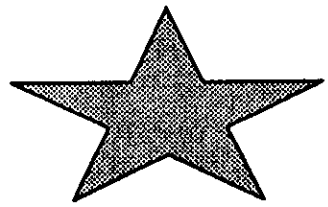
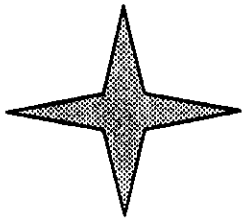


Results & Discussion



(IV)RESULTS AND DISCUSSION

1- Obtaining metaphase chromosomes from pupal testes

Male pupae were separated from females by their narrow thorax. After dissection of the pupae, the testes were recognized as white or yellowish oval organs, usually attached to their ducts and located in the 4th abdominal segment. Each testes consists of a group of follicles, in which the sperms are produced.

In this work a trial was made to prepare metaphase chromosomes from *Ae. aegypti, Ae. albopictus, Ae. triseriatus* and *Cx. pipiens* following the standard procedures of ***French et al., (1962)*** without pretreatment with colchicine which was considered essential for obtaining good and rapid preparations of mosquito chromosomes, and helps to accumulate arrested metaphases ***(French et al., 1962 ; Rai, 1966; Baker and Aslamkhan, 1969; Baker et al., 1971, 1977 and Rao & Rai 1987).***

Good results were obtained from individuals which were collected after 10-12 hours post pupation. These findings disagree with ***French et al., (1962)*** who stated that the best results were obtained from individuals which were collected immediately after pupation.

It was difficult to get good preparations of metaphase chromosomes without using colchicine solutions. About 2000 pupae were dissected to get about 20 slides with good metaphase chromosomes.

Good preparations of metaphase chromosomes were obtained when dissection was made in the morning, at spring and summer seasons as compared to the preparations made in the afternoon and at winter season.

The reason(s) for these periodicity, seasonality, humidity and temperature is unknown.

The squash technique was used by many investigators to study chromosomes in different tissues of mosquitoes. ***Breland(1959)*** suggested the use of this technique for the study of the chromosomes of mosquito larvae of *Orthopodomyia signifera*, *O. alba* Baker, *Culiseta inornata*, *Toxhorynchites rutilus septentrionalis* and *Cx. quinquefasciatus* Say. ***Breland, (1961),*** also used the so-called squash technique on the brains of prepupae to study the chromosomes of 16 mosquito species; whereas ***Akestein (1962)*** used a squash preparations of the gonads to prepare a chromosomes of some species of mosquito. ***Mc Donald & Rai (1970)*** used a squash preparation of mosquito's tissues for cytological examination included brain and limb buds from early fourth instar larvae, testes from pupae and ovaries from newly emerged adults.

Good results were obtained when the slides were dehydrated in the same day or during 24 hours of preparation. More than 24 hours dehydration may cause loss of tissues from the slide, the most critical part of the whole process. All division figures (and in many cases stages) from early prophase I to anaphase II were seen. The metaphase chromosomes were relatively the largest and were easily identified.

2- Estimation and cutting DNA with restriction endonucleases

The concentration of the DNA was estimated by spectrophotomeric analysis at 260 to 280 nm using spectrophotometer. The best results are obtained when the ratio is between 1.7-2.0. In the present work, different kinds of cosmid DNA [K6.3D9 - K6.2H2 - K6.2E6 - K6.2D2 - K6.3F11 - K6.50A4 - K6.3G9 - K6.3F4 - K6.172C4 - K6.122G5 and K6.3B3] were prepared and estimated by spectrophotometer, the ratio between 260 and

280nm was between 1.8 to 2.0 (table 1). This result agrees, with that of ***Sinibaldi and Cummings (1981)*** who stated that the purity of the DNA was checked by dissolving the precipitate in 0.01 M Tris, 0.001 M EDTA, pH 8.0 and checking the optical density. 260 / 280 ratio and they demonstrated that the 260 / 280 ratio of 1.9 was deemed acceptable. Also ***Shermoen and Kiefer (1975)*** used the 260 / 280 ratio to estimate the DNA, and they demonstrated that DNA was extracted with equal volumes of chloroform / isomyl alcohol until the 260 / 280 ratio was 1.95 or greater.

In this study, *Not* I restriction endonuclease (restriction enzyme) was used to cut the cosmid probes to fragments of different sizes, using agarose gel electrophoresis, this experiment allowed us to identify or estimate the size of each probe as bp or Kb. The estimated size of each DNA fragment based upon mobility relative to the marker on the electrophoresis gel. This result agrees with that of ***Birnboin and Doly (1979)*** who stated that the size of the DNA fragment may be estimated by agarose gel electrophoresis, and the estimated range of integrated DNA fragments based upon the mobility of DNA relative to marker and the second important method for characterizing recombinant plasmids is restriction enzyme digestion. Their alkaline extraction procedure gives a preparation of plasmid DNA which is pure enough to be susceptible to digestion by several restriction enzymes. Also ***Lapitan et al., (1997)*** demonstrated the digestion of the vector with HindIII, and checked for digestion on an agarose gel. Also the result agrees with that of ***Hanson et al., (1995)*** who mentioned that the BAC inserts were excised with *Not* I restriction endonuclease digestion prior to electrophoresis.

Table (1) Estimation of the DNA concentration by spectrophotomeric analysis.

DNA Sample	260 nm	280 nm	Ratio	µg/ µl	Total (µg)
K6.3D9	0.019	0.010	1.9	9.8	10.2
K6.2H2	0.007	0.004	1.8	26.5	3.8
K6.2E6	0.016	0.009	1.8	7.8	12.8
K6.2D2	0.014	0.009	1.6	13.3	7.5
K6.3F11	0.011	0.007	1.6	18.9	5.0
K6.50A4	0.008	0.004	2.0	23.2	5.4
K6.3G9	0.025	0.014	1.8	4.3	23.3
K6.3F4	0.005	0.003	1.7	37.2	2.7
K6.172C4	0.007	0.005	1.8	26.5	4.7
K6.122G5	0.007	0.004	1.8	17.7	5.6
K6.3B3	0.014	0.008	1.8	13.3	7.5

The results of the CHEF gel electrophoresis of restricted cosmid DNA indicated a separation of fragments of different sizes which were produced by the restriction enzymes Fig (6). The first lane of the gel contained DNA ladder (high molecular weight DNA marker), the other lanes contained cosmid DNA as described in table (1). The band which migrated the shortest distance however, is not a fragment of the cosmid DNA, it is undigested cosmid. The next bands which migrated the longest distance are fragments of the cosmid DNA (table 1).

Table (2) and (3) show another kinds of cosmid DNA estimated by spectrophotomeric analysis at 260 and 280 nm using spectrophotometer, the ratio was between 1.7-2.0. Fig (7) as an example of 1 % agarose gel shows several kinds of cosmid DNA to check the presence of the probes. The first lane of the gel contained 1 Kb DNA ladder. The cosmids DNA were loaded onto the other lanes of gel. The loading of this gel was shown in table (2). In this instance a clear bands were produced in the gel, indicating that there is DNA in the vector.

Fig. (8) shows a restricted centric heterochromatin probe BAC K20.1A5 which produced by *Not* I restriction enzyme using CHEF gel electrophoresis. The first lane of the gel contain a high molecular weight marker, the second lane of gel contain BAC K20.1A5 sample. The band in lane 2 which migrated the shortest distance , is the undigested BAC vector while the next band is a fragment of the BAC K20.1A5.

