993). Thelytokous individuals, carrying the *Wolbachia*, have been found sympatrically with arrhenotokous conspecifics in at least six species of *Trichogramma* (*T. deion, T. brevicapillum, T. pretiosum, T. chilonis, T. platneri, T. nr. deion* from Mojave Desert). The frequency of thelytokous reproduction in populations can be very low. Most populations have thelytokous forms occurring at frequencies of less than 5 per cent, only one population is known at present where only thelytokous individuals are found and two other populations have thelytokous individuals occurring at rates between 10 per cent and 20 per cent (Stouthamer, 1991).

Our studies involve three species of *Trichogramma* with microbe-associated thelytoky, *Trichogramma deion, T. pretiosum* and *T. nr. deion* from the Mojave Desert. In each species, thelytokous individuals were collected in predominantly arrhenotokous populations. Using genetic markers we tested the ability of arrhenotokous males to father thelytokous offspring and subsequently tracked the inheritance of paternal and maternal genes in thelytokous lines. The results of these studies, coupled with a cytogenetic analysis of gametogenesis in thelytokous females, allowed us to determine the cytogenetic mechanisms of microbe-associated thelytoky and to test for gene flow to thelytokous lines in field populations of one species.

Materials and methods

Origin of cultures

Thelytokous lines were started with a single female originating from a field-collected host. The following thelytokous lines were examined: *T. deion* from Sanderson, TX, USA (tSAN) and Belle Fourche, SD, USA (tBEL), *T. pretiosum* from Nuevo Leon, Mexico (tNLM) and Lara State, Venezuela (tLSV) and *T. nr. deion* from Danby, CA (tDAN1). Arrhenotokous lines

were established from these lines using antibiotic treatment and initiated with a single mated female (Stouthamer *et al.* 1990a). The derived arrhenotokous lines extracted from the latter are referred to as aSAN, aBEL, aNLM and aDAN1, respectively.

Arrhenotokous lines were started with a single female that had mated with a male originating from the same field-collected host. The following arrhenotokous lines were examined: *T. deion* from Seven Pines, CA (SVP), *T. pretiosum* from the University of California's South Coast Field Station in Orange County, CA (SCFS) and *T. sp near deion* from Danby, CA (DAN2). Collection details of these cultures are given in Stouthamer *et al.* (1990b), Pinto *et al.* (1991) and Kazmer (1992). Voucher specimens are placed in the collection of the Department of Entomology of the University of California, Riverside, California, USA.

Culture maintenance

Wasp cultures were maintained on irradiated *Trichoplusia ni* (Cabbage Looper) eggs as described in Stouthamer *et al.* (1990b).

Genetic markers

Allozymes and a visible mutant marker allele were used to track maternal and paternal genetic contributions in the F_1 and F_2 generations. Allozymic variants were detected in single individuals using the isoelectric focusing procedures described in Kazmer (1991). Allozymic variants in four enzyme systems were used: phosphoglucomutase (PGM, E.C. 2.7.5.1), glucose-phosphate isomerase (PGI, E.C. 5.3.1.9), fumerase (FUM, E.C. 4.2.1.2) and nonspecific esterases (EST, E.C. 3.1.1.1). A single locus was apparent for PGM, PGI and FUM whereas three EST loci (*EST-I, EST-II* and *EST-III*) were distinguished. Simple Mendelian inheritance patterns with allelic codominance have been observed for all loci except *EST-III* (Kazmer, 1991, 1992; Pinto *et al.* 1992; this paper). The *EST-III* polymorphism consists of high and low esterase activity phenotypes in a zone anodal to that of the *EST-I* and *EST-II* electromorphs. Crossing studies indicate that a single locus with a partially or completely dominant 'high' *EST-III* activity allele and a recessive 'low' (wild-type) activity allele underlies the *EST-III* polymorphism (D. J. Kazmer, unpublished data). High *EST-III* activity alleles have only been observed in *T. pretiosum*.

The visible mutant marker allele was 'wing-unfold' (*wu*), a recessive mutation that segregates as a simple Mendelian trait (R. Stouthamer, unpublished data). In contrast to wild-type wasps, females homozygous for *wu* and (hemizygous) males carrying the *wu* allele hold their wings perpendicular to the long axis of their body immediately after emergence and usually do not completely unfold their wings.

