A NEW MEMBER OF THE *ANOPHELES QUADRIMACULATUS* COMPLEX, SPECIES C

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ABSTRACT. A new member, species C, of the *Anopheles quadrimaculatus* complex was recently found in collections from the northwest coast of Florida. This new species cannot be differentiated from the other 2 species with available taxonomic keys. Evidence for this taxon as a sibling species includes data on hybrid sterility and distorted sex ratios in the progeny of crosses to species A and B, chromosomal differences, and diagnostic allozymes.

INTRODUCTION

Previously, 2 sympatric sibling species within the taxon of *Anopheles quadrimaculatus*, provisionally designated species A and B, were described from populations in the southeastern United States (Lanzaro[1] 1986, Kaiser et al. 1988a). Taxonomic keys are not suitable for the identification of the 2 species, but they can be identified by diagnostic allozymes (Lanzaro[1] 1986, Narang et al. 1988) and polytene chromosomes (Kaiser et al. 1988b). When strains of the 2 species were crossed, the hybrid females were semisterile and the hybrid males were sterile (Lanzaro et al. 1988, Kaiser et al. 1988a). They noted polymorphic incompatibility in species B, in that some lines propagated F_1 hybrid males and other lines did not.

Further investigation of the distribution of species A and B led to the identification of a third species in a population along the Gulf coast of northwest Florida. In this report, we describe the results of hybridization crosses to species A and B, and also electrophoretic and cytogenetic criteria used to identify the new form, tentatively designated as species C.

MATERIALS AND METHODS

Anopheles quadrimaculatus s.l. adults were aspirated from tree holes at 2 locations (ca. 5 km apart) in a hardwood hammock next to a saltmarsh in Levy County (LEV), Florida, on the northwest Gulf coast. Subsequent transfer to the laboratory, rearing, and handling were done according to established procedures (Kaiser et al. 1988a). Cytological analysis of the ovarian nurse cell polytene chromosomes of adult females was done according to the tech-

niques of Kaiser and Seawright (1987). When chromosomes that were different from those of species A and B were observed, eggs were collected from additional individual LEV females in vials. As is often the case for anopheline females, it was necessary to traumatize the females by tearing a wing from the thorax to stimulate oviposition. Isofemale lines were established, and the X chromosome pattern was used to distinguish species A and B from the third cytotype (provisionally named species C).

Previous work by Lanzaro[1] (1986) indicated that 2 isocitrate dehydrogenase (IDH, E.C.1.1.1.42) loci, *Idh-1* and *Idh-2*, were diagnostic for identifying species A and B. Therefore, starch gel electrophoresis (Steiner and Joslyn 1979) was used to examine the *Idh* allozymes in isofemale lines of species C for comparison to species A and B using standard reference strains: species A. Q2 was homozygous for *Idh-1^{100}* and *Idh2-^{100}*; species B. MON, an inbred stock from Montgomery County, AL was homozygous for *Idh-1^{86}* and *Idh-2^{162}* (Lanzaro[1] 1986). The results were used as a basis for subsequent analysis and determination of the frequencies of species A, B and C in the LEV population.

Induced copulation techniques (Baker et al. 1962) were used for crossing species C to species A STU (a 3-year old stock from Stuttgart, Arkansas) and species B MON (Kaiser et al. 1988a). The results obtained from crosses of STU to MON were reported previously by Kaiser et al. (1988a). Gravid females were traumatized and placed in vials for oviposition, egg hatch (%) was determined and families were reared separately. The fertility of hybrid progeny was assessed by dissection of the testes and ovaries according to the method described by Lanzaro et al. (1988). Salivary gland polytene chromosomes from 4th stage hybrid larvae were prepared by established procedures (Kaiser et al. 1982). When fertile hybrids from crosses of species B and C were found, backcrosses were performed to ascertain whether gene flow between the 2 species was possible. Due to the considerable difficulties encountered in syn-

[1] Lanzaro, G. C. 1986. Use of enzyme polymorphism and hybridization crosses to identify sibling species of the mosquito *Anopheles quadrimaculatus* Say. Ph.D. dissertation, University of Florida, Gainesville, FL, 92 pp.

chronizing the rearing of the F_1 hybrids and the MON and species C strains, backcrosses were conducted to the parental type that was available.