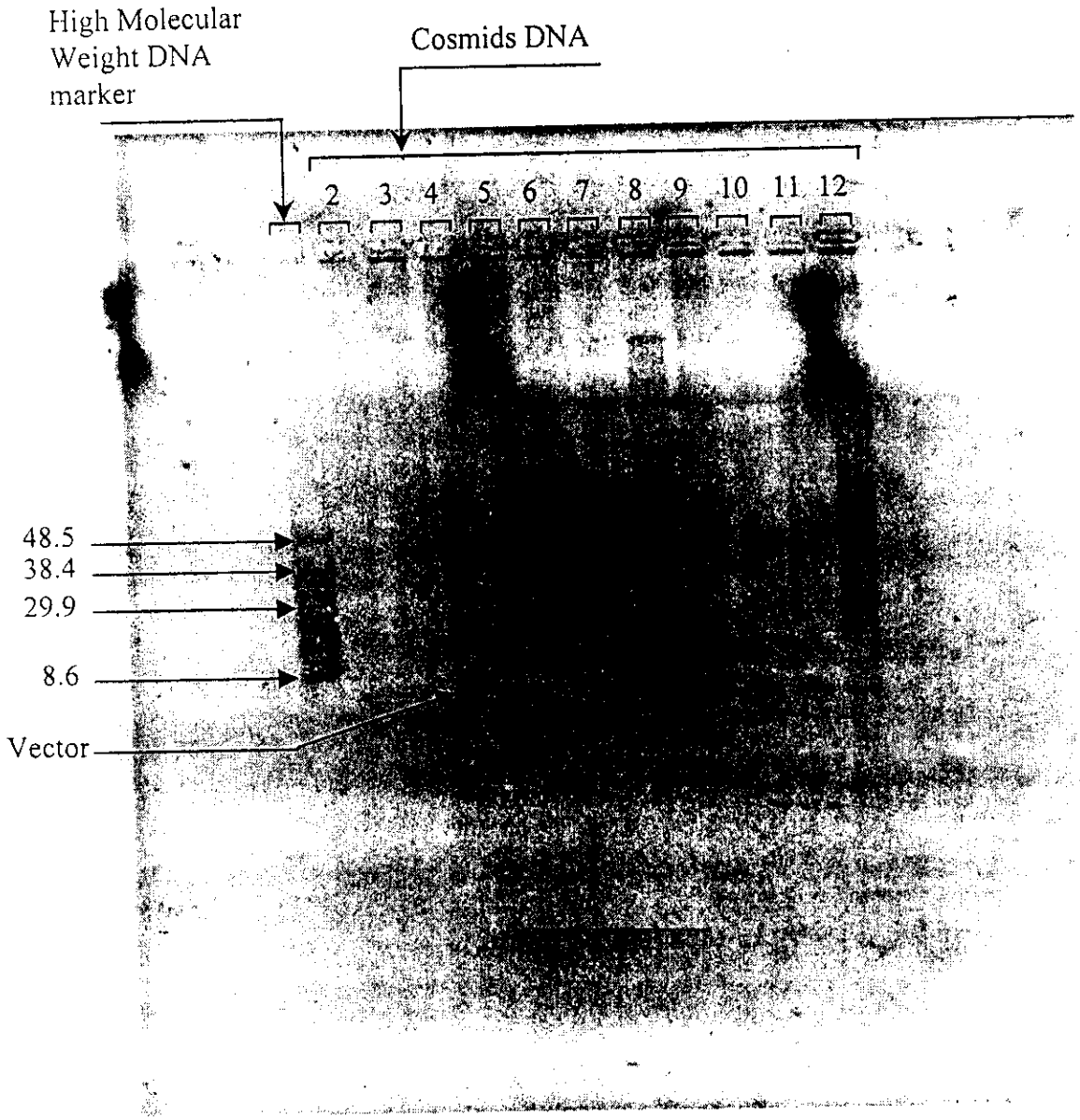


Fig. (6):Digital images of 1% CHEF gel showing several kinds of cosmid restricted by *Not* I restriction endonuclease. A high molecular weight DNA marker is located in the first lane of gel. The cosmids DNA are located in the other lanes of gel.

Lane 2 :	K6.3D9
Lane 3 :	K6.2H2
Lane 4 :	K6.2E6
Lane 5 :	K6.2D2
Lane 6 :	K6.3F11
Lane 7 :	K6.50A4
Lane 8 :	K6.3G9
Lane 9 :	K6.3F4
Lane 10:	K6.172C4
Lane 11:	K6.122G5
Lane 12:	K6.3B3

Table (2) Estimation of the DNA concentration by spectrophotomeric analysis.

DNA Sample	260 nm	280 nm	Ratio	µg /µl	Total (µg)
K2.33	0.014	0.005	2.8	8.9	11.2
K6.168B1	0.915	0.008	1.9	8.3	12.0
K6.3F2	0.029	0.015	1.9	4.3	14.0
K6.2G12	0.020	0.011	1.8	6.2	9.7
K6.153C10	0.010	0.005	2.0	12.4	8.1
K6.6C2	0.051	0.027	1.9	2.4	25.0
K6.172C2	0.007	0.004	1.8	17.7	5.6
K6.4E3	0.011	0.007	1.7	11.3	8.8
K6.50A11	0.035	0.018	1.9	3.5	17.1
K6.4D11	0.002	0.001	2.0	62.1	1.6
K6.168C3	0.012	0.007	1.7	10.4	5.8
K6.2G8	0.053	0.030	1.8	2.5	24.0
K6.168B10	0.041	0.022	1.9	3.1	19.4
K6.50A5	0.017	0.010	1.7	7.3	8.2
K6.2H8	0.012	0.008	1.7	10.4	5.8
K6.168B11	0.027	0.013	2.0	4.6	13.0
K6.168E11	0.018	0.010	1.8	6.9	8.7
K6.2A4	0.024	0.012	2.0	5.2	11.5
K6.168E10	0.016	0.007	2.3	7.8	12.8
K6.2H3	0.014	0.008	1.8	8.9	11.2

Table (3) Estimation of the DNA concentration by spectrophotomeric analysis.

DNA Sample	260 nm	280 nm	Ratio	µg /µl	Total (µg)
K6.172C11	0.009	0.007	1.3	13.8	7.2
K6.3B3	0.022	0.012	1.8	3.2	31.3
K6.122G5	0.003	0.003	1.0	41.0	2.4
K6.3E10	0.005	0.003	1.7	24.8	4.03
K6.3F11	0.003	0.002	1.5	41.0	2.4
K2.163	0.056	0.033	1.7	2.2	45.5
K6.52C3	0.002	0.001	2.0	62.1	1.6
K6.3F1	0.006	0.003	2.0	20.7	4.8
K6.3B6	0.017	0.012	1.5	7.3	13.7
K6.3C4	0.002	0.001	2.0	62.0	1.6
K6.2A7	0.017	0.010	1.7	7.3	8.2
K6.2F6	0.002	0.001	2.0	62.1	1.6
K6.2B11	0.007	0.004	1.8	17.7	3.4
K6.4B12	0.007	0.004	1.8	17.7	3.4
K6.5A3	0.004	0.002	2.0	31.3	1.9
K6.50A1	0.004	0.002	2.0	20.7	4.8

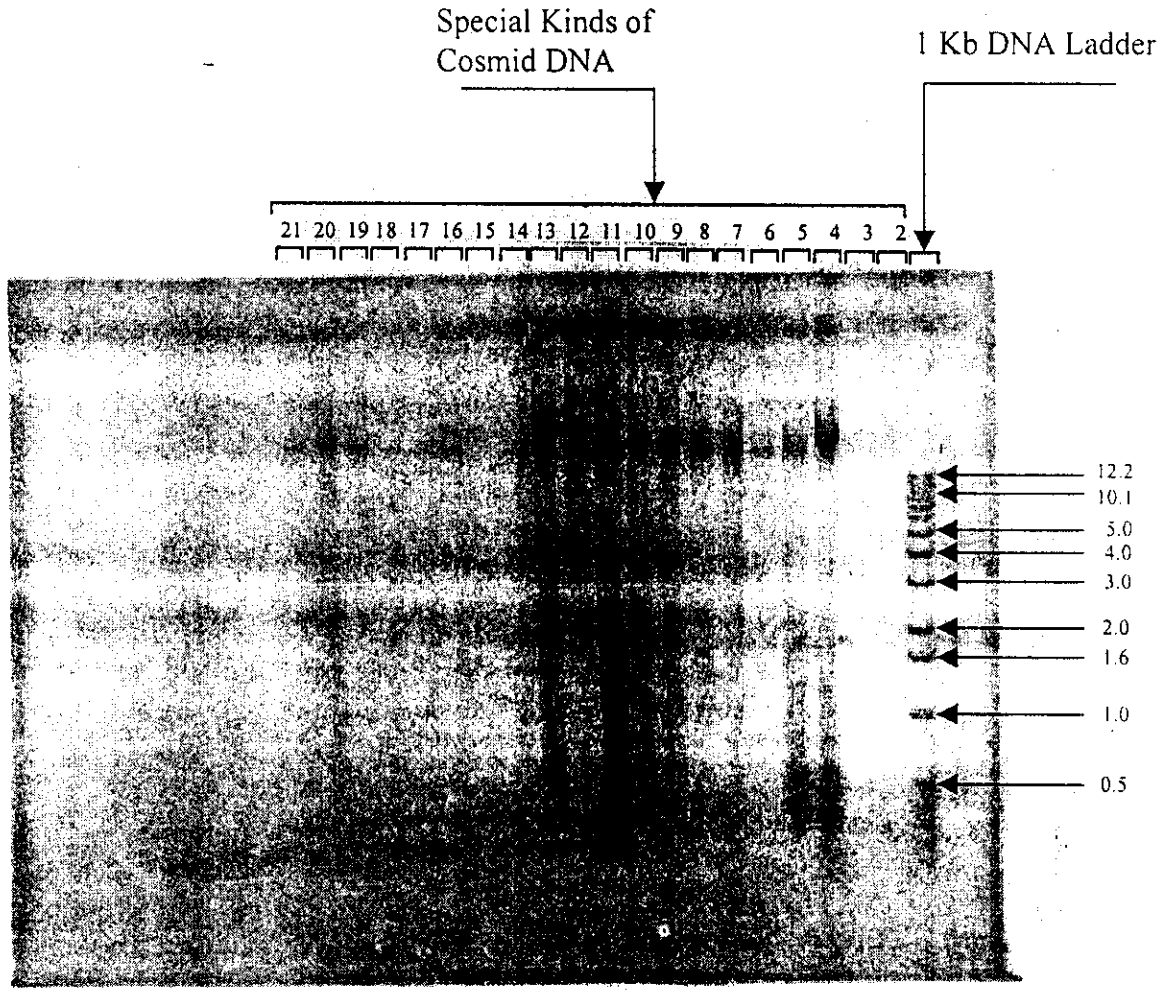


Fig. (7): **Digital images of 1% agarose gel showing several kinds of cosmid DNA. A 1 Kb DNA ladder is located in the first lane. All twenty lanes of gel are cosmid DNA.**