Sperm utilization in thelytokous females

Preliminary observations indicated that thelytokous females copulate with conspecific males and that sperm is transferred and stored by the females as a result of these copulations. To test if thelytokous females use sperm of conspecific males to produce hybrid offspring, thelytokous females were paired with conspecific males and the F_1 offspring were obtained. The maternal and paternal lines were chosen such that they carried different alleles at one or more marker loci. The F_1 offspring were then analysed electrophoretically to determine if maternal or paternal alleles were expressed.

Segregation and independent assortment in thelytokous females

Some of the F_1 offspring examined in the sperm utilization test were hybrids, i.e. they expressed both maternal and paternal alleles (see Results). These hybrid females were allowed to produce offspring as virgins. Subsequently, the segregation and assortment of maternal and paternal alleles in their female offspring were examined electrophoretically.

Frequency and mating of thelytokous females in field populations

Field collections of arrhenotokous and thelytokous *T. nr. deion* were made near the former town of Danby (San Bernardino Co., CA, USA) and in Last Chance Canyon (El Paso Mnts., Kern Co., CA, USA). Host eggs were collected from *Eriogonum inflatum* Torr. & Frem (Polygonaceae) on 30 April and 7 May 1988 at Danby and 14 May and 21 May 1988 at Last Chance Canyon. The most common host species of *T. nr. deion* at these sites was *Apodemia mormo deserti* (Lepidoptera: Rhiodinidae). The eggs of an unidentified geometrid moth were also parasitized but such parasitism was rare (less than 5 per cent of all 'patches', see below). Field-collected hosts were held in the laboratory for wasp emergence, if any. Following Kazmer (1992), a 'patch' was defined to consist of those wasps that emerged from host eggs on the same node or internode on the same day in the laboratory.

To estimate the frequency of thelytokous females in the study populations, the reproductive mode of females that emerged in all-female patches was tested.

These tests consisted of exposing the females individually to an excess of *T. ni* eggs for 24 h and later determining the sex of the progeny. Because females from all-female patches were not exposed to adult males, only two types of progeny broods, all-female (indicative of thelytoky) and all-male (indicative of arrhenotoky), were possible. The reproductive mode of females that emerged in mixed-sex patches was not tested because such females were exposed to adult males on emergence and all-female progeny could be produced by thelytokous and arrhenotokous females under these conditions. Data on the distribution of thelytokous and arrhenotokous females among and within the all-female patches sufficed for making rough estimates of the overall frequency of thelytokous females in the populations (see Results).

To determine if thelytokous females mate and use sperm to produce offspring in the field, females used in the reproductive mode tests were frozen in liquid nitrogen and subsequently electrophoresed. Electrophoretic phenotypes were determined at two polymorphic loci (*PGI* and *FUM*). The presence of heterozygous phenotypes in these samples is evidence that thelytokous females mate and sexually produce offspring in the field (see Results).

Cytogenetic techniques

Female wasps were fed honey for one to several days after emergence. From 50 to several hundred females were given an egg sheet containing 10–20 host eggs to parasitize for 30–60 min. After this time the egg sheet was removed and wasps remaining on the egg sheet were brushed off. The egg sheet was kept for a variable timespan to allow the *Trichogramma* eggs to develop. Wasp eggs were removed from the host egg by opening the host in a drop of *Drosophila* Ringers solution on a siliconized microscope slide. Each host egg contained up to 25 wasp eggs that sank and adhered to the microscope slide. The host egg debris was removed by washing with Ringers. The wasp eggs were dechorionated for three min in bleach-Ringer (1:100 household bleach: *Drosophila* Ringer), fixed for 3 min in Carnoy's fixative (3:2.5:1 of 99 per cent ethanol:chloroform:glacial acetic acid), rinsed in 70 per cent ethanol for 3 min and then stained with a stain (2 per cent lacmoid (Lacmoid Pfaltz & Bauer, Research Chemical Division, Stamford, CT, USA) in a 1:1:1 mixture of water:lactic acid:glacial acetic acid). The sample was immediately covered with a glass coverslip and the excess of lacmoid was removed from under the coverslip. The coverslip was sealed with clear acrylic nail-polish and studied under oil emersion at 1000× on a phase contrast microscope.

