

Cytogenetic mechanism and genetic consequences of thelytoky in the wasp *Trichogramma cacoeciae*

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In Hymenoptera, complete parthenogenesis, that is thelytoky, is a common phenomenon where virgin females produce only daughters. Thelytoky is often induced by bacteria of the genus *Wolbachia*, but can also be genetically determined by the insect itself, as in the genus *Trichogramma* where both forms exist. In order to compare these two forms of thelytoky, chromosome behaviour analysis in young eggs and genetic analysis of microsatellite markers were carried out in the wasp *Trichogramma cacoeciae*, where thelytoky is genetically determined. Microscopic studies revealed that during female gamete formation meiotic cells undergo only a single equational division followed by the expulsion of a single polar

body. This absence of meiotic recombination and reduction corresponds well with the high levels of heterozygosity observed in females collected from the field and a nonsegregation pattern in the offspring of heterozygous females. We therefore concluded that diploidy in *T. cacoeciae* is maintained through an apomictic cloning mechanism and that the incidence of thelytoky under genetic control of the wasp differs entirely from the mechanism induced by *Wolbachia* infection, where thelytoky is restored through gamete duplication.

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Introduction

The most common form of reproduction in the insect order Hymenoptera is arrhenotoky, where haploid males develop from unfertilised eggs and diploid females develop from fertilised eggs. However, numerous species exhibit thelytoky, a form of complete parthenogenesis where unfertilised eggs develop into diploid females (Luck *et al.*, 1992). Suomalainen *et al.* (1987) and Gottlieb *et al.* (2002) described different mechanisms accounting for such parthenogenetic development, but differing in their consequences for genetic diversity: (1) Apomixis resembles a normal mitosis with only a single maturation division occurring in the oocyte. Recombination is generally absent, resulting in the conservation of heterozygosity in the offspring and clonal reproduction of females. (2) Automixis, where meiotic reduction is compensated, produces diploid gametes by premeiotic doubling, fusion of two of the four meiotic nuclei, or fusion of cleaved nuclei and results in decline of heterozygosity in the offspring, except for the case of premeiotic doubling where offspring is genetically identical to the mother. While apomixis has been observed in several orders of insects, it has only been observed in a few species in Hymenoptera where

automixis seems to prevail (Suomalainen *et al.*, 1987; Beukeboom and Pijnacker, 2000).

Thelytoky can be under the control of the insect itself or its endosymbionts (Stouthamer *et al.*, 1990a, 1997). Among the different microorganisms that have been identified (Stouthamer *et al.*, 1993; Weeks *et al.*, 2001; Zchori-Fein *et al.*, 2001), bacteria of the genus *Wolbachia* appear to be the most common parthenogenesis inducers. This symbiotic form of thelytoky can easily be distinguished from the thelytoky under direct control of the insect by its revertibility. In microbe-induced parthenogenesis, elimination of the symbiont through antibiotic or heat treatment allows females to produce males, whereas in the nonrevertible thelytoky, induction of male production is not possible (Stouthamer *et al.*, 1990b).

In the tiny wasps of the genus *Trichogramma* (Hymenoptera: Trichogrammatidae), both forms of thelytoky are known (Stouthamer *et al.*, 1990b). It is not known whether they are related, but one can imagine that nonrevertible parthenogenesis could have arisen from the integration of one (or more) bacterial gene(s) in the insect genome. This scenario has already been proposed in Isopods where both *Wolbachia* infection and nuclear factors, potentially acquired from *Wolbachia*, can cause feminisation (review in Rigaud, 1997). Recently, Kondo *et al.* (2002) found evidence for transfer of *Wolbachia* genes into the host genome.

One possible way to explore if the two forms of thelytoky are related is to compare their cytogenetic mechanisms. In *Trichogramma*, restoration of diploidy in *Wolbachia*-induced thelytoky arises from the nonsegregation of chromosomes

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during the first mitotic division of the development by fusion of cleaved nuclei and consequently produced totally homozygous females (Stouthamer and Kazmer, 1994).

Here, we describe the cytogenetic mechanism of thelytoky in *Trichogramma cacoeciae* and the consequences for its genetic diversity. The species exhibits complete parthenogenesis and males are absent in natural populations. Pinto (1998) observed only five males out of approximately 15 000 individuals under laboratory conditions. Males cannot be induced by antibiotic or heat treatment, and neither *Wolbachia* nor other symbionts have ever been found (Stouthamer *et al.*, 1990b). Our results show that, in *T. cacoeciae*, the cytogenetic mechanism of thelytoky and the consequences of asexuality for genetic diversity and heterozygosity clearly differ from what is observed in *Wolbachia*-induced thelytoky, suggesting an independent evolutionary origin of the two forms of thelytoky.