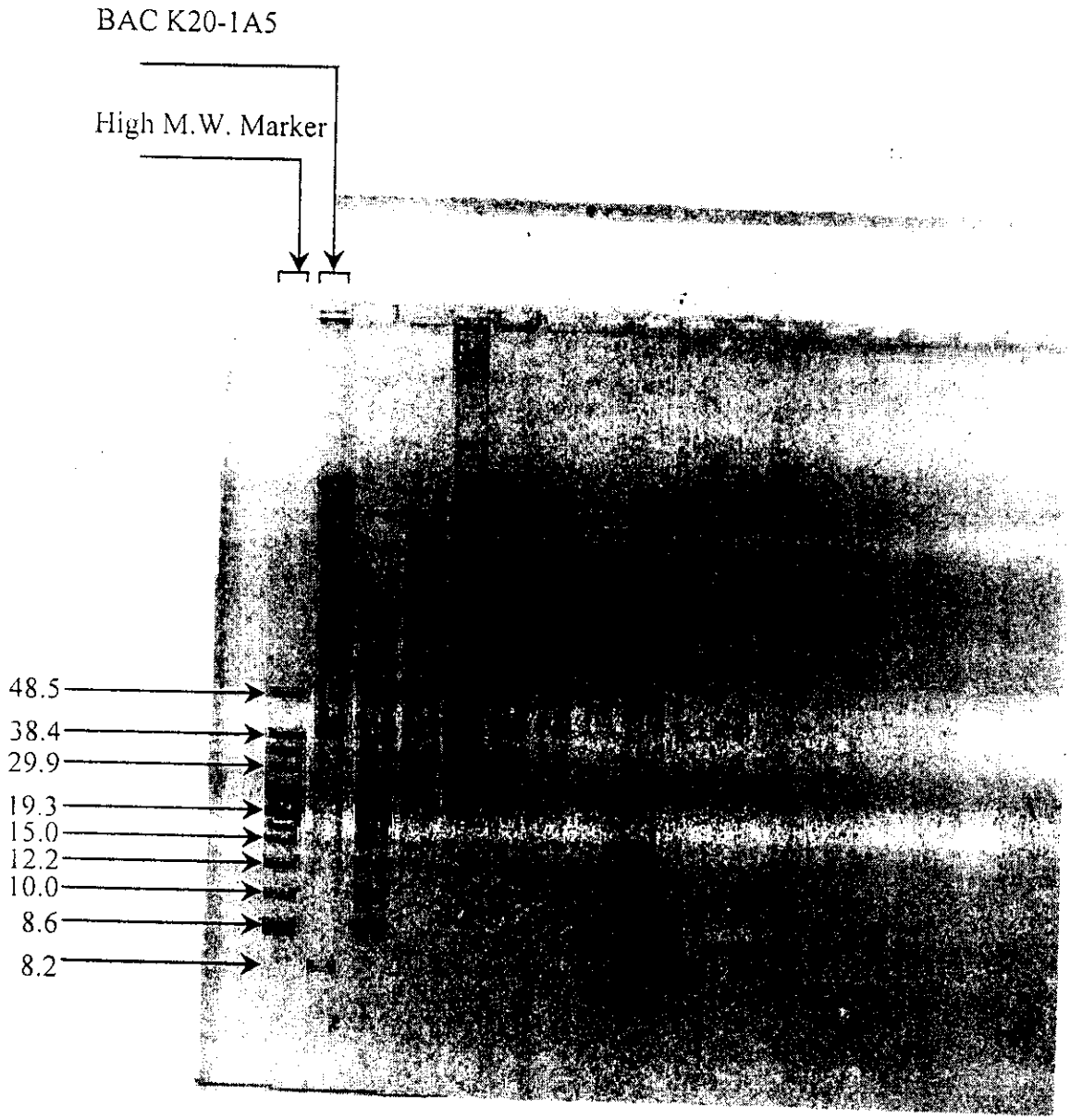


Fig. (8): Digital images of 1% CHEF gel showing restricted BAC K20.1A5. A high molecular weight marker is found in the first Lane. The BAC K20.1A5 is located in the second lane.

Fig (9) shows a preparation of K20.1A5 as a probe by nick translation technique to make sure that the digested DNA is between 100-500 bp. The first lane of gel contain 1 Kb DNA ladder, the second lane contained DNA sample (K 20.1A5). A two wide bands produced in the second lane of gel, the band which migrated the shortest distance is undigested DNA, the next wide band is a fragment of the digested DNA. The size of this probe was between 100-500 bp. Fig (9) is an example of Nick-translation.

In this study, Nick translation technique and 1% agarose gel electrophoresis were used to determine the size range of digested DNA, and the target size range was between 100-500 bp. The BAC K20.1A5 was an example to use this technique and it was found that the size of this probe was between 100-500 bp. The result of the obtained target size of the DNA agrees with that of ***Brown et al., (1995)*** who used the same technique and obtained similar result. Also ***Britten and Kohne (1968)*** stated that the optimal length for use of DNA in hybridization experiments is approximately 400 nucleotides long. ***Rigby et al., (1977)*** stated that the quantity of the DNase I which used in the nick translation reaction was chosen to promote substantial rates of nucleotide incorporation and to give a product with an average single-strand length of 400 nucleotides. ***Ried et al., (1992)*** stated that the cosmid and phage clones were labeled by Nick translation reactions. ***Nederlof et al., (1990)*** also labeled the probes with AAF precipitated and dissolved in nick-translation buffer for labeling with bio-11-dUTP. The obtained results also agree with that of ***Hanson et al., (1995).*** They stated that the BAC DNA was labeled with biotin 14-dATP using the Gibco BRL Bio Nick labeling system, and the probes produced had an average fragment size of 200-500 base pairs (bp).

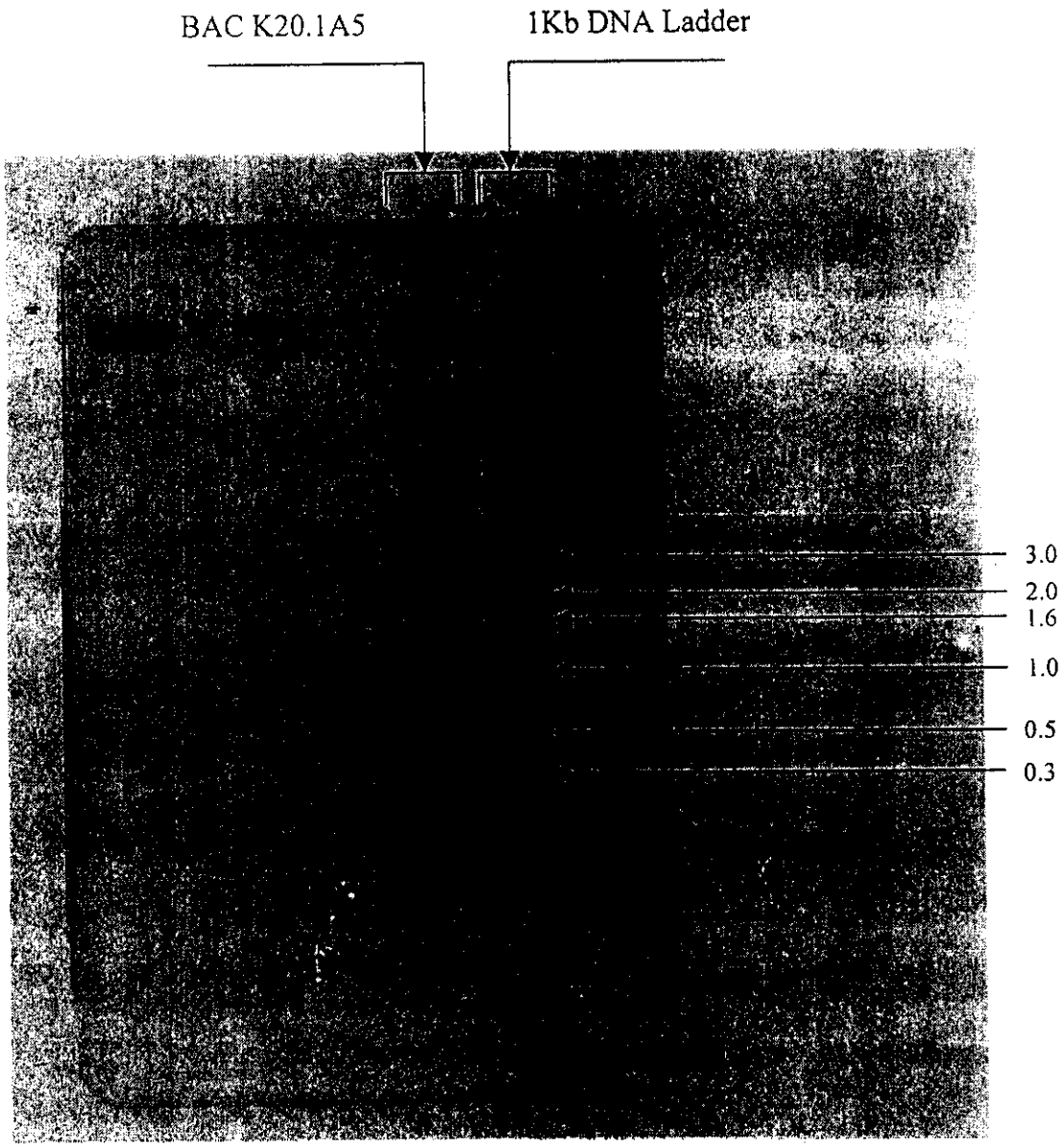


Fig. (9): **Digital images of Nick-translation profile showing BAC K20.1A5. A 1 Kb DNA ladder is in the first Lane. The second lane is K20.1A5** probe.

Also ***Jiang et al., (1995)*** showed that the purified BAC DNA was labeled by standard nick-translation reaction mixtures. ***Whiting et al., (1989)*** labeled the probes by nick-translation procedure with biotin-11-dUTP.