Results

Sperm utilization by thelytokous females

Thelytokous females (Maternal (=M) phenotype, *MM* genotype) paired and allowed to copulate with conspecific arrhenotokous males (Paternal (=P) phenotype, *PO* genotype) produce female offspring either hybrid, MP phenotypes or nonhybrid, M phenotypes (Table 1). The electrophoretic banding patterns of the MP and M phenotype thelytokous offspring are indistinguishable from those of arrhenotokous females with *MP* and *MM* genotypes, respectively (not shown). The expression of the paternal genes in the thelytokous offspring indicates that thelytokous females can still fertilize eggs with sperm of conspecific males and that paternal genes are incorporated into the genome of offspring arising from fertilized eggs. Moreover, the electrophoretic similarity of MP phenotype thelytokous offspring and *MP* genotype arrhenotokous females is consistent with the hypothesis that the hybrid thelytokous offspring are diploid.

Table 1 Electrophoretic evidence of sperm utilization by thelytokous females when paired with conspecific males

Wasp species	Lines crossed female × male	Marker locus	No. of females tested	Proportion of females producing hybrid MP offspring	Proportion of offspring with phenotype[a]			
					MP	M	P	No. tested
T. nr. deion	tDAN × DAN	*PGI*	9	0.89	0.89	0.11	0	63
		FUM	9	0.89	0.89	0.11	0	
T. pretiosum	tNLM × CAP	*PGM*	4	1.00	0.65	0.35	0	31
T. deion	tBEL × SVP	*PGM*	8	0.63	0.61	0.39	0	28
	tSAN × SVP	*PGM*	5	1.00	0.65	0.35	0	34

[a] Pooled over females that produced ⩾ one hybrid (MP) offspring.
M and P designate the maternal and paternal alleles, respectively.

Most of the thelytokous females paired with conspecific males produced at least one hybrid, *MP* phenotype offspring (63–100 per cent, Table 1). Among those females that produced at least one hybrid offspring, a high proportion of the offspring were hybrid (61–89 per cent, Table 1). These hybrid, thelytokous offspring were used in the tests for independent assortment (see below). Thus, strong barriers to mating and 'hybridization' do not exist among the thelytokous and arrhenotokous forms used in this study. In addition, the bias in mated thelytokous females towards producing a majority of offspring from fertilized eggs (61–89 per cent) has an analogue in the typically female-biased sex ratio (41–81 per cent) of arrhenotokous *Trichogramma* (Stouthamer *et al.*, 1990b).

Segregation and independent assortment in hybrid thelytokous females

As expected, heterozygous arrhenotokous virgins produced only sons carrying the grandparental or the grandmaternal marker in a 1:1 ratio (Table 2). Similarly, the hybrid thelytokous virgins (MP phenotype) produced only daughters with M or P phenotypes in a 1:1 ratio (Table 2). No MP phenotype thelytokous F_2 offspring were detected in a total of 646 individuals examined. Thus, the cytogenetic mechanism of thelytoky in these wasps leads to complete homozygosity in one generation.

Among pairs of marker loci the ratio of recombinant:nonrecombinant offspring produced by hybrid thelytokous females did not differ from 1:1 in three of seven locus pairs (Table 3). An excess of nonrecombinant offspring was observed in the four locus pairs where significant deviations from a 1:1 ratio occurred. For one locus pair (*PGM-EST-1*), a significant deviation from 1:1 with an excess of nonrecombinant offspring was also observed in a cross between two arrhenotokous lines derived from thelytokous lines (aBEL × aSAN; Table 3). The simplest interpretation of these results is that the four cases of nonindependent assortment occur between linked locus pairs and the three cases of independent assortment occur between unlinked locus pairs. Consequently, the products of gametogenesis in hybrid thelytokous females are identical to those expected from normal meiosis with recombination.

Frequency of thelytokous females in field populations

Tests on the reproductive mode of female *T. nr. deion* emerging from all female patches show that such patches consist either of thelytokous females (TH only), arrhenotokous females (AR only) or both

Table 2 Segregation of maternal (M) and paternal (P) marker alleles in the offspring of hybrid (MP phenotype) thelytokous females. Comparative data for the arrhenotokous lines extracted from the thelytokous lines are also shown