RESULTS AND DISCUSSION

The method used previously (Kaiser and Seawright 1987, Kaiser et al. 1988b) for preparation of the ovarian nurse cell polytene chromosomes of species A and B was not suitable for species C. As shown in Fig. 1, the chromosomes of species C had diffuse bands and large areas of asynapsis (Fig. 1b). Varying the incubation time after the blood meal (before dissection) or the fixation and staining time of the ovaries failed to improve the results. This method, which provided excellent preparations of *Anopheles culicifacies* Giles for Saifuddin et al. (1978), has been used successfully in our laboratory to study the ovarian polytene chromosomes of *Anopheles freeborni* Aitken, *Anopheles punctipennis* Say, and *Anopheles crucians* Wiedemann (unpublished data). The technique of Green (1972), which was used for *Anopheles gambiae* Giles, was also tried unsuccessfully. However, it was possible to use cytological criteria for identifying species C because of the peculiar appearance of the chromosomes with diffuse bands in all of the 100 females analyzed. As indicated by Narang et al. (1988), there was a positive correlation between allozyme and chromosome analyses in the identification of the 3 sibling species.

The salivary gland chromosomes from 20 larvae of species C were fragile and the band patterns were not easily interpreted. Salivary gland polytene chromosome preparations from larvae of species A and B were of inferior quality which precluded comparisons with C.

When electrophoretic patterns of *Idh-1* and *Idh-2* were analyzed comparatively, species C was distinguishable from both species A and B (Fig. 2). Species A and C, though polymorphic for *Idh-1*,[100] contained *Idh-1*[100] as the most common allele; species B was homozygous for *Idh-1*[86]. For *Idh-2*, species B and C were homozygous for *Idh-2*[162]. Species A was polymorphic, with a low frequency of *Idh-2*[162] (0.05 to 0.33) in natural populations (Lanzaro[1] 1986, Narang et al. 1988). Analysis of field collections by the electrophoretic method (2 *Idh* loci) from LEV showed that the adults of species A and C occurred sympatrically at both sites. Of the LEV adults analyzed, the majority were species C, with species A present at frequencies of 20% and 6% at the LEV-1 (n = 102) and LEV-2 (n = 118) sites, respectively. The results indicated reproductive isolation between species A and species C (Narang et al. 1988). Species B indi-

viduals were not seen in the electrophoretic samples from LEV, but 2 B females were found during cytological analysis of adults from the same collection.

In a subsequent more detailed study it was determined that the analysis of additional loci was necessary for the precise identification of the 3 sibling species. About 4% of the species A individuals in the LEV population were homozygous for *Idh-1*[100] and *Idh-2*[162], and therefore would be incorrectly scored as species C. Other diagnostic loci, viz., Acon-1 (ACON, E.C.4.2.1.3) *Est-2* (EST, E.C.3.1.1.1), *Had-1* and *Had-3* (HAD, E.C.1.1.1.8), *Got-2* (GOT, E.C.2.6.1.1), *Pgi-1* (PGI, E.C.5.3.1.9), *Pep-2* (PEP, E.C.3.4.1.1), *Mpi-1* (MPI, E.C.5.3.1.8) and *Pgm-3* (PGM, E.C.2.7.5.1) (unpublished data) can be used in conjunction with the *Idh* loci for an accurate identification of species C.

The results of interspecific crosses and backcrosses (Tables 1 and 2) confirmed postmating reproductive isolation between species A, B and C. The F_1 progeny of the reciprocal crosses, C × A and A × C, consisted of sterile hybrid males (Table 1). There were no hybrid female progeny. Mortality of female progeny occurred in the embryonic and larval stages, as indicated by the low fertility and survival rates, respectively.