Material and methods

Strain and rearing

One line of *T. cacoeciae* (Y190) was established from a single female originating from a field-collected host in Wageningen (The Netherlands) in 1994. Wasp culture was maintained on irradiated *Ephestia kuehniella* eggs (supplied by Koppert bv, Berkel & Rodenrijs, The Netherlands).

Cytogenetic techniques

Female wasps were fed honey for 1 day after emergence. Several hundreds of females were given an egg sheet containing 10 *Mamestra brassicae* eggs. After 30–45 min, the egg sheet was removed and wasps remaining on the egg sheet were brushed-off. The egg sheet was kept for a variable time span (from 0 to 4 h) to allow the *Trichogramma* eggs to develop. Host eggs were opened in a drop of water on a microscope slide allowing the recovery of up to 30 wasp eggs. Host debris was then removed, and wasp eggs were dechorionated in bleach-water (1:250 commercial bleach solution:water) for 3 min, fixed in 60% glacial acetic acid for 3 min and finally covered with freshly prepared Carnoy's fixative (3:1 of absolute ethanol:glacial acetic acid). After air drying, slides were carefully rinsed with 70% ethanol and air dried again. DNA was then stained with DAPI (100 ng/ml) in Vectashield® (Vector Laboratories, Burlingame, CA, USA). Slides were covered with a coverslip and studied under an epifluorescence microscope with DAPI filter and ×100 Plan Apo objective. Selected images were

photographed on 400 ISO negative film and scanned for digital processing.

Genetic markers and PCR reaction

Four sets of microsatellite markers were used (Table 1), either to study segregation in the offspring of isolated females or to study the genetic diversity in the field (see below). For DNA extraction, one individual was ground in 50 µl of 5% Chelex solution. Samples were kept for 6 h at 56°C. After 10 min at 95°C, samples were centrifuged and 2–4 µl of the supernatant were used for PCR reactions in 25 µl final volume containing 200 mM dNTP, 10 pM primers (forward primer labelled with Cy3 fluorochrome), 1 IU taq DNA polymerase, and 2–4 µl of template DNA. PCR conditions were 5 min at 95°C, then 35 cycles: 1 min at 95°C, 1 min at annealing temperature (Table 1) and 1 min at 72°C. PCR reaction was ended by a 10 min elongation at 72°C. The amplified DNA samples were analysed on a polyacrylamide gel using an automatic sequencer and GeneScan software (Applied Biosystems).

Segregation experiments

In total, 15 females from the line Y190 were isolated and allowed to oviposit on *E. kuehniella* eggs for 5 days. DNA from mothers and daughters was then extracted, and all individuals were genotyped using the TAC47 microsatellite primers.

Genetic diversity in the field

Collection in the field was performed around Wageningen, The Netherlands using *Mamestra brassicae* eggs glued on cards (van Rijswijk *et al.*, 2000). Despite the absence of males, identification of *T. cacoeciae* is straightforward using the sequence of the internal transcribed spacer 2 (Pinto *et al.*, 2002). The emerged *T. cacoeciae* individuals were females, which were used to initiate isofemale lines. Since one egg card could have been parasitised by one or several females, we only studied a single line per egg-card. In the end, we could analyse the genotype of 14 field females. Despite the low number of individuals, we performed a parsimony analysis using PAUP 4.0b2a (Swofford, 2001) to reconstruct the history of these 14 genotypes.

Results

Cytogenetic analysis

Each parasitized *M. brassicae* egg contains about 30 *T. cacoeciae* embryos. This allowed us to look at several hundreds of embryos and consequently each stage of

Table 1 Microsatellite loci studied and genetic diversity measured on fourteen field-collected females

	TTG 46	TAC 47	TTC 53	CT 122
Forward primer	5'-GATGTTTACT TCGCAGGCCGC	5'-GGGCATGTGC AAAAGACTAG	5'-GTGACTGCCT TATTCTGCATAC	5'-CTACGGCGAC AATTGCCAC
Reverse primer	5'-CTACGGGGCA TACGATATGTG	5'-CGAGAGGGTTT CTTCAATGG	5'-TCGGGTCGTT GTAGCGGGC	5'-CATCTTGGTC GAACCGAGCAG
Annealing temp. (°C)	66	58	58	66
Number of alleles	5	10	4	2
min–max freq.	0.14–0.29	0.04–0.25	0.05–0.65	0.07–0.93
Heterozygosity	0.5	0.93	0.4	0.14

Only the frequencies of the least and the most common alleles are given for each locus.

