

GENETIC AND PHENOGENETIC STUDIES ON THE
DYNAMIC NATURE OF THE
CYTOPLASMIC INHERITANCE SYSTEM IN *CULEX PIFIENS*

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ABSTRACT

Analysis by a progeny test system has permitted the selection for different cytoplasmic sterility factors in two strains of mosquitoes derived from the progeny of a single female. Reciprocal interstrain crosses show full fertility and viability. However, testcrosses to a third cytoplasmic type show highly significant, reproducible differences in the cytoplasmic systems of the selected strains. A direct relationship between teratological growth patterns and the degree of heterozygosity of cytoplasmic factors was observed. In testcrosses, cytoplasmic sterility factors, when selected to homozygosity, produce non-teratological inviable haploid, or exceptional viable gynogenetic diploid larvae. Heterozygosity in the cytoplasmic system in testcrosses produced a broad spectrum of teratological growth patterns related to the degree of heterozygosity of the multiple factors present in the cytoplasm. Formal genetic crosses involving the selected strains show that teratological growth and cytoplasmic sterility, both partial and complete, are inherited through the germ plasm of the maternal parent. This work suggests that the interactions of cytoplasmic factors that result in teratological growth and cytoplasmic sterility may not be restricted to *Culex pipiens*, where in some cases it is prominent and obvious, but may be a much more general hereditary mechanism of major significance in other eukaryotic animals.

ONE of the best documented cases of cytoplasmic inheritance in eukaryotic animals is the cytoplasmic incompatibility system in the *Culex pipiens* complex. In crosses between mosquitoes of different geographical origins, cytoplasmic incompatibility (sterility) may result in neither, in one, or in both crossing directions of the reciprocal crosses. At least seventeen different crossing types have been identified. This sterility is thought to be due to failure of syngamy in the egg after sperm penetration (LAVEN 1957, 1967).

Initial experimental results indicated that the cytoplasmic system in any given population is quite uniform and that crosses between individuals from two allopatric populations should show complete incompatibility or essentially complete fertility depending on the cytoplasmic type and the crossing direction in the reciprocal crosses. This cytoplasmic system has been reviewed in detail by LAVEN (1967).

Later studies indicated that cytoplasmic incompatibility shows uniform and predictable results when certain populations are tested under laboratory condi-

tions. However, other populations show considerable variability in their cytoplasmic systems. This variability results in the appearance of a variable number of viable progeny in crosses that would otherwise be considered cytoplasmically incompatible. This phenomenon has been designated partial compatibility and indicates a polymorphism in the cytoplasmic mating types of many mosquito populations (BARR 1970; FRENCH 1970; FAUST 1971; KRISHNAMURTHY and LAVEN 1974; SUBBARAO *et al.* 1974, 1975).

Although the references cited indicate that this partial compatibility is hereditary, no systematic genetic analysis has been presented to elucidate the fundamental mechanisms of inheritance of this dynamic system. The term dynamic is used in this paper, as it is in physics, to convey the concept of changes in motion (phenotypic expression) and equilibrium in a system under the action of forces (biological factors) both intrinsic and extrinsic to the system itself. The present paper is a systematic genetic analysis of the intrinsic factors of the cytoplasmic inheritance system in *Culex pipiens*.

MATERIALS AND METHODS

Strains: Three strains of *Culex pipiens pipiens* were used in these experiments. The strain designated as Aw-193-B was collected in the Aswan dam area of Egypt and selected for cytoplasmic compatibility to the Thies strain. A second strain, also from the Aswan dam area of Egypt, designated As-209-A was selected for cytoplasmic incompatibility to the Thies strain. The Thies strain, Th, collected in the vicinity of Thies, Senegal was used as a standard reference strain for crossing with Aw-193-B and As-209-A to determine the cytoplasmic mating types of the Aswan strains. The Th strain carried the sex-linked recessive eye color rot (WILD 1963) in the homozygous condition.

The phenotype of the homozygous recessive rot, an orange eye color, was clearly distinguishable in well-developed, unhatched larvae from either the heterozygous or homozygous normal phenotypes, which have a bright-red eye color during later stages of larval development. These phenotypes were utilized to distinguish, unambiguously, whether a given Th larva had developed gynogenetically or after a normal process of fertilization. The original strains utilized in this research were graciously provided by Dr. Hannes Laven.

Before the initiation of these experiments, the original Aswan and Thies strains were continuously inbred by brother-sister matings and were selected for fertility in the intrastain crosses. Egg rafts from individual females in intrastain crosses showed greater than 95% embryonation of the eggs within a single raft, and no less than 98% of the embryonated eggs hatched when the genetic crosses were initiated. There was no teratological growth in any progeny in the intrastain crosses. This level of fertility and normal development was maintained throughout the course of this work in all intrastain crosses.