In the crosses between species B and C, polymorphic incompatibility was observed (Table 1). There were 3 types of results for the cross, B × C, and 2 types for the reciprocal cross, C × B. For the crosses involving C males, the types of results were: (1) C_1, the survival was very low (12.3%) and only hybrid females, with no ovaries, survived; (2) C_2, the survival was 11.2%, and only hybrid males that lacked testes survived; (3) C_3, the survival was 43.2% and the F_1 hybrids had reproductive organs that qualitatively appeared to be normal in size (with large quantities of mature sperm) in the males and slightly smaller, (i.e., fewer ovarioles) than normal in the females. For the crosses involving C females, there were 2 distinct types: (1) C_1, the results were similar to the C_1 male cross, except that the survival was extremely low (1.3%); and (2) C_3, the results were similar to the C_3 (male) cross.

Results of previous crosses (Kaiser et al. 1988a) between species A and B also revealed a polymorphic incompatibility, in that the F_1 male progeny of type B_1 males died in the pupal stage and type B_2 produced sterile F_1 hybrid males with atrophied testes. The F_1 hybrid females produced from both types of males were semisterile. Strains of B_1 and B_2 were maintained in our laboratory and crossed to A females each generation with consistent results. Eventually, the 2 strains were combined and called MON-B, and the males from this strain mated to
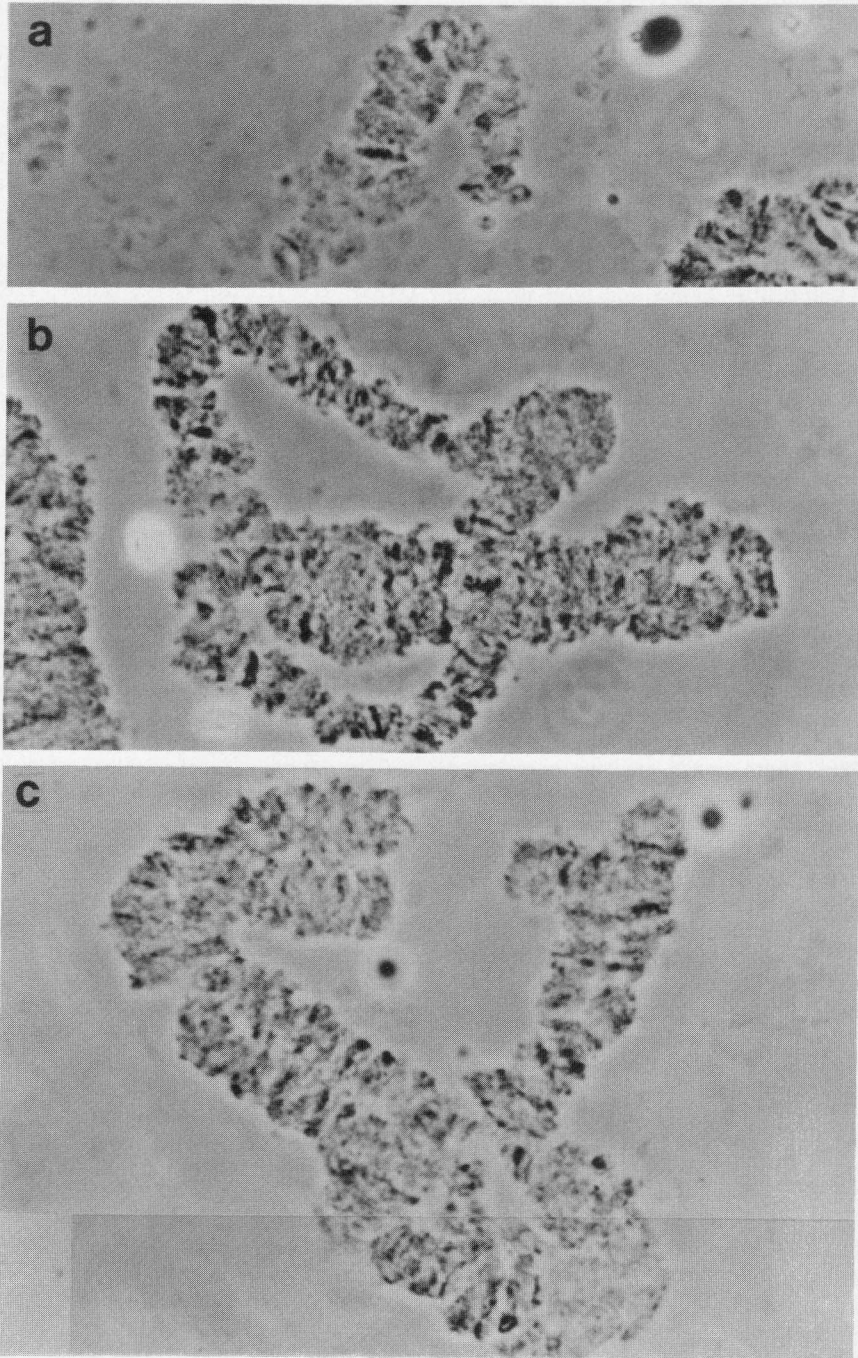
Fig. 1. Ovarian nurse cell polytene chromosomes of species C of the *Anopheles quadrimaculatus* complex. (a) X chromosome. (b) Autosome. (c) Autosome. The chromosomes were photographed under phase contrast (900×) using oil immersion and standard magnification printing (1,562×).