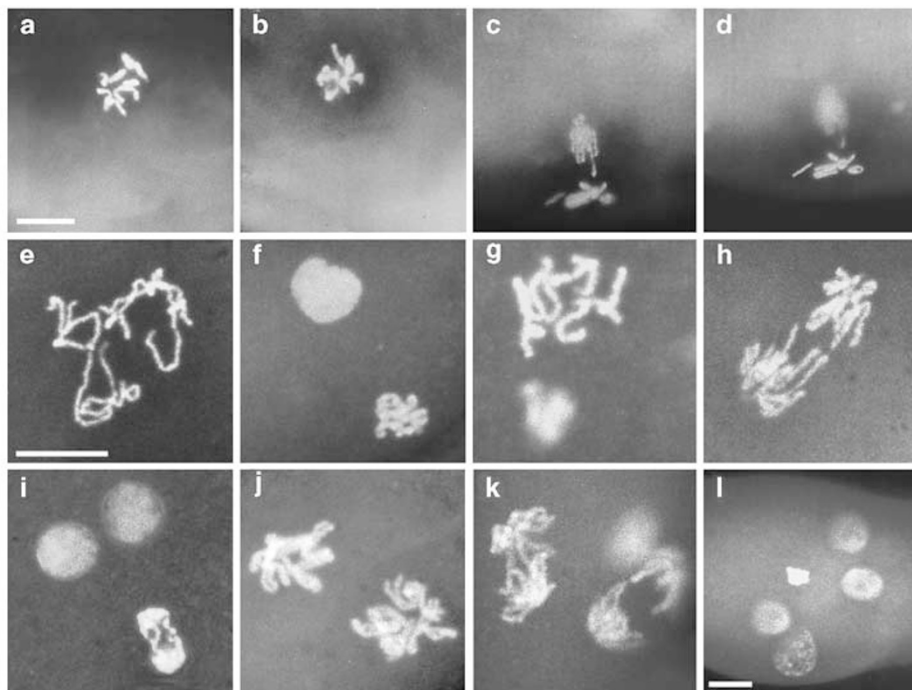


Figure 1 Meiosis and first mitotic divisions in *Trichogramma cacoeciae*. Bars indicates 10 μm . (a–d) same scale, (e–k) same scale. (a and b) Compact first metaphase. (c and d) First anaphase. (e) Central nucleus after the first anaphase showing 10 chromosomes. (f) Central nucleus in interphase indicating the end of meiosis and a single polar body. (g) Central nucleus in metaphase. (h) Central nucleus in anaphase of the first mitotic division. (i) Two central nuclei in interphase and a single polar body showing partial division. (j) Two central nuclei in metaphase of the second mitotic division. (k) Anaphase of the second mitotic division. (l) Four nuclei in interphase and a single polar body.

development has been observed several times. Early embryonic development of *Trichogramma* eggs have recently been characterized in *T. kaykai* (Van Vugt *et al*, 2003). We observed roughly the same time scale for development in *T. cacoeciae*. In newly laid eggs, we observed condensed chromosomes at meiotic prophase I (Figure 1a). The clumped chromosome mass did not allow us to study chromosome pairing. In approximately 30-min-old eggs, the nuclei decondense and orient towards the equatorial plate (Figure 1b). Although individual chromosomes are difficult to assess, we counted about 10 chromosomes in the complement, a diploid chromosome number also known from other *Trichogramma* species (Stouthamer and Kazmer, 1994; Van Vugt *et al*, 2003). Therefore, there is no doubling of the chromosome number before meiosis. Anaphase cells were rarely observed, which suggest that the stage lasts a very short time. One set of chromosomes lies close to the periphery of the egg and forms the polar body I, while the other set, with about 10 discernable chromosomes, lies in the central part (Figure 1c–d). We never observed cell complements with the haploid chromosome number ($n = 5$) indicating that meiosis is exclusively nonreductional (Figure 1e). After a short lasting interphase the central nucleus went through a mitotic division with 10 doubled chromosomes at metaphase (Figure 1f) and two sets of chromatids at anaphase (Figure 1g–h), indicating that meiosis is replaced by a single equational division followed by the expulsion of a single polar body. None of the two nuclei that entered interphase (Figure 1i) resulted in the polar body II, instead they divided again mitotically giving rise to four nuclei (Figure 1j–l). At the

same time, the chromosomes of the polar body I separated without displaying a clear mitosis.