The cosmids DNA shown in table (1) , (2) and (3) can be used to hybridize the three chromosomes of mosquito species, some of these DNA samples can hybridize chromosome 1, some of them can hybridize chromosome 2, and the other can hybridize chromosome 3. Plasmids p1887, p2405 and p2056 were chosen because in the first generation FISH physical map studies, three cosmids (K2.9, K2.40 and K2.61) were identified each of which produced a strong signal on paired metaphase chromosome derived from cell line culture of *Ae. aegypti* species. A single plasmid, p 2392, which contained the three chromosome tag sequences, was constructed to yield an *Ae. aegypti* chromosome tagging reagent which has been used to simplify FISH physical mapping of unknown probes ***(Brown and knudson 1997).***

3- Microscopy and digital -imaging

By probe combination, pairs (or more) are mapped at the same time on the same slide. This is accomplished by labeling the probes with fluorochromes which emit light at different wavelengths. For example, one probe may be labeled with biotin-11 dUTP and the other labeled with digoxigenin-11-dUTP. The biotin can be detected by avidin-FITC, which is observed with the green filter, and the digoxigenin was detected with anti-digoxigenin-rhodamine antibody. The *Ae. aegypti* chromosome-specific tags (plasmids p1887, p2405 and p2056) and a centric heterochromatin probe K20.1A5 were labeled with biotin-fluoroscein, the tagging plasmid p2392 containing the chromosomal tags or landmarks was detected using anti-digoxigenin-rhodamine, and the chromosomes

identification was generally done by DAPI counterstain. This allows three different probes to be hybridized and detected on one slide.

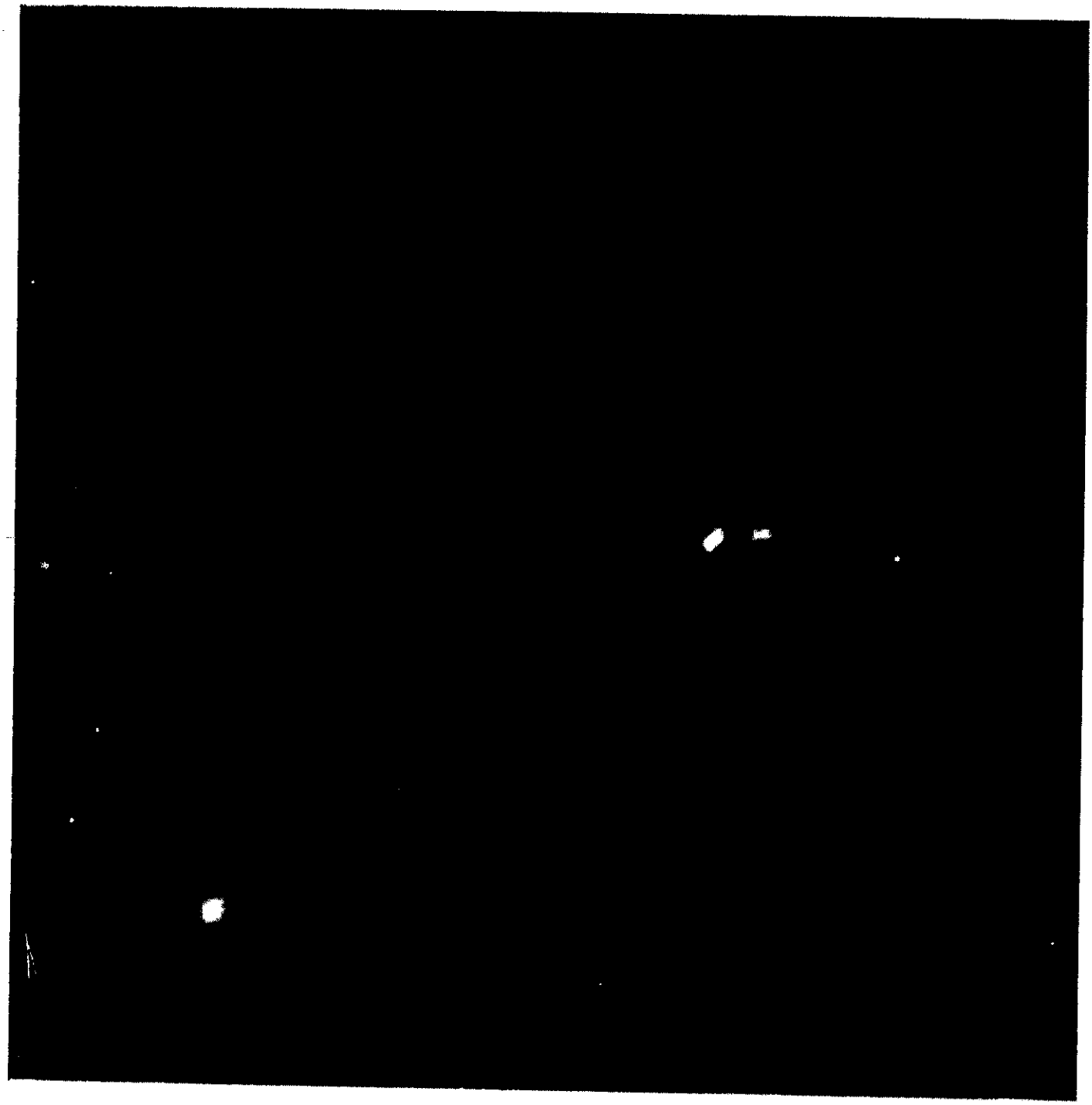
Fluorophore images were captured as grayscale and pseudocolored to match the emission wavelength: rhodamine to red, fluoroscein to green, and DAPI counterstain to blue. The images were superimposed. The DNA counterstain DAPI [4.6-diamidine - 2- phenylindole] delineates the chromatids of each chromosome. The specific signal would be detected using different optical filters resulting in 2 separate images which may be enhanced and merged to yield a composite with both signals present. Images of hybridized probes can be collected with high resolution into computer memory via charge coupled device (CCD) cameras which is a powerful instrument for biological researches. Identification of chromosomes was generally done by DAPI-banding, both the hybridization signal and the propidium iodide counterstain were imaged electronically using a cooled array CCD collector mounted on a Zeiss Axioskop microscope equipped with compatible band-pass filters.

It should be noted that when multiple fluorescence images of a given specimen are taken, each fluorophore signal is generally recorded using a different set of optical filters. It is imperative that each image be obtained in precise registration in order to guarantee the correct spatial representation of each after they are merged to construct the final image.

Figure (10-a) shows the result of hybridization of a double labeled probe for chromosome 1 of *Ae. aegypti* (p1887 - p2392) with DAPI counterstain. In this figure the DAPI can be used to delineate the three chromosomes of *Ae. aegypti*. Figure (10 - b) shows the result of hybridization of p1887 probe for chromosome 1 of *Ae. aegypti*, with

Figure (10-a): **Photomicrograph representing FISH with 1887/ 2392 to *Ae. aegypti* metaphase chromosomes stained by DAPI.**

Figure (10-b): Photomicrograph representing FISH with 1887/2392 to *Ae. aegypti* metaphase chromosomes detecting 1887 stained by FITC.



biotin-fluoroscein (FITC) stain. In this figure two lighter signals can be observed in chromosome one. The FITC stain can be used to delineates the p1887signals. Figure (10-c) shows the result of hybridization of plasmid p2392 probe for *Ae. aegypti* with anti-dioxigenin-rhodamine. In this figure there are signals located in all three chromosomes. The rhodamine stain was used to delineate the p2392 signals in the chromosomes. Figure (11-a) represents FISH with 2405 / 2392 to *Ae. aegypti* metaphase chromosomes stained by DAPI, while figure (11-b) shows the FISH with 2405 / 2392 to *Ae. aegypti* metaphase chromosomes detecting p2405 stained by FITC. These signals were seen in all six chromosomes as a lighter signals. Figure (11-c) represents the FISH with 2405/2392 to *Ae. aegypti* metaphase chromosomes detecting p2392 stained by anti-dioxigenin-rhodamine. Figure (12- a) shows the result of hybridization of a double labeled probe for chromosome 3 of *Ae. aegypti* (2056/2392) stained with DAPI counterstain. Figure (12- b) shows the result of hybridization of 2056 probe for chromosome 3 of *Ae. aegypti* stained by FITC. In this figure lighter signals can be observed in chromosome 3. Figure (12-c) shows the result of hybridization of plasmid p2392 probe for *Ae. aegypti* chromosomes, stained with anti-dioxigenin-rhodamine. The signals were seen in all six chromosomes.

Figure (13- a) represents FISH with K20.1A5 / 2392 to *Ae. aegypti* metaphase chromosome stained by DAPI, while figure (13 - b) represents FISH with K20.1A5 / 2392 to metaphase chromosomes of *Ae. aegypti* detecting the position of a centric heterochromatin probe (K20.1A5) stained by FITC. In this figure there are lighter signals in all six chromosomes, these signals are the positions of probe K20.1A5 in each chromosome.

Figure (10-c): Photomicrograph representing FISH with 1887 / 2392 to *Ae. aegypti* metaphase chromosomes detecting 2392 stained by rhodamine.