Culture techniques: Egg rafts were collected individually in shell vials and first instar larvae from single rafts were reared to adulthood in quart milk bottles. After emergence, the adults were placed into cages made from round half-gallon ice cream cartons. Both larvae and adults were reared at 25°, with a relative humidity of 80%. The adults were fed on cotton pads saturated with a 10% honey solution.

Selection for cytoplasmic factors: A progeny-test procedure was required for a systematic selection for cytoplasmic sterility factors. This procedure is a method of estimating the breeding value of an animal by the performance or phenotype of its offspring. The average percentage of viable offspring from individual egg rafts, produced when several of the male progeny from a single Aswan female are crossed to a number of partially compatible Thies females, gives an excellent index (cytoplasmic index) of the cytoplasmic composition of the individual maternal Aswan female.

If the cytoplasmic index were low for a given female (her male progeny on testing produced a predominance of inviable offspring), the daughters of this female were brother-sister mated and individually tested. A single female who showed a lower cytoplasmic index than her mother was selected as a parent for the subsequent generation of selection. Three generations were required to establish a completely cytoplasmically incompatible strain of Aswan mosquitoes to the reference Thies cytoplasm.

To establish a cytoplasmically compatible Aswan strain to the reference Thies cytoplasm, a single female who showed a higher cytoplasmic index than her mother was selected as a parent in the next and subsequent generations of selection.

Crosses to elucidate the hereditary mechanism of partial cytoplasmic sterility: After selection of the As-209-A strain for complete incompatibility and the Aw-193-B strain for a high degree of compatibility to the Thies cytoplasm, the two strains (As-209-A \times Aw-193-B) were reciprocally crossed, and all appropriate F_1 , F_2 , and backcross mosquitoes were produced. At least 20 male progeny in each generation were crossed to Thies females to determine the properties of the cytoplasmic factors present. Formal genetic crosses between the two Aswan strains in all generations produced normal, fully fertile, and viable offspring; differences in results were evaluated by testcrossing males produced in each generation to Thies females.

RESULTS

Selection for cytoplasmic factors: The selection for cytoplasmic genes was initiated with the progeny of a single female, and was continued for three generations. Data from the (Aswan) population from which the initial female was selected are given in Table 1. In this population, the cytoplasmic index (the mean percentage of hatching larvae from the individual egg rafts) and the mean and standard error after arcsin transformation were 3.83% (7.35 ± 9.26). The cytoplasmic index is not appropriate for direct statistical analysis since, as it

TABLE 1

*Results of selection for cytoplasmic factors*Part I. Results of testcrosses of $\delta \delta$ from each strain to Thies $\varphi \varphi$

	Rafts	Eggs	Embryo-nated eggs	Unembryo-nated eggs	Larvae	Cytoplasmic index*	
Aswan	10	1116	677	398	41	$\bar{Y} = 3.83\% (7.35 \pm 9.26) \dagger$	
Aw-193-B	10	1846	89	599	1158	$\bar{Y} = 57.09\% (49.25 \pm 11.12)$	
As-209-A	10	1565	956	608	1	$\bar{Y} = 0.04\% (0.358 \pm 1.13)$	
Part II. Contingency tables showing the results of selection							
Part II-A	Eggs	Larvae			Part II-B	Eggs	Larvae
Aw-193-B	1846	1158	3004		Aswan	1121	36 1157
As-209-A	1565	1	1566		As-209-A	1565	1 1566
	3411	1159	4570			2686	37 2723
$\chi^2 = 805.41 \gg 12.1$	[χ^2 0.0005(1)]				$\chi^2 = 46.11 \gg 12.1$	[χ^2 0.0005(1)]	
	Part II-C	Eggs	Larvae				
	Aswan	1121	36 1157				
	Aw-193-B	1846	1158 3004				
		2967	1194 4161				
	$\chi^2 = 512.66 \gg 12.1$	[χ^2 0.0005(1)]					

* The mean percentage of matching larvae from the individual egg rafts.

$\dagger \bar{Y} \pm SE$ after arcsin $\sqrt{\text{percentage}}$ transformation of data (SNEDECOR 1946).

approaches its limits of 0% or 100%, it is not normally distributed. The data after arcsine transformation are normally distributed and provide useful information about the mean and its variability (SNEDECOR 1946).

After selection for cytoplasmic sterility, the strain As-209-A showed complete fixation of the cytoplasmic factors, with the cytoplasmic index and its standard error approaching zero. The single larva produced in the testcrosses was a gynogenetic female. Such females are expected at this frequency in stable cytoplasmically incompatible crosses (LAVEN 1967).