Wasp species	Cross producing hybrid females	Locus	Proportion of offspring with phenotype			No. of mothers, offspring tested	χ^2 heterogeneity among mothers[a]	χ^2: proportion of M and P phenotype offspring equal[b]
			MP	M	P			
T. nr. deion	tDAN × DAN	*FUM*	0	0.54	0.46	16, 192	6.8 NS[c]	1.3 NS
		PGI	0	0.48	0.52		16.5 NS	0.3 NS
T. pretiosum	tNLM × SCFS	*PGM*	0	0.51	0.49	9, 92	10.0 NS	<0.1 NS
		EST-2	0	0.55	0.45		1.6 NS	1.1 NS
		EST-3	0	0.50	0.50		10.1 NS	0.0 NS
T. deion	tSAN × SVP	*PGM*	0	0.49	0.51	20, 209	14.4 NS	0.1 NS
T. deion	tBEL × SVP	*PGM*	0	0.44	0.56	16, 153	8.2 NS	1.9 NS
T. deion	aSAN × aBEL[d]	*EST-1*	0	0.50	0.50	7, 127	7.6 NS	<0.1 NS
		PGM	0	0.47	0.53		9.2 NS	0.4 NS
T. deion	aSAN × SVP[d]	*EST-1*	0	0.51	0.49	9, 106	4.1 NS	<0.1 NS
		PGM	0	0.49	0.51		4.0 NS	<0.1 NS

[a] χ^2 values were corrected for continuity due to the small number of offspring tested per mother. The degrees of freedom in each case is two less than the number of mothers tested.

[b] Each case has one degree of freedom.

[c] NS, not significant at the $p = 0.05$ level.

[d] Crosses were performed reciprocally in cases involving two arrhenotokous lines. The *M* allele is that of the SAN line.

Table 3 Recombination and independent assortment in thelytokous and arrhenotokous females of *Trichogramma*

Wasp species	Cross producing double or triple heterozygous females	Locus pair	Fraction of recombinant offspring (N)	χ^2: proportion of recombinant and nonrecombinant offspring equal[a]
T. nr. deion	tDAN × DAN	*FUM-PGI*	0.46 (192)	1.3 NS
T. pretiosum	tNLM × SCFS	*PGM-EST-2*	0.48 (92)	0.2 NS
		PGM-EST-3	0.01 (92)	88.0*
		EST-2-EST-3	0.51 (92)	< 0.1 NS
T. deion	tBEL × aSAN	*WU-PGM*	0.06 (102)	79.4*
		WU-EST-1	0.00 (150)	150.0*
		PGM-EST-1	0.09 (22)	14.7*
T. deion	aBEL × aSAN	*PGM-EST-1*	0.13 (127)	68.1*

[a] Each test has one degree of freedom.
NS, not significant as the $p = 0.05$ level.
*$P < 0.001$.

Table 4 Frequency of host egg patches producing thelytokous (TH) and arrhenotokous (AR) female *T. nr. deion* and the estimated frequency of TH foundresses in two field populations

Population	No. of patches	Percentage of patches: Untested (≥ 1 male produced)	Tested (no males produced): TH only	TH and AR	AR only	Estimated frequency of TH foundresses: Min. 1 foundress per patch ($s = 1$)	Max. 2 foundresses per patch ($s = 0$)
Danby	112	76.8	7.1	2.7	13.4	0.071	0.267
Last Chance	78	80.8	6.4	7.7	5.1	0.064	0.253

Min, minimum; Max, maximum.

arrhenotokous and thelytokous females (mixed; Table 4). The mixed all-female patches presumably result from at least one thelytokous female and at least one arrhenotokous female successfully parasitizing the same host patch. Analysis of the genetic structure of these populations indicates that most host patches are successfully parasitized by one or two females (Kazmer, 1992).

The foregoing data can be used to estimate the frequency of thelytokous females in the study populations if several assumptions are made. First, assume that patches are successfully founded by either one or two females. Let r equal the proportion of patches successfully founded by one female and $1 - r$ equal the proportion of patches successfully founded by two females. Next, assume that thelytokous and arrhenotokous females are equally likely to parasitize once-parasitized patches and that they do not differentially parasitize patches according to the type (arrhenotokous or thelytokous) of the first foundress. Lastly, assume that all of the untested patches (i.e. those which produced more than one male) were successfully parasitized by at least one arrhenotokous female. This may not be true in all cases because thelytokous females are known to produce male offspring on rare occasions (Stouthamer *et al.*, 1990b). With this assumption, the observed proportion of patches containing only thelytokous females, s is given by:

$$s = rt + (1 - r)t^2,$$

where t is the proportion of thelytokous females in the population and $1 - t$ is the proportion of arrhenotokous females in the population. By setting $r = 1$ and $r = 0$ minimal and maximal estimates of t are given by s and $\sqrt{s}$. For the two field populations of *T. nr. deion*, these estimates range from 0.064 to 0.267 (Table 4).