Genetic analysis

Genetic analyses of females from line Y190 revealed heterozygosity for the TAC47 locus in all 15 mothers and 64 daughters tested. This suggests suppression of recombination of the locus due to its position in the achiasmatic centromere region or by the total absence of meiotic recombination during the 2 years this isofemale line was maintained in the laboratory.

Tests on the genetic variability and heterozygosity of the four microsatellite loci in 14 field-collected females are presented in Table 1. Number of alleles per locus varies from 2 to 10, and heterozygosity ranged from 0.14 for CT122 to 0.93 for TAC47. Moreover, in the 14 females that were tested we detected 11 different profiles, showing that populations are composed of several different genotypes, and that genetic diversity in these markers is relatively high. To gain insight into the history of these fourteen genotypes, we used parsimony to analyse their relationships. We obtained 12 equally parsimonious trees. These trees have a length of 22, while the minimum length is 20, indicating that only two homoplasies are necessary to reconstruct the phylogenetic relationships among the 14 females. Even though based on only 14 individuals, this analysis suggests that recombination does not take place, that sex is either absent or extremely rare, and that asexuality probably arose from a single event in this species.

Discussion

Our results strongly suggest achiasmatic meiosis in the thelytokous *T. cacoeciae* because: (i) meiosis is achiasmatic with only a single equational division and expulsion of a single polar body; (ii) heterozygous females do not segregate for microsatellite markers in their offspring; (iii) field-collected females display moderate to high levels of heterozygosity. This mechanism is thus typically apomictic and differs substantially from *Wolbachia*-induced parthenogenesis insect systems where diploidy is restored through an automixis resulting in homozygous females in a single generation (Stille and Dävring, 1980; Stouthamer and Kazmer, 1994; Gottlieb et al., 2002) and therefore suggests that *Wolbachia* and genetically determined thelytoky in *Trichogramma* do not have a common evolutionary origin.

Thelytoky in *T. cacoeciae* could have originated from interspecific hybridisation (Pinto and Stouthamer, 1994), which has been described for *T. voguelei* × *T. brassicae* (Pintureau and Babault, 1981) and *T. exiguum* × *T. californicum* (Nagarkatti, 1970). Hybridisation-induced parthenogenesis is likely caused by epistatic interactions between parental genomes. Lack of recombination (as observed in *T. cacoeciae*) between the parental genomes can make epistatic interactions fixed and could allow the maintenance of thelytoky.

The great genetic diversity, based on the large number of different genotypes in this small population sample, can be explained by different factors. Firstly, heterozygosity resulting from incidental sexual reproduction may be maintained through generations in the absence of recombination, as suggested in thelytokous species of the genus *Lysiphlebus* (Belshaw et al., 1999; Belshaw and Quicke, 2003). However, recombination has not been detected among the 14 females tested, and moreover apomixis should lead in an increase of ploidy if rare sex occurs (Belshaw and Quicke, 2003), which has not been observed. Secondly, mutation-drift equilibrium could be in favour of mutations, which implies that the effective size of the population is sufficiently high to maintain an important allelic diversity (Kimura, 1983). It would be very interesting to have a clearer picture of the genetic diversity in *T. cacoeciae* at a larger geographical scale. Comparison between mitochondrial and nuclear markers could help in resolving this issue, since the same patterns are expected for the two compartments only if rare sex does not occur.

In any case, the mechanism that maintains diploidy prevents the formation of homozygotes and thus the expression of recessive lethal mutations, reducing the negative impact of the lack of sexual reproduction. To what extent this may explain why *T. cacoeciae* shows such a wide geographic distribution remains to be studied (Pintureau, 1997). However, since the genotypes are fixed in the course of generations (no recombination occurs), there is little space for the creation of new genetic combinations and *T. cacoeciae* can be expected to respond to selection slower than sexual taxa, unless rare sex occurs and then creates new combinations.

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