A simple 2×2 contingency table is presented in Table 1, Part II-B, which compares the number of viable larvae hatching from the eggs laid in testcrosses of males, from both the unselected Aswan strain and the As-209-A strain, to the Thies cytoplasm. The experimental χ^2 value of 46.11 exceeds the standard χ^2 value of 12.1 at the 0.0005 probability level. Selection for cytoplasmic sterility was obviously very effective.

The data in Table 1, Part II-C, show that the selection for compatibility to the Thies cytoplasm in the Aw-193-B strain was successful. The null hypothesis that there is no difference between the unselected Aswan and the Aw-193-B males in compatibility to Thies females under the given experimental conditions can be rejected with high confidence. The experimental χ^2 value of 512.66 exceeds the standard value of χ^2 of 12.1 at the 0.0005 probability level, with one degree of freedom, by a very comfortable margin.

The third 2×2 contingency test (Table 1, Part II-A) compares the As-209-A strain with the Aw-193-B strain and indicates whether selection for cytoplasmic compatibility (Aw-193-B) and selection for cytoplasmic incompatibility (As-209-A) has differentiated these strains with respect to the Thies cytoplasm. The experimental χ^2 value of 805.41 for this test significantly exceeds the standard χ^2 value of 12.1 at the 0.0005 probability level, with one degree of freedom.

Therefore, selection of factors for cytoplasmic compatibility and for cytoplasmic incompatibility, initiated with the progeny of a single female, has been very effective in producing significant changes in the cytoplasmic systems of the mosquitoes.

Observations on the inheritance of teratological growth: A direct relationship was observed during the course of selection for the cytoplasmic factors between teratological embryonic development and cytoplasmic sterility in the testcrosses. Before selection, the Aswan strain showed a significant amount of abnormal embryonic development in the progeny of males from this strain when crossed to Thies females. This abnormal growth was most readily observed in the development of the larval eyes, which were clearly visible through the egg membranes of unhatched eggs. Other readily distinguishable abnormalities were found in the formation of the head, the egg tooth, the thorax, and the abdominal tergites and sternites.

During selection for cytoplasmic incompatibility of the As-209-A strain, teratology diminished progressively until at the end of selection, when cytoplasmic incompatibility was complete and stable, no teratological larvae could be found among the embryonated unhatched eggs of the test progeny.

Selection for partial cytoplasmic compatibility in the As-193-B strain resulted in an increase in the variability of the expression of larval teratology in the progeny of males from this strain when tested to Thies females. Although progressively more normal larvae hatched in the test crosses during the course of this selection, many of those larvae that did not hatch showed an extreme degree of teratological development.

In the formal genetic crosses to be presented next, all crosses between the Aswan strains resulted in normal embryonic development. Only when the males from the selected strains were testcrossed to the Thies cytoplasm were pronounced differences between the strains observed in embryonic development.

Crosses to elucidate the hereditary mechanism of partial cytoplasmic sterility: The results of crosses involving As-209-A (selected for cytoplasmic sterility) and Aw-193-B (selected for a high degree of cytoplasmic compatibility) are presented in Tables 2 and 3. The F_1 data show that the Aw-209-A females transmit to their F_1 males the stable cytoplasmic sterility established during selection. The F_1 data also clearly show that the partial cytoplasmic compatibility present in the As-193-B females is transmitted to their male progeny. It is also clear from the data that the degree of cytoplasmic compatibility in this case has been significantly reduced, in the absence of selection, from that found in the As-193-B population immediately after selection.

Ten egg masses per cross were classified as either partially compatible or incompatible in the F_2 and all backcrosses. To be classified as incompatible, embryonated eggs must be unhatched and all the unhatched larvae (or any rare hatched larva) in the egg mass must have the rot eye phenotype. This phenotype provides direct evidence for gynogenetic development and cytoplasmic

TABLE 2

Results of crosses of As-209-A ♀♀ by Aw-193-B ♂♂, data from testcrosses of progeny ♂♂ to Thies ♀♀

Crosses: $P_1 \times P_1$ As-209-A ♀♀ \times Aw-193-B ♂♂
 F_1 (F_1 ♂♂ \times r/r Th ♀♀)

Rafts	Eggs	Unembryo- nated eggs	Rot-eye embryonated not hatched	Results: + eye embryonated not hatched	Hatching larvae	Cytoplasmic index*
14	1421	506	915	0	0	0% (0.0 \pm 0.0)†
	$F_1 \times F_1$					
	F_2 (F_2 ♂♂ \times r/r Th ♀♀)			Results: 10 rafts—		incompatible
	F_1 ♀♀ \times As-209-A ♂♂			Results: 10 rafts—		incompatible
	‡ (BC ♂♂ \times r/r Th ♀♀)			Results: 10 rafts—		incompatible
	F_1 ♂♂ \times As-209-A ♀♀			Results: 10 rafts—		incompatible
	(BC ♂♂ \times r/r Th ♀♀)			Results: 10 rafts—		incompatible

* The mean percentage of hatching larvae from the individual egg rafts.