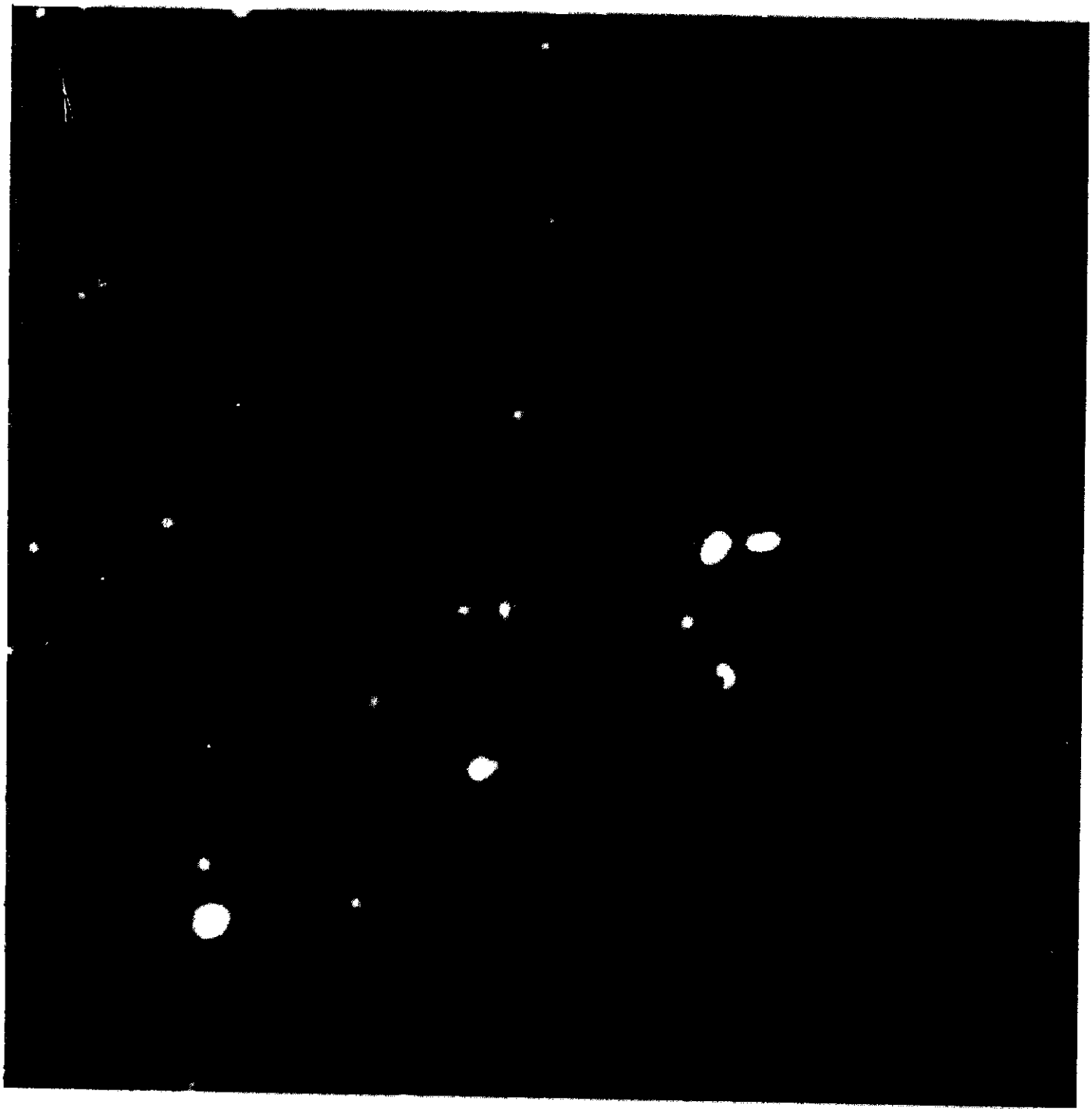


Figure (11-a): Photomicrograph representing FISH with 2405 / 2392 to *Ae. aegypti* metaphase chromosomes stained by DAPI.

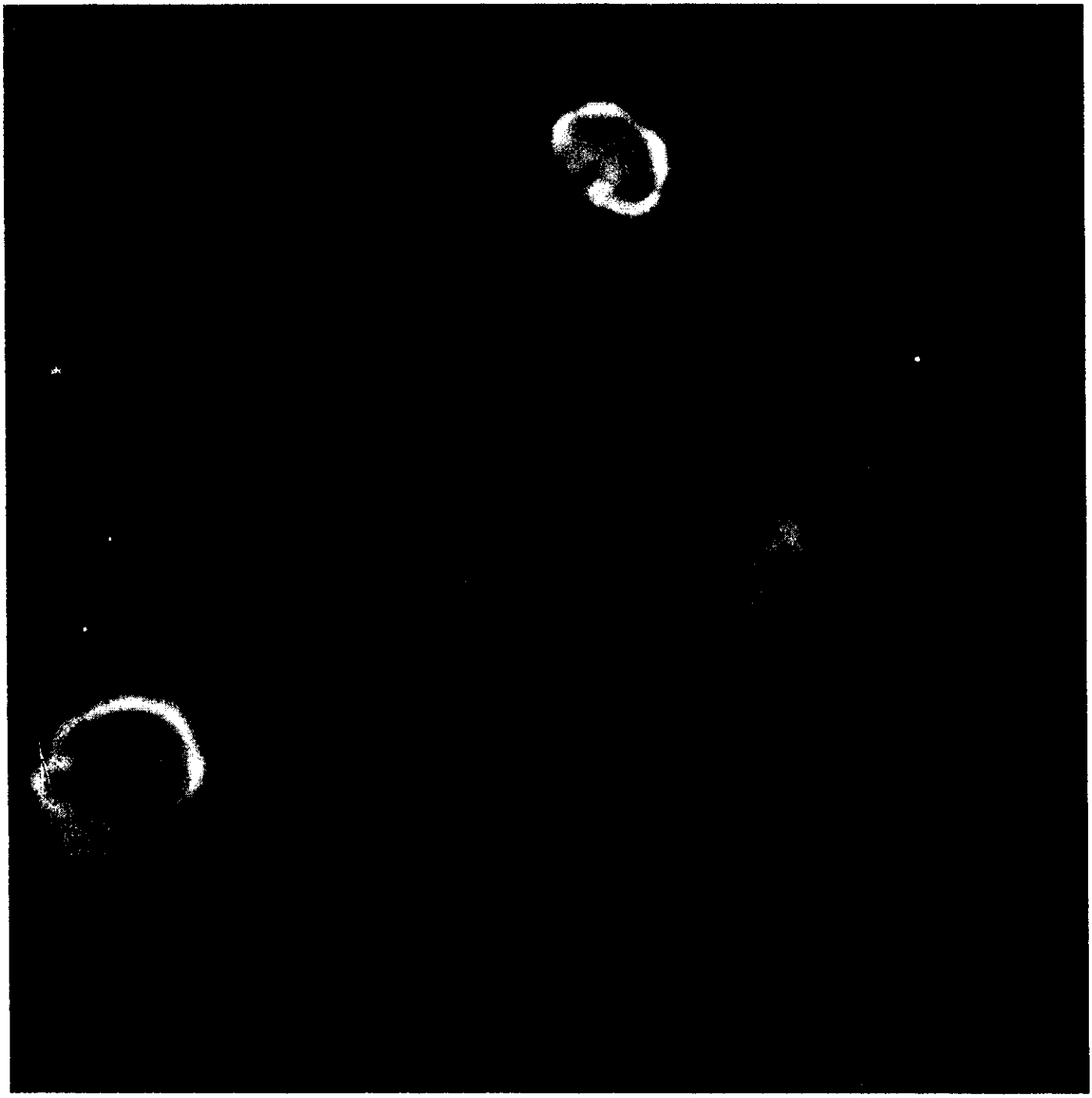


Figure (11-b): Photomicrograph representing FISH with 2405 / 2392 to *Ae. aegypti* metaphase chromosomes detecting 2405 stained by FITC.