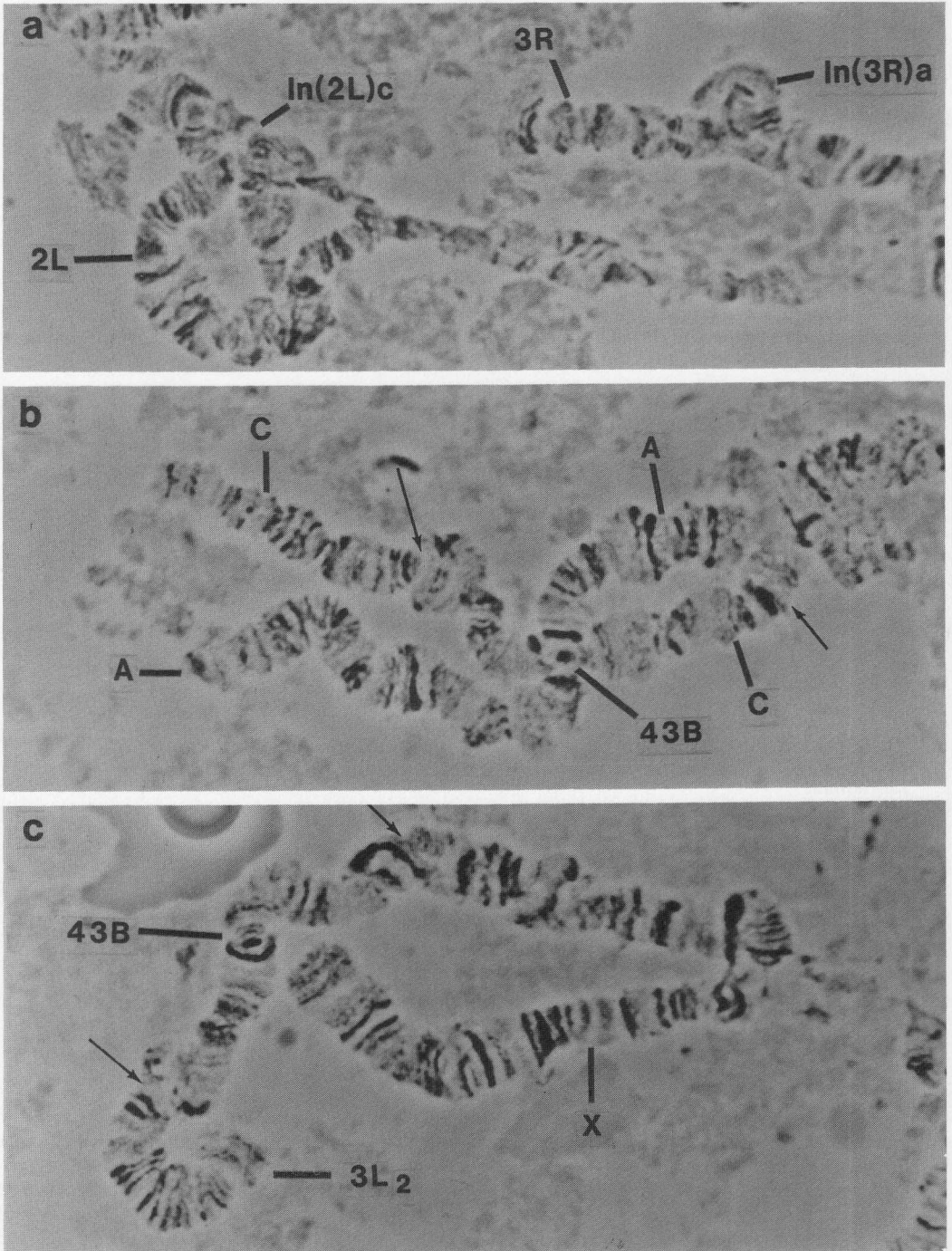Fig. 3. Salivary gland polytene chromosomes of F_1 hybrids of species A × B (a), A × C (b), and B × C (c). a) Synapsed 2L and 3R with species B fixed inversion, In(2L)c, and species A floating inversion, In(3R)a, shown. b) Asynaptic $3L_2$ homokaryotype with species A and C homologues identified; arrows indicate breakpoints of species C inversion. c) Synapsed X chromosome and $3L_2$ homokaryotype; arrows indicate inversion breakpoints and 43B identifies location of "crescent and dot" (1,562×).
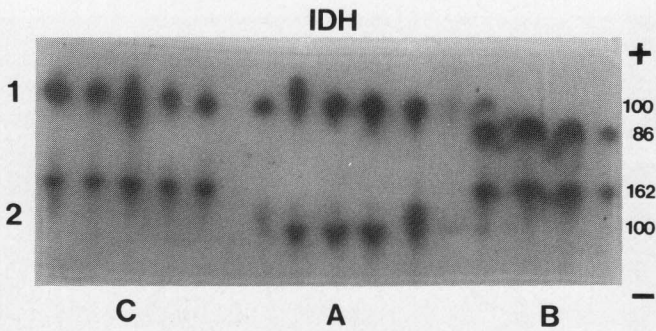
Fig. 2. Isocitrate dehydrogenase (Idh-1, Idh-2) electromorphs of the 3 sibling species, A, B and C, of the *Anopheles quadrimaculatus* complex. The migration values are relative (%) to anodic migrations of respective electromorphs of the standard reference strain.