† $\bar{Y} \pm$ SE after arcsin $\sqrt{\text{percentage}}$ transformation of data (SNEDECOR 1946).

‡ BC ♂♂ indicates the male progeny from a backcross of F_1 mosquitoes to one of the parental types.

TABLE 3

Results of crosses of Aw-193-B ♀♀ by As-209-A ♂♂, data from testcrosses
of progeny ♂♂ to Thies ♀♀

Crosses: P₁ × P₁ Aw-193-B ♀♀ × As-209-A ♂♂
F₁ (F₁ ♂♂ × r/r Th ♀♀)

Rafts	Eggs	Unembryo- nated eggs	Rot-eye embryonated not hatched	Results: + eye embryonated not hatched	Hatching larvae	Cytoplasmic index*
10	995	670	227	31	67	6.73% (14.26 ± 5.32)†
	F ₁ × F ₁					
	F ₂ (F ₂ ♂♂ × r/r Th ♀♀)			Results: 10 rafts—partially compatible		
	F ₁ ♀♀ × As-209-A ♂♂					
	‡ (BC ♂♂ × r/r Th ♀♀)			Results: 10 rafts—partially compatible		
	F ₁ ♂♂ × As-209-A ♀♀					
	(BC ♂♂ × r/r Th ♀♀)			Results: 10 rafts—incompatible		

* The mean percentage of hatching larvae from the individual egg rafts.

† $\bar{Y} \pm SE$ after arcsin $\sqrt{\text{percentage}}$ transformation of data (SNEDECOR 1946).

‡ BC ♂♂ indicates the male progeny from a backcross of F₁ mosquitoes to one of the parental types.

incompatibility. To be classified as partially compatible, all hatched larvae from an egg raft must have a normal eye color and at least some of the embryonated eggs containing unhatched larvae must show normal eye pigmentation.

The data clearly show that in all crosses between the As-209-A and the Aw-193-B strains, the cytoplasmic gene composition of the progeny is directly dependent on the genetic composition of the cytoplasm of the maternal parent of the males tested to the Thies cytoplasm.

DISCUSSION

YEN and BARR (1973) proposed a hypothesis in which the etiological agent for cytoplasmic incompatibility in *Culex pipiens* is a rickettsia-like symbiote; *Wolbachia pipientis*. The results of this work are interpreted as indicating that each geographical form of *C. pipiens* has developed its own clone of microorganisms. It is these coadapted combinations of mosquitoes and *Wolbachia* that are interpreted as producing the spectrum of mating types found when different strains of *C. pipiens* are crossed.

The evidence of YEN and BARR (1973) is well presented and convincing. The existence of a system of hereditary, self-replicating cytoplasmic endosymbiotes in these mosquitoes is in complete accord with the genetic data presented in the present paper.

Other hypotheses concerning cytoplasmic incompatibility, however, are not consistent with these genetic data.

The first (LAVEN 1969), although not proposed as an alternative to incomplete cytoplasmic compatibility, could account for the variable number of viable progeny in a cross that was otherwise sterile. In that case, the transmission of

semisterility is restricted to males as a consequence of a translocation between the male-determining chromosome and one or the other of the autosomes. If the males were permanent heterozygotes, they would constantly give rise to reduced numbers of offspring. The males in that type of system must be permanent heterozygotes, and the inheritance must be from father to son. The data (Tables 2 and 3) clearly show that this is not the case in the present work. The data show that the sterility mechanism follows a strict maternal inheritance pattern and do not show the pattern required if male-linked translocations were responsible for the partial sterility.

The phenomenon of partial compatibility is widespread in *Culex pipiens*. It is important that adequate genetic data be analyzed before assuming, even in the presence of visible cytological aberrations, that any hereditary sterility is due to a complex mechanism such as permanent male-linked translocations.