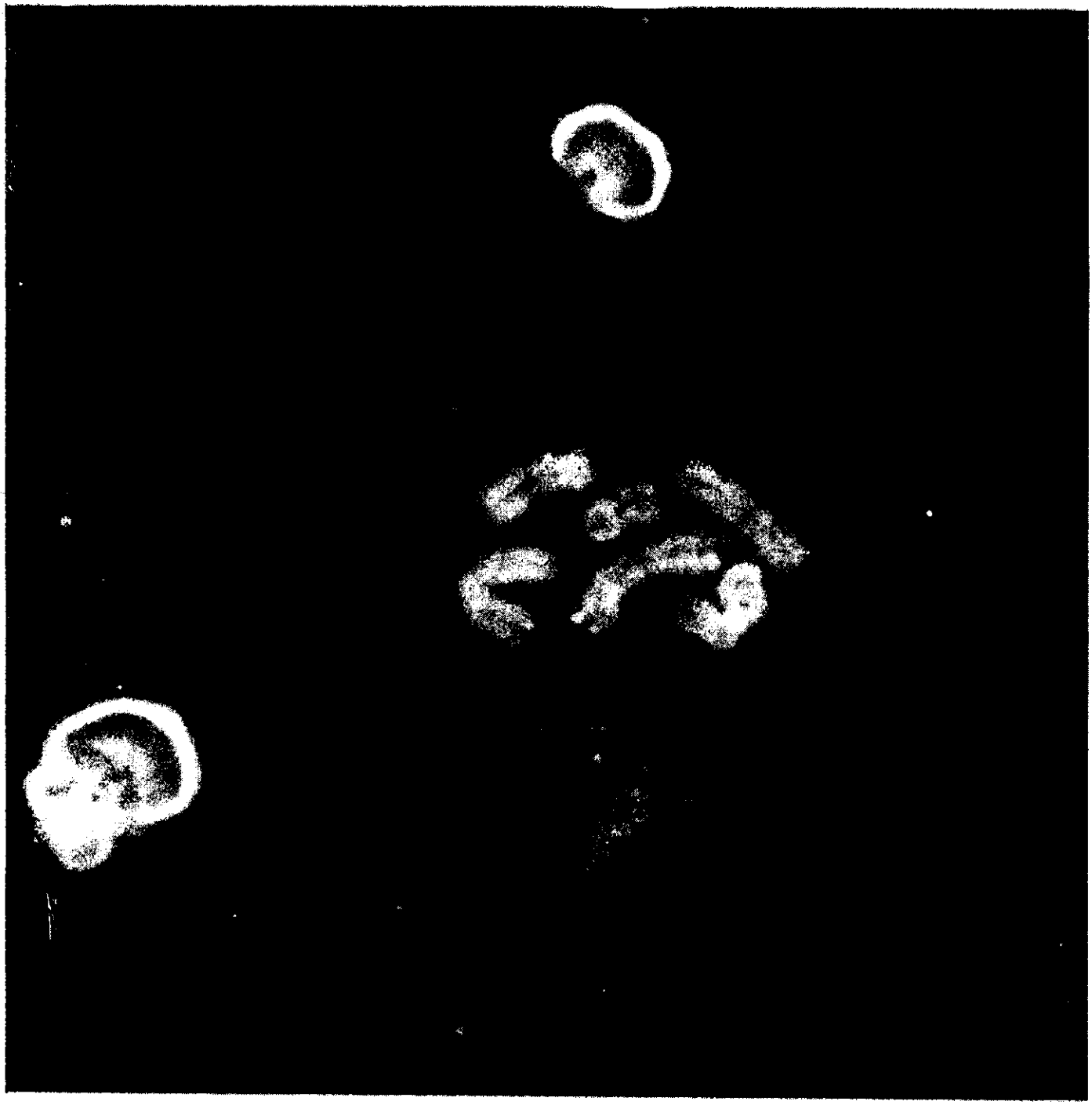


Figure (11-c): Photomicrograph representing FISH with 2405 / 2392 to *Ae. aegypti* metaphase chromosomes detecting 2392 stained by rhodamine.

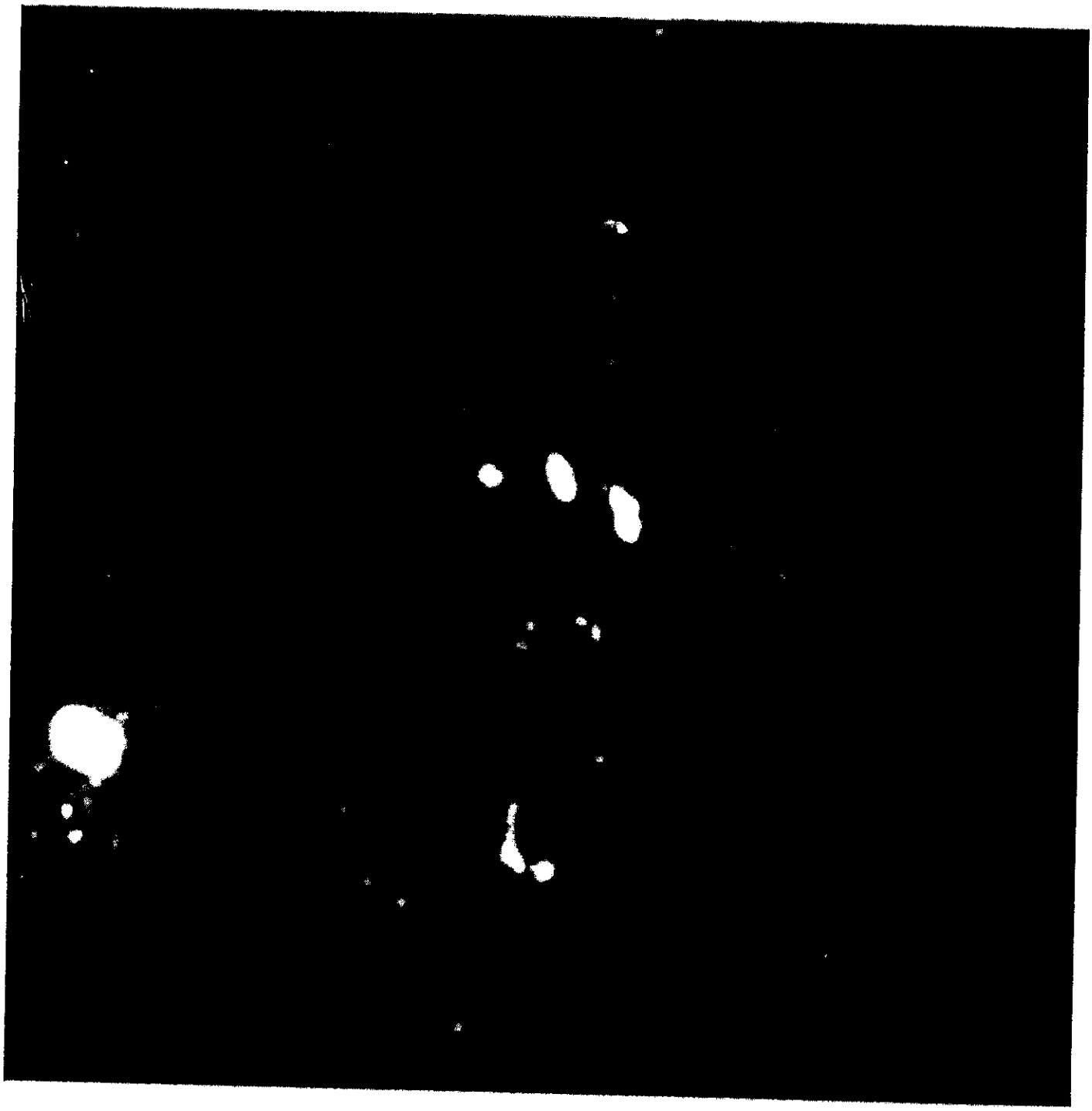


Figure (12-a): Photomicrograph representing FISH with 2056 / 2392 to *Ae. aegypti* metaphase chromosomes stained by DAPI.

Figure (12-b):**Photomicrograph representing FISH with 2056 / 2392 to *Ae. aegypti* metaphase chromosomes detecting 2056 stained by FITC.**

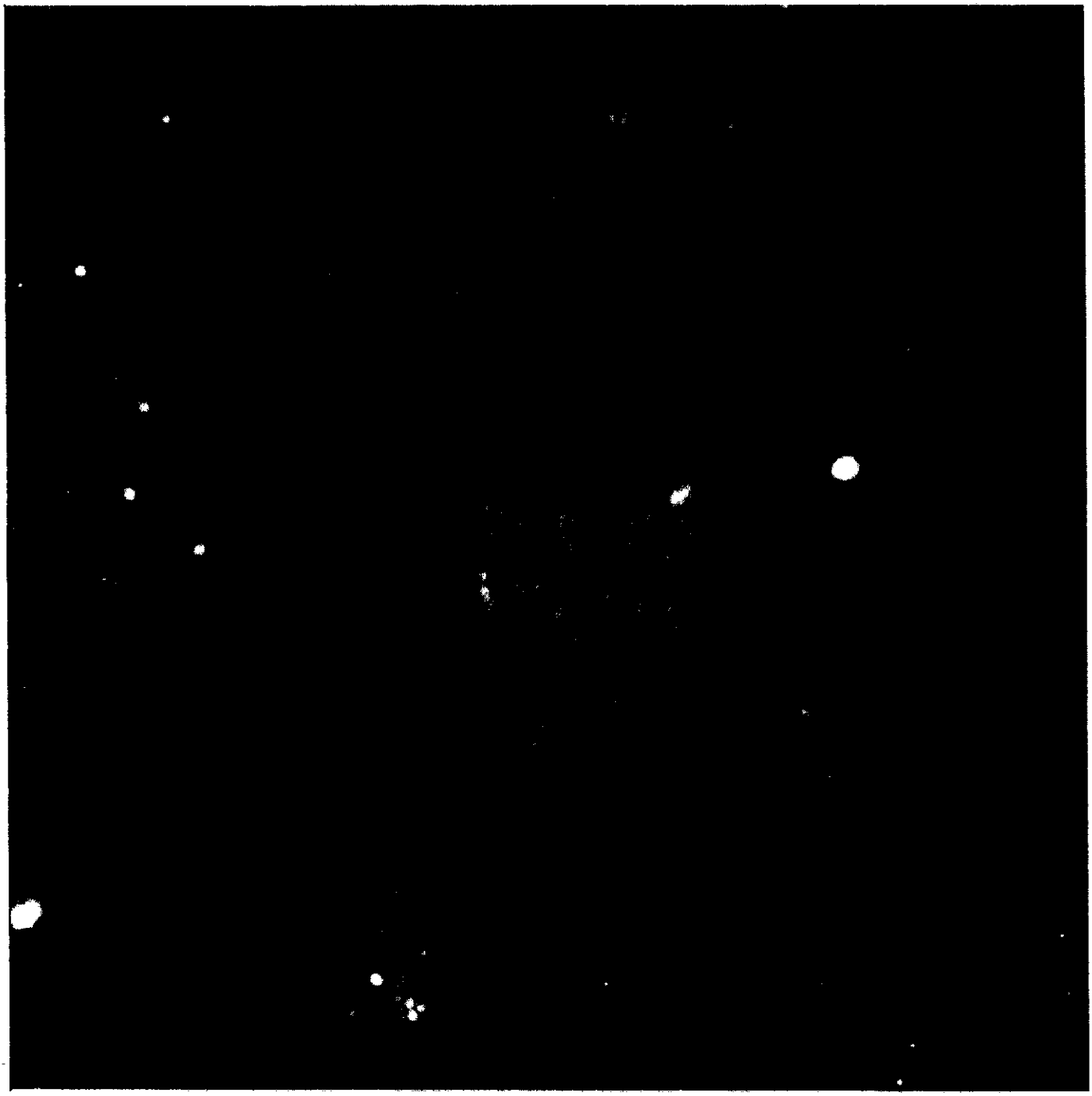


Figure (12-c): **Photomicrograph representing FISH with 2056 / 2392 to *Ae. aegypti* metaphase chromosomes detecting 2392 stained by rhodamine.**