Mating of thelytokous females in field populations

A high proportion of the thelytokous females reared from TH only patches had heterozygous phenotypes at one or both marker loci (32 per cent at Danby and 81 per cent at Last Chance Canyon; Table 5). The laboratory studies show that thelytokous females with hetero-

Table 5 Frequency of heterozygous thelytokous (TH) females in patches producing only TH females in two field populations of *T. nr. deion*

Population	No. of patches	Locus	No. of individuals	Frequency of common allele	Individuals heterozygous (%)
Danby	8	*FUM*	22	0.95	9.1
	8	*PGI*	19	0.63	31.6
	8	Both	25	—	32.0
Last chance	5	*FUM*	21	0.74	52.4
	5	*PGI*	16	0.65	68.8
	5	Both	21	—	81.0

zygous phenotypes arise only as the sexual offspring of mated thelytokous females. It is important to note that the proportion of heterozygous females underestimates the frequency of sexual reproduction because not all sexual offspring will be detected using just two markers each with just two alleles. Consequently, the high proportion of heterozygous thelytokous females observed in these field samples suggests that mating and sexual reproduction by thelytokous females may be common in natural populations.

Cytogenetics of restoration of diploidy in Trichogramma deion *(BEL)*

In newly laid eggs the chromosomes are tightly wound in a fusiform nucleus. Meiosis in these eggs is arrested in anaphase I. Shortly after the egg is laid, the fusiform nucleus decondenses (Fig. 1.1) and two metaphase plates are formed, each containing five metaphase chromosomes (Figs 1.2, 1.3). One of the metaphase plates lies close to the periphery of the egg (Fig. 1.3b), near the vitelline membrane whereas the other lies inward from the surface (Fig. 1.3a). This central metaphase plate will go into anaphase II (Fig. 1.4a) while the peripheral group remains in metaphase I (Fig. 1.4b). One set of the anaphase II chromosomes moves towards the centre of the egg (Fig. 1.4a), while the other moves towards the periphery where it will be positioned very close to the peripheral metaphase plate (Fig. 1.4a,b). The peripheral group of metaphase chromosomes becomes the polar body I and the single set of anaphase chromosomes originating from the central metaphase plate becomes polar body II. Polar body I in the meantime has not undergone a clear anaphase II but the chromosomes have separated and condense before the second polar body condenses (Fig. 1.5b). The central nucleus goes into an interphase and reappears as 5 metaphase chromosomes (Fig. 1.5a), while at the periphery 15 polar chromosomes are visible (Fig. 1.5b). This stage is not followed by a normal anaphase of the first mititotic division, but the chromosomes separate without being pulled to opposite poles (Figs 1.6a, 1.7a), resulting in a single nucleus with 10 chromosomes. The polar bodies are now one group of 15 condensed polar chromosomes that lies close to the periphery (Figs. 1.6b, 1.7b). Next the central nucleus goes into an interphase (Fig. 1.8a) and reappears as a single nucleus with 10 metaphase chromosomes (Fig. 1.9). This is followed by an anaphase (of the second mitotic division) resulting in two nuclei each with 10 chromosomes (Figs 1.10, 1.11). From then on these mitotic divisions continue (Fig. 1.12). This cytogenetic mechanism was also observed in all other microbe-associated thelytokous forms that were studied: *T. deion* (IRV), *T. deion* (SAN) and *T. pretiosum* (NLM) and (LSV).

Discussion

Cytogenetic mechanism in microbe-associated thelytoky

The electrophoretic studies indicate that thelytokous females use the sperm to fertilize their eggs in approximately the proportions that sexual females do. The resulting hybrid females when allowed to reproduce as virgins produce female offspring that are homozygous for the marker of either the grandfather or the grandmother in a ratio of 1:1. This distribution of offspring types is consistent with the cytogenetic mechanisms of (i) gamete duplication, i.e. normal meiosis followed by a fusion of the two nuclei of the first mitotic division, or (ii) terminal fusion combined with a suppression of recombination during meiosis, i.e. at the end of the meiosis two sets of chromosomes that originated from the same set of chromosomes fuse, if there is no recombination two identical sets of chromosomes fuse, leaving the offspring completely homozygous. We could distinguish between these two possibilities by determining whether recombination occurred during the egg maturation of thelytokous eggs. This was