A second hypothesis, which has been proposed by McCLELLAND (1967) to explain the nonreciprocal compatibility phenomena in *Culex*, assumes that there exists at least one pair of cytoplasmic conditioning alleles. One of these alleles must be incompatible with the cytoplasm associated with the other allele. A second assumption essential to rationalize LAVEN's (1957) data to this hypothesis is that polarized segregation during spermatogenesis, and not necessarily at oogenesis, is required in which the compatible allele is effectively eliminated in functional sperm. If the required highly efficient meiotic drive mechanism were present in the cytoplasmically stable Aswan strain (As-209-A), then the pattern of inheritance in the experimental crosses (Tables 2 and 3) would show a strong paternal influence. This is in all ways contrary to the data presented.

The possibility that cytoplasmic incompatibility in these mosquitoes is the result of the loss or gain of infectious microbial agents is not supported by the genetic data. In the formal genetic crosses between the two selected Aswan strains, reciprocal P_1 , F_1 , F_2 and backcrosses afforded maximal opportunity for any possible infection by microbial agents, yet the cytoplasmic systems remained unaltered within the parameters of these experiments. The possibility of infectious agents being the causative agents of cytoplasmic incompatibility is contrary to the evidence presented by YEN and BARR (1973) and BARR (personal communication) in which noninfectious, coadapted, cytoplasmic endosymbiotes are implicated as the etiological agents of cytoplasmic incompatibility.

That different hereditary cytoplasmic factors exist between populations of *Culex pipiens* is well documented (LAVEN 1957, 1967). That this heterogeneity also exists within populations is also well documented (BARR 1970; FRENCH 1970; FAUST 1971; KRISHNAMURTHY and LAVEN 1974; SUBBARAO *et al.* 1974, 1975). The present paper provides evidence to show that heterogeneity of cytoplasmic factors can exist within individuals of a population. Genetic analysis of these individuals provides insight into the nature and significance of alternative hereditary cytoplasmic factors in these eukaryote animals.

When heterogeneity of cytoplasmic factors exists in the germ plasm of an individual female, a segregation of these factors occurs. This segregation is most obviously observed using the progeny-test system presented earlier and most

readily documented by the effectiveness of selection where this segregation provides the requisite cytoplasmic variability.

When all available evidence is considered, the following interpretation of the results on teratological development appears to be the most reasonable.

When both compatible and incompatible cytoplasmic alleles are introduced by the sperm during the fertilization process, fertilization may or may not take place depending on the relative proportion of the compatible factors present in the sperm cell. If the proportion of factors coadapted in the sense of YEN and BARR (1973) is sufficiently high, fertilization can occur. However, the resulting embryo will contain within its cells varying proportions of the alternative incompatible cytoplasmic factors, introduced by the sperm, when the individual cell membranes are formed during subsequent differentiation of the fertilized egg. Subsequent development of the individual cells reflect this heterogeneity of cytoplasmic factors in their efficiency of cell metabolism and cell division. It is most likely this differential efficiency in cellular metabolism and mitosis that is observed phenotypically as teratological growth in these mosquitoes.

Irrespective of the above interpretation, the results of this work show that non-teratological, gynogenetic development is most characteristic of cytoplasmically incompatible crosses, and teratological diploid embryonic development is the most characteristic property of partially compatible crosses.

It was possible to evaluate the relative frequency of alternative cytoplasmic factors causing partial cytoplasmic compatibility during the course of selection and analysis by observing larval viability. The relative frequencies of these alternative factors constitute intrinsic forces operating on this cytoplasmic system. The dramatic changes in compatibility and teratology resulting when these various intrinsic forces were systematically evaluated in the cytoplasm of the testcrosses reveal the dynamic nature of these cytoplasmic systems.

Teratology diminished progressively during selection for both the compatible and the partially compatible strains when tested as indicated in this text. Teratology was completely eliminated in the progeny of testcrosses of the incompatible strain, and its frequency was greatly reduced in the testcross progeny of crosses involving partial compatibility.

Cytoplasmic factors causing teratology and sterility can exist at variable frequencies in populations where their expression is completely repressed, and these factors are expressed only when outcrossed to a cytoplasmic system to which they are not coadapted.

Under the above conditions, especially if the frequencies of the detrimental cytoplasmic factors were low, the hereditary nature of cytoplasmic types of sterility, abnormal growth and teratology in other eukaryote organisms would defy precise genetic analysis. It is extremely unlikely that *Culex pipiens* is the only eukaryotic species during evolution that has acquired coadapted hereditary cytoplasmic endosymbiotes (cytoplasmic factors) such as the rickettsia-like *Wolbachia pipientis* of YEN and BARR (1973). These cytoplasmic phenomena are most probably of fundamental importance in other eukaryote animals.

Systematic procedures for cytoplasmic genetic analysis in eukaryote animals have been presented, and some properties of the cytoplasmic inheritance system in *Culex pipiens* have been elucidated.

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