Table 1. Results of crosses between members of the *Anopheles quadrimaculatus* sibling species complex.

Cross ♀ ♂	No. of families	Mean % hatch ± SD	Mean % survival ± SD	Female	Male	Ovary	Testes
A × A[a]	14	82.5 ± 17.7	61.2 ± 8.9	314	314	normal	normal
B × B[b]	24	87.1 ± 14.7	68.1 ± 19.5	713	804	normal	normal
C × C[c]	10	65.9 ± 15.4	73.2 ± 8.9	512	593	normal	normal
A × C	4	36.0 ± 23.1	62.9 ± 26.6	–	103	–	absent
C × A	5	65.5 ± 17.9	24.4 ± 20.5	–	29	–	absent
B × C₁	3	80.6 ± 10.7	12.3 ± 7.9	40	–	absent	–
B × C₂	7	42.6 ± 24.7	11.2 ± 5.2	–	67	–	absent
B × C₃	2	52.9 ± 35.5	43.2 ± 9.6	39	22	normal	normal
C₁ × B	4	75.3 ± 17.0	1.3 ± 1.0	6	–	absent	–
C₃ × B	3	76.0 ± 28.8	30.1 ± 23.4	43	75	normal	normal

[a] STU strain.
[b] MON strain.
[c] LEV strain.