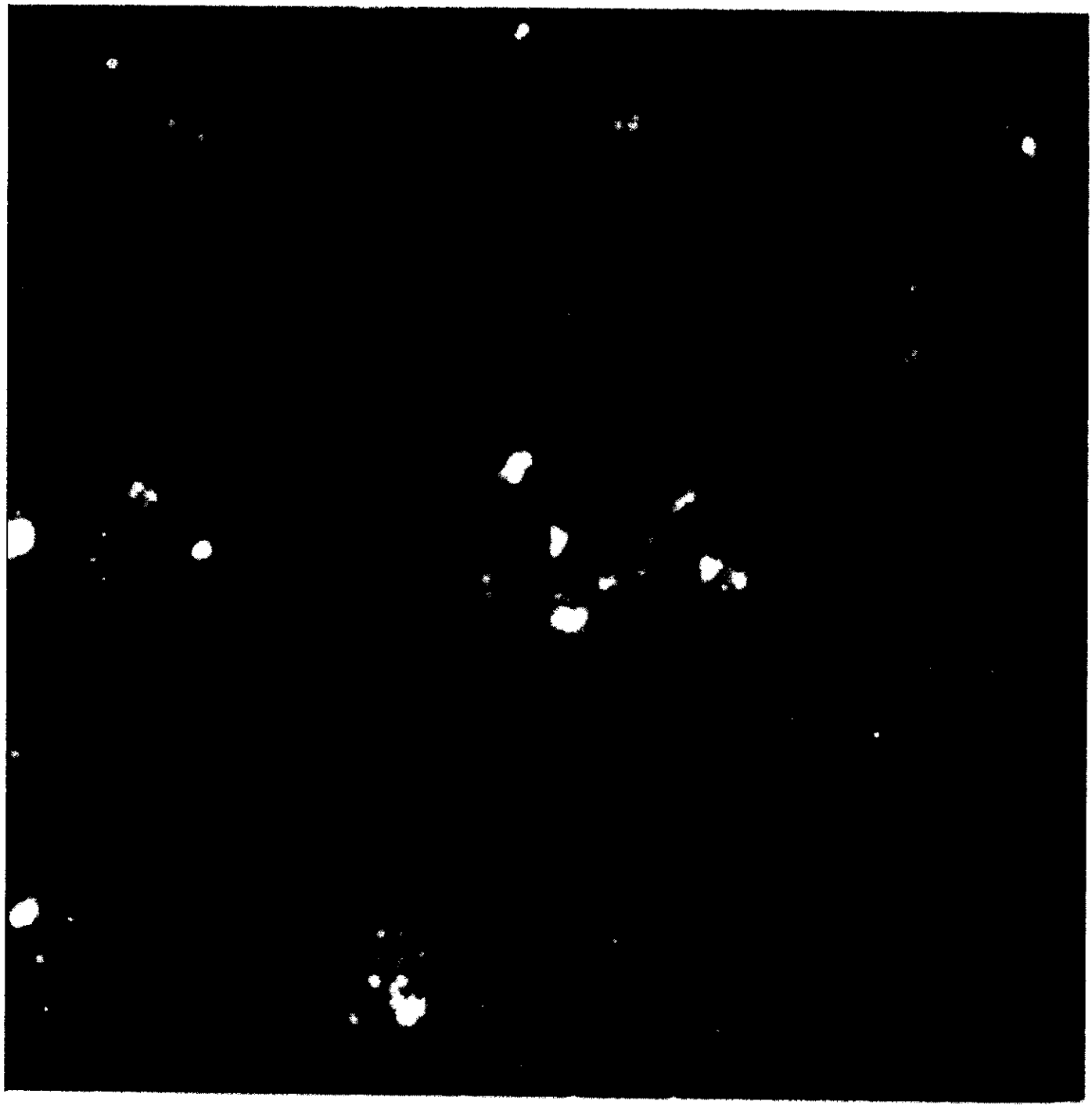


Figure (13 - a): Photomicrograph representing FISH with K20.1A5 / 2392 to *Ae. aegypti* metaphase chromosomes stained by DAPI.

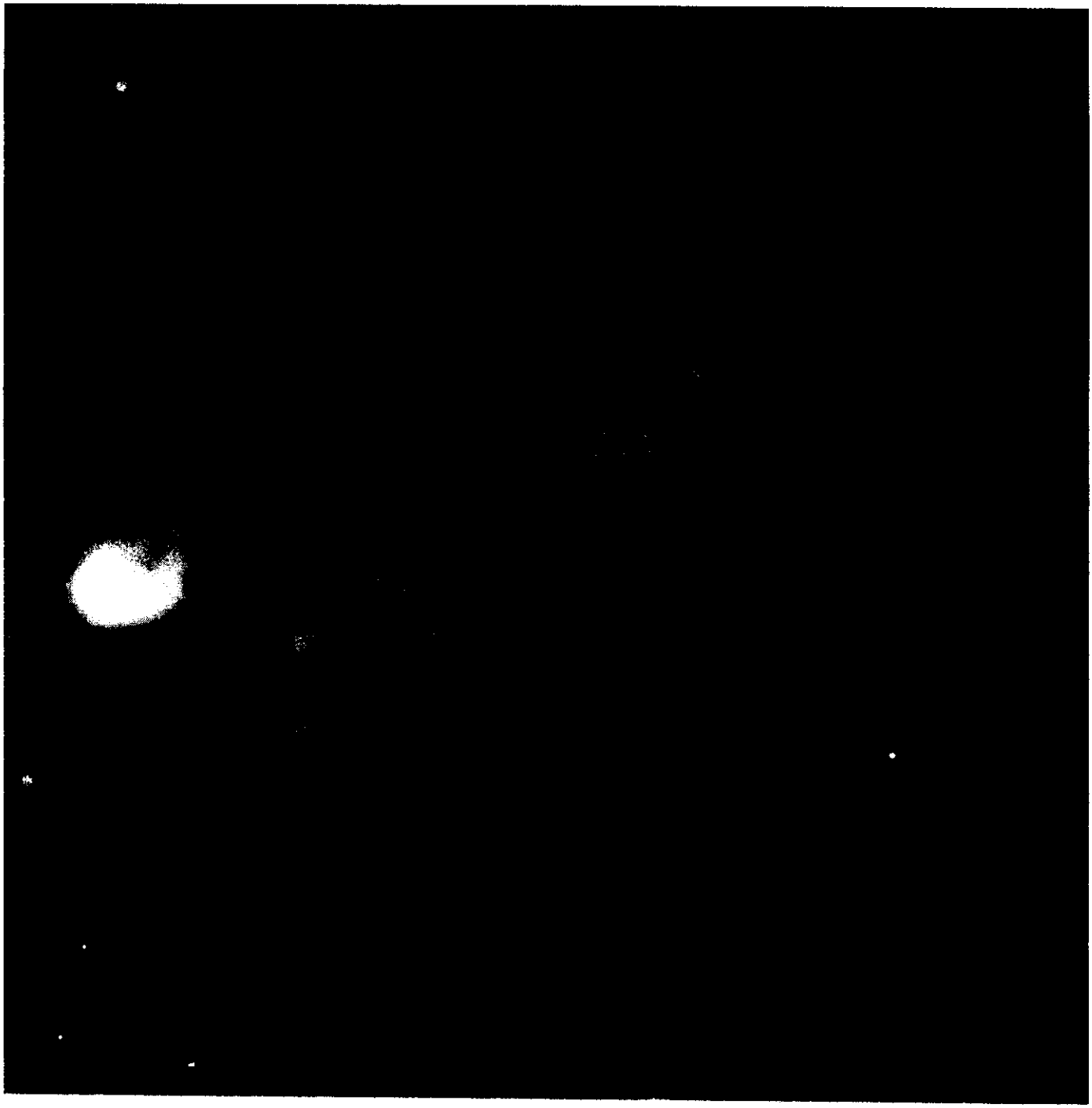


Figure (13 - b): **Photomicrograph representing FISH with K20.1A5 / 2392 to *Ae. aegypti* metaphase chromosomes detecting K20.1A5 stained by FITC.**