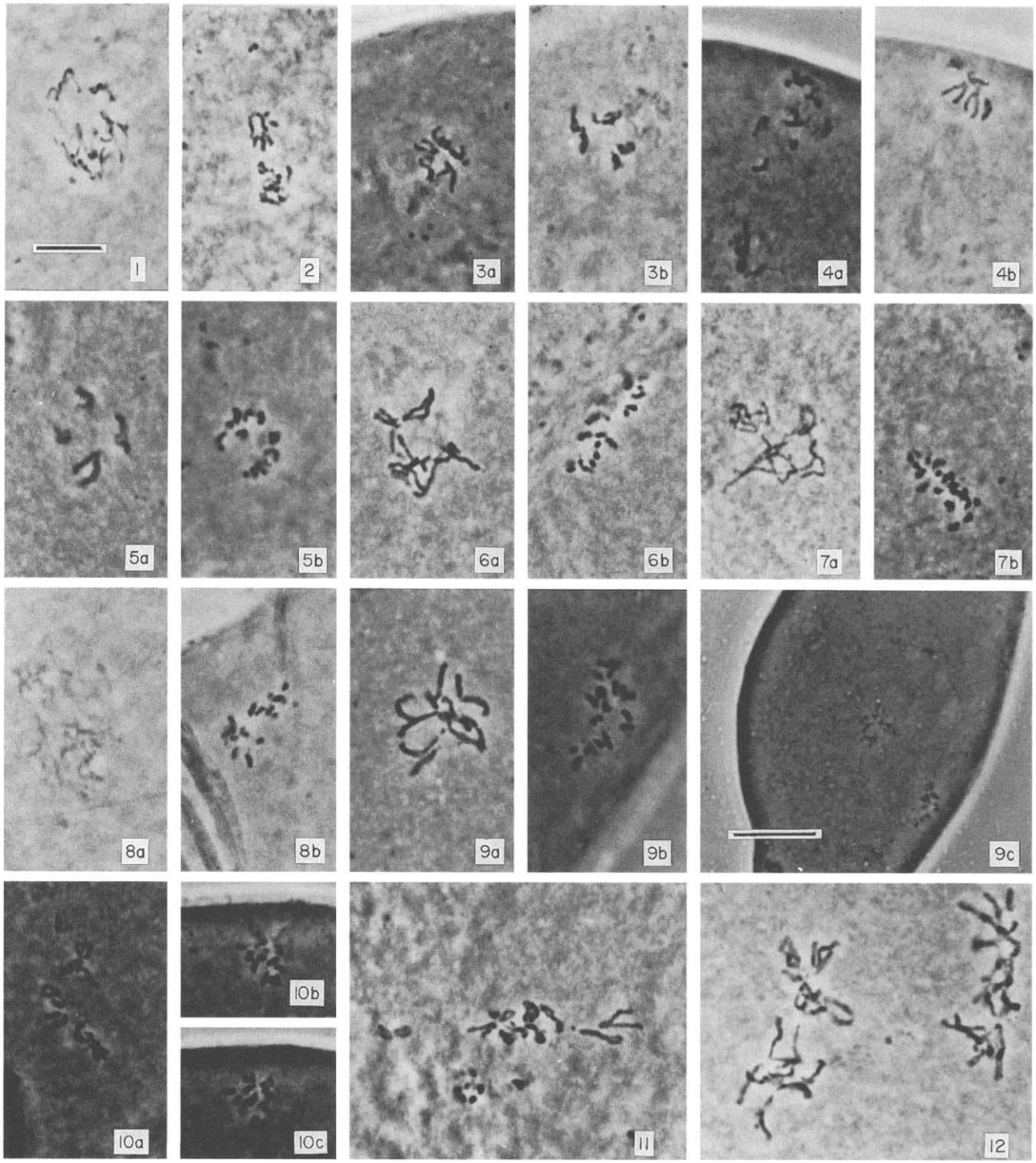

Fig. 1 Overview of the different stages of egg maturation after oviposition of microbe-associated thelytokous *Trichogramma deion* (tBEL, see text). The size bar in 1(a) equals 10 μ and applies to all figures with the exception of 9(c), where the bar equals 50 μ. **1** Anaphase I of egg nucleus. **2** Early metaphase I; two metaphase plates are forming. **3** Two metaphase I plates of the same egg: (a) metaphase plate located in the interior of the egg. (b) metaphase plate located in the periphery of the egg. **4** (a) Anaphase II of the chromosomes originating from the interior metaphase plate. One set of anaphase chromosomes moves to the centre of the egg whereas the other group moves toward the periphery where it will become polar body II. (b) At this stage the peripheral metaphase plate is still intact and will later become polar body I. **5** (a) Five metaphase chromosomes of the first mitotic division of the central nucleus; (b) polar bodies I and II consisting of 15 chromosomes located at periphery. **6** (a) Aborted anaphase of first mitotic division of central nucleus, chromosomes are separating without being pulled to opposite poles by a spindle. (b) 15 polar chromosomes visible at periphery of egg. **7** (a) Similar to 6 (a) but in a later stage of chromosome separation. (b) 15 polar chromosomes visible at periphery of egg. **8** (a) Interphase of the central nucleus between the first and second mitotic divisions. (b) 15 polar chromosomes visible at periphery of egg. **9** (a) Metaphase of second mitotic division in which ten metaphase chromosomes are visible. (b) 15 polar chromosomes at periphery of egg. (c) Overview of egg showing both the metaphase of the central nucleus (a) and the polar chromosomes (b). **10** (a) Ten metaphase chromosomes of the third mitotic division in the egg (2 nuclei stage). (b,c) Different focal planes of polar body showing 15 polar chromosomes. **11** Ten metaphase chromosomes of the two nuclei in the third mitotic division in the egg. **12** Four metaphase plates of the fourth mitotic division in the egg (4 nuclei stage).

carried out by following the segregation of two linked markers. The markers *EST-1* and *PGM* have a crossing-over frequency of 13–15 per cent in arrhenotokous females. If no recombination occurs the markers from a grandparent should always segregate together whereas if recombination occurs crossing-over should result in the presence of a marker from the grandfather together with one from the grandmother in the same progeny at a frequency approximately equal to the crossing-over rate. This latter case appeared to be true: we found a crossing-over between the *EST-1* and *PGM* markers in thelytokous females of 10 per cent and, therefore, the cytogenetic mechanism should be a form of gamete duplication. Therefore the two identical mitotic products fuse after the first mitotic division or at some later stage. Our cytogenetic observations showed that the meiosis results in a haploid pronucleus consisting of five chromosomes and a total of 15 polar chromosomes. This stage was followed, after an interphase, by a metaphase in which five sets of metaphase chromosomes were visible. This metaphase was not followed by a complete anaphase, but the chromosomes just seemed to separate without a clear spindle formation resulting in a nucleus containing 10 chromosomes. After an interphase the nucleus reappeared consisting of 10 sets of metaphase chromosomes that underwent a normal mitosis. Throughout this process the 15 polar chromosomes remained at the periphery. Therefore, the cytogenetic mechanism is gamete duplication.

When a sperm fertilizes a thelytokous egg, several possible processes can take place. Smith (1955) suggested that the doubling of chromosomes would take place normally and would be followed by the fusion of this diploid nucleus with the sperm nucleus. This would result in triploid offspring. The segregation of markers in the offspring of these triploid females may be expected to be 2:1 for grandmaternal and grandpaternal markers, respectively. We find a segregation resulting in a 1:1 ratio of markers. Alternatively, the fusion of the sperm nucleus with the egg pronucleus may take place before gamete duplication. This would result in tetraploid eggs. Normal meiosis in eggs of such tetraploid females would result in diploid eggs. However, our finding that recombination takes place in eggs of heterozygous females (i.e. those females that are the offspring of a thelytokous mother and an arrhenotokous male), together with the fact that all offspring of such females are homozygous for all markers, excludes tetraploidy for these females. Therefore, the most probable scenario taking place in fertilized thelytokous eggs is that the fusion of the sperm nucleus with the egg pronucleus leads to the formation of a diploid nucleus and no subsequent gamete duplication takes place.

The frequent occurrence of gynandromorphs in the offspring of thelytokous females that have been reared at temperatures around 28 °C (Wilson & Woolcock, 1960; Wilson, 1962; Bowen & Stern, 1966; Laraichi, 1978; Babi & Pintureau, 1983; Vargas & Cabello, 1985) can also be explained from this cytogenetic mechanism. If the fusion of mitotic products takes place not during the first mitotic division but in only one nucleus of one of the later divisions, both haploid and diploid nuclei will be found in an embryo (Cooper, 1959). In the resulting adult those tissues orginating from the haploid cells will show male characteristics whereas those from the diploid cells will give rise to female tissues.

Genetic isolation of thelytokous lines

Under gamete duplication the presence of heterozygous thelytokous females indicates that they emerged from fertilized eggs. The population of *Trichogramma nr. deion* from the Mojave Desert, California, consists of both thelytokous and arrhenotokous forms; at least 31–81 per cent of the thelytokous females collected in the field had emerged from fertilized eggs. This indicates that a high proportion of the thelytokous females in the field mate, and consequently there is a substantial gene flow between the arrhenotokous and thelytokous populations in the field.