Table 2. Results of backcrosses among members of the *Anopheles quadrimaculatus* sibling complex.

Cross ♀ ♂	No. of families	Mean % hatch ± SD	Mean % survival ± SD	Female	Male	Ovary	Testes
B × BC₃	5	22.6 ± 23.4	21.9 ± 21.5	–	50	–	absent
BC₃ × B	1	51.8 ± 0	0	–	–	–	–
B × C₃B	5	50.3 ± 26.5	18.2 ± 16.2	–	93	–	absent
C₃B × B	1	1.2 ± 0	0	–	–	–	–
C₃B × C	1	37.2 ± 0	11.3 ± 0	–	8	–	small

species A females resulted in a normal sex ratio with sterile males and semisterile females.

The results of the backcrosses involving the fertile hybrids from C₃ × B (and reciprocal) are shown in Table 2. The F₁ females and males from B × C₃ were backcrossed only to species B because species C adults were not available at the time. The only adult progeny resulting from the backcrosses were sterile males, and these were observed in only 3 of the 6 crosses. As shown in Table 2, most hybrids were infertile, both in terms of egg hatch and the number of females that laid eggs. For example, of 20 F₁ (B

× C₃) females backcrossed to B males by induced copulation, only 2 laid egg batches; one of these hatched but all of the larvae died. For comparison, the fertility of the 4 backcrosses (Kaiser et al., unpublished data) of F₁ females from A × B and B × A ranged from 10.0 ± 5.6% to 17.1 ± 14.4%, and the progeny consisted of sterile males and semisterile females, with the sex ratios skewed toward a surplus of females.

The ovarian nurse cell polytene chromosomes of the B × C and C × B hybrid females were similar to the species C chromosomes (diffuse bands). There were no hybrid female progeny

from the crosses A × C or C × A. We attempted to study the salivary gland polytene chromosomes of the hybrids (Fig. 3), but difficulty encountered in making readable chromosome preparations limited the sample size for each of the hybrid types. (1) A × B. In contrast to the asynapsis observed for ovarian polytene chromosomes (Kaiser et al., unpublished data), most of the chromosome arms were synapsed (Fig. 3a). The exceptions were the completely asynaptic X chromosomes and the left arms of chromosome 3. However, asynapsis was expected for 3L because of the dimorphic forms ($3L_1$ and $3L_2$) for this arm that occur in all populations of Species A and B (Kaiser et al. 1988b). (2) A × C. Ten larvae were analyzed, and all the arms were asynaptic except for the "dot and crescent" landmark (Kitzmiller et al. 1967) on the left arm of chromosome 3 (43B in Fig. 3b). There is a large inversion, covering regions 45E-42A (arrows in Fig. 3b), on 3L of species C. (3) B × C. Based on the study of chromosomes of only 2 hybrid female larvae, all the arms, including the X chromosome, were synapsed and homosequential (Fig. 3c). This is significant, because the X chromosomes do not synapse in A × B hybrids (N = 40), and moreover, the fixed inversions, In(X)a and the inversion for regions 42A-45E on 3L, occur in both species B and C.

Though the cytological analysis of the salivary chromosome banding patterns in the 3 species and their interspecific hybrids were not conclusive, the results of the hybrid crosses and allozyme analysis (Narang et al. 1988) indicated a closer relationship between species A and B.

ACKNOWLEDGMENTS

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