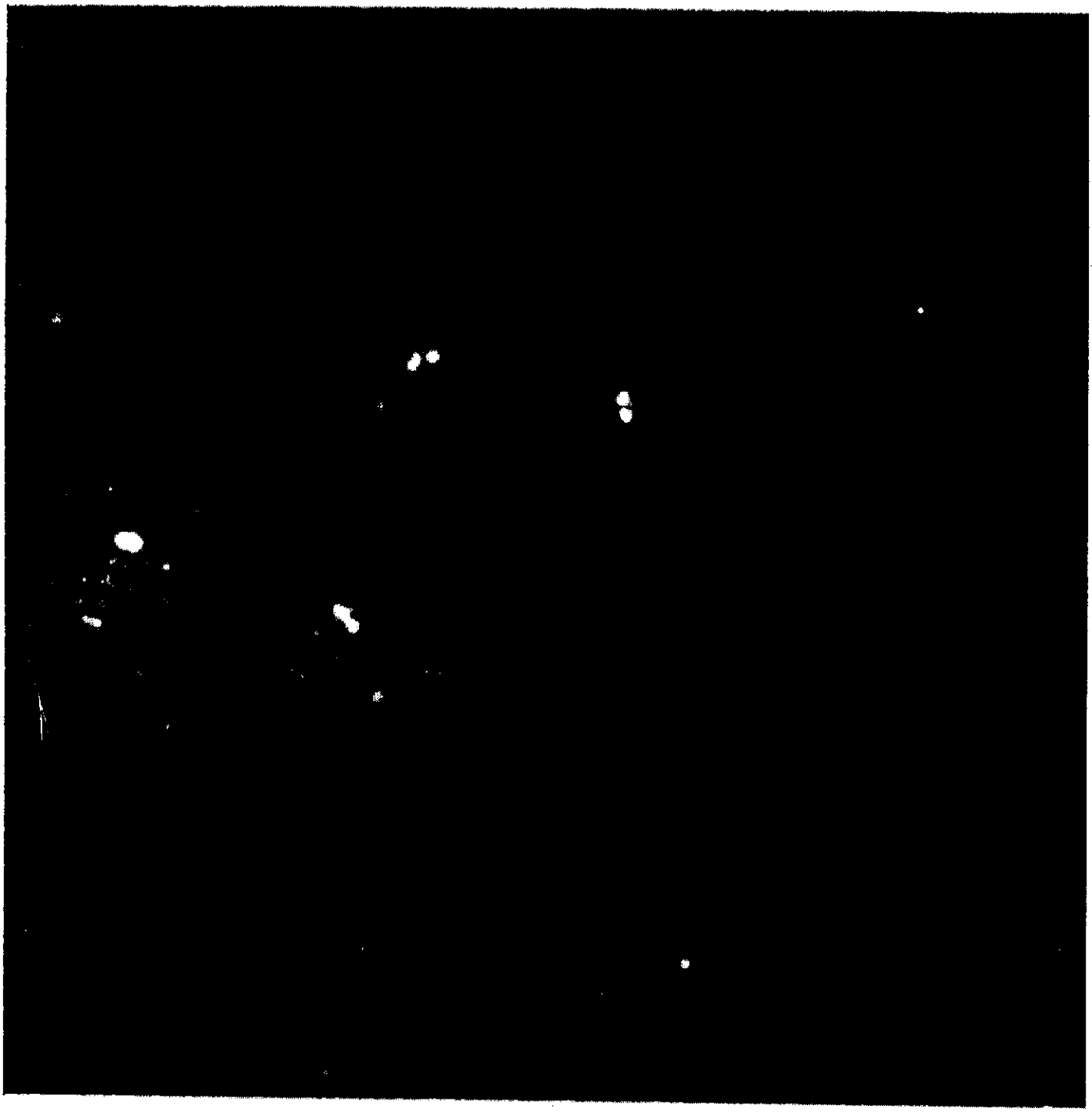


Figure (14-a), represents FISH with 1887/ 2392 to *Ae. triseriatus* metaphase chromosomes stained with DAPI counterstain, while figure (14-b) shows the result of hybridization of 1887 probe for *Ae. triseriatus* chromosomes stained by FITC . In this figure there are two lighter signals on two different chromosomes. Figure (14-c) shows the result of hybridization of 2392 probe for *Ae. triseriatus* chromosomes, stained by anti-dioxigenin-rhodamine, in this figure there are two lighter signals observed on two different chromosomes. Figure (15-a) represents FISH with K20. 1A 5 / 2392 to *Ae. triseriatus* metaphase chromosomes stained by DAPI and figure (15-b), shows the result of hybridization of K20.1A5 for *Ae. triseriatus* chromosomes stained with FITC, where no signals were observed in the chromosomes.

Figure (16-a) showed the results of hybridization of a double labeled probe for chromosome I of *Ae. albopictus* (1887 / 2392) stained with DAPI counterstain while figure (16 - b) represents FISH with 1887 / 2392 to *Ae. albopictus* chromosomes detecting 1887 stained by FITC. Figure (16-c) represents FISH with 1887 / 2392 to *Ae. albopictus* chromosomes detecting 2392 stained by anti-dioxigenin-rhodamine. Figure (17-a) represents FISH with K20.1A5 / 2392 to *Ae. albopictus* chromosomes stained with DAPI, but figure (17- b) shows the result of FISH with K20.1A5 / 2392 to *Ae. albopictus* chromosome detecting K20.1A5 stained by FITC, in this figure there are no signals observed on any chromosomes.

Figure (18-a) represents FISH with 1887/2392 to *Cx. pipiens* metaphase chromosomes stained by DAPI. Figure (18-b) shows the results of hybridization of 1887 probe for chromosome 1 of *Cx. pipiens*

Figure (14 -a): **Photomicrograph representing FISH with 1887/2392 to *Ae. triseriatus* metaphase chromosomes stained by DAPI.**

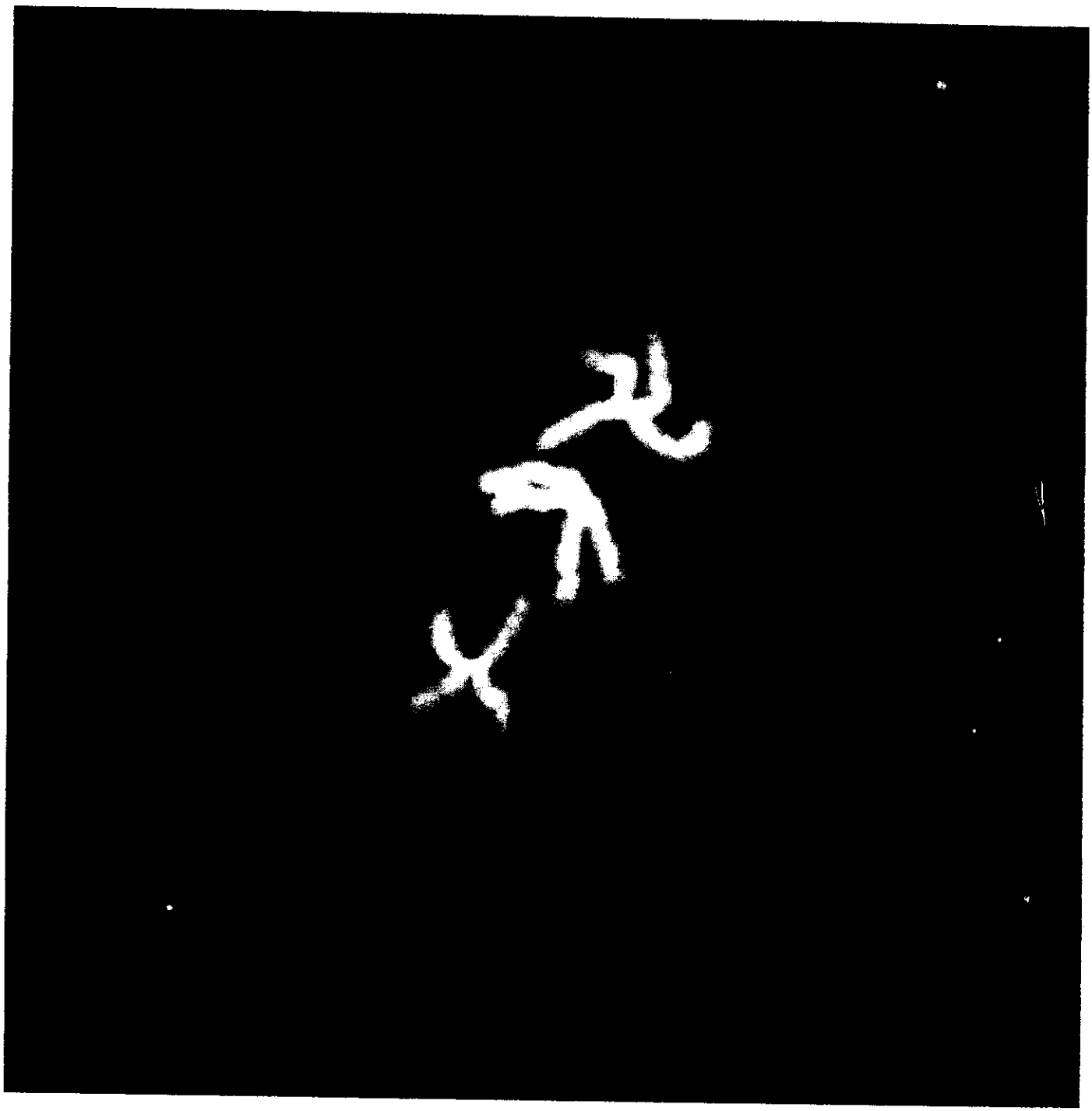


Figure (14 - b): **Photomicrograph representing FISH with 1887 / 2392 to *Ae. triseriatus* metaphase chromosomes detecting 1887 stained by FITC.**

Figure (14 - c): Photomicrograph representing FISH with 1887/2392 to *Ae. triseriatus* metaphase chromosomes detecting 2392 stained by rhodamine.