Implications for sex determination in Hymenoptera

The finding of gamete duplication as the mechanism for restoration of diploidy has important implications for sex determination in Hymenoptera. One of the currently accepted models is the single locus sex determination model developed by the Whitings and their collaborators (Whiting, 1961), in which the sex of an individual is determined by the alleles at the sex locus. Diploid individuals that are heterozygous at the sex locus become females whereas individuals homozygous at the sex locus become diploid males. Haploid individuals are always males. This model has been shown to be correct for several species of Hymenoptera (Cook, 1993b). Under this model diploid males should always be found in crosses between a mother and her haploid sons. Failure to detect diploid males in such crosses has resulted in the suggestion that possibly not one locus but several loci are involved in determining the sex of an individual (Snell, 1935). A diploid individual becomes a female when she is heterozygous at only one of these loci. Under this model it would take several generations of inbreeding before diploid males would be found in crosses between mother and son. However, even such inbreeding has not led to the

discovery of diploid males (Cook, 1993a), leading to the suggestion that homozygosity at certain of these sex loci would lead to lethality and consequently diploid males under this multiple locus sex determination model would not be detected (Crozier, 1971). Our finding that thelytokous lines of *Trichogramma* restore their diploid number by gamete duplication leads to the conclusion that no variant of the single or multiple locus sex determination models applies to these species. One could still hypothesize that in these thelytokous lines the symbiont produces some substance that would over-ride the nuclear genetic sex determination of wasps. This hypothesis can also be refuted, however, because arrhenotokous lines derived by antibiotic treatment from an isofemale line of thelytokous *Trichogramma* also produce female offspring from fertilized eggs (Stouthamer *et al.*, 1990b). These females are also completely homozygous and could not exist under any multiple locus sex determination mechanism. Additional cases of gamete duplication have been reported for *Muscidifurax uniraptor* (Legner, 1985) in which parthenogenesis is also associated with microbial infection (Stouthamer *et al.*, 1993) and in the gallwasp *Diplolepsis rosae* (Stille & Dävring, 1980), where possible microbial involvement in parthenogenesis has not been studied. In other haplo-diploid arthropods, such as thrips, whiteflies and spider mites, little is known about sex determination. However, in the whiteflies one case of gamete duplication is known, i.e. the thelytokous form of the greenhouse whitefly *Trialeurodes vaporariorum* (Thomson, 1927). Consequently, here too the sex determination cannot be a form of the multiple locus models.

The known cases in which microbes are thought to cause thelytoky all have gamete duplication as the mechanism of restoration of diploidy. Whiteflies are also known to harbour many symbionts and it is therefore conceivable that in the greenhouse whitefly symbionts are also associated with thelytoky. If this relationship between symbiont-induced thelytoky and gamete duplication holds then the cases of thelytoky in those species with a single or multiple locus sex determination cannot be attributed to microbial infection. Single locus sex determination may be the rule in Ichneumonoidea, Tenthredoidae and Apoidae (Stouthamer *et al.*, 1992; Cook, 1993b). In these cases the cytogenetic mechanism of thelytoky must be such that heterozygosity is retained in at least that part of the genome where the sex locus is found.

The alternative to the single or multiple locus models is the genetic balance theory in which the sex of an individual is determined by the noncumulative male-determining loci and cumulative female-determining loci. In a diploid individual the cumulative female-determining loci outweigh the male-determining loci but in haploid males the situation is reversed. The presence of diploid males in the species *Nasonia vitripennis* could be explained by using this model. The diploid males come about after a mutation that inactivates one of the main female-determining loci. Consequently, a diploid individual with one of these female-determining loci inactivated becomes a male. We can hypothesize that there are at least two different sex-determining systems in the Hymenoptera, one in which the sex is determined by a single locus and another which possibly works through a balance system.

Acknowledgements

We thank R. F. Luck for providing us with the opportunity to undertake part of this work during our PhD studies. J. D. Pinto and G. R. Platner provided us with most of the cultures used in these studies as well as their identity. U. Nur, J. A. J. Breeuwer and J. H. Werren advised us on the various staining procedures and U. Nur helped interpreting the cytogenetic results. We are grateful to J. M. Cook and an anonymous reviewer for comments on the manuscript. This work was supported in part by a USDA grant 91-37302-6206 (R.S) and grants from the chancellors patent fund of the University of California, Riverside to D. J. K. and R. S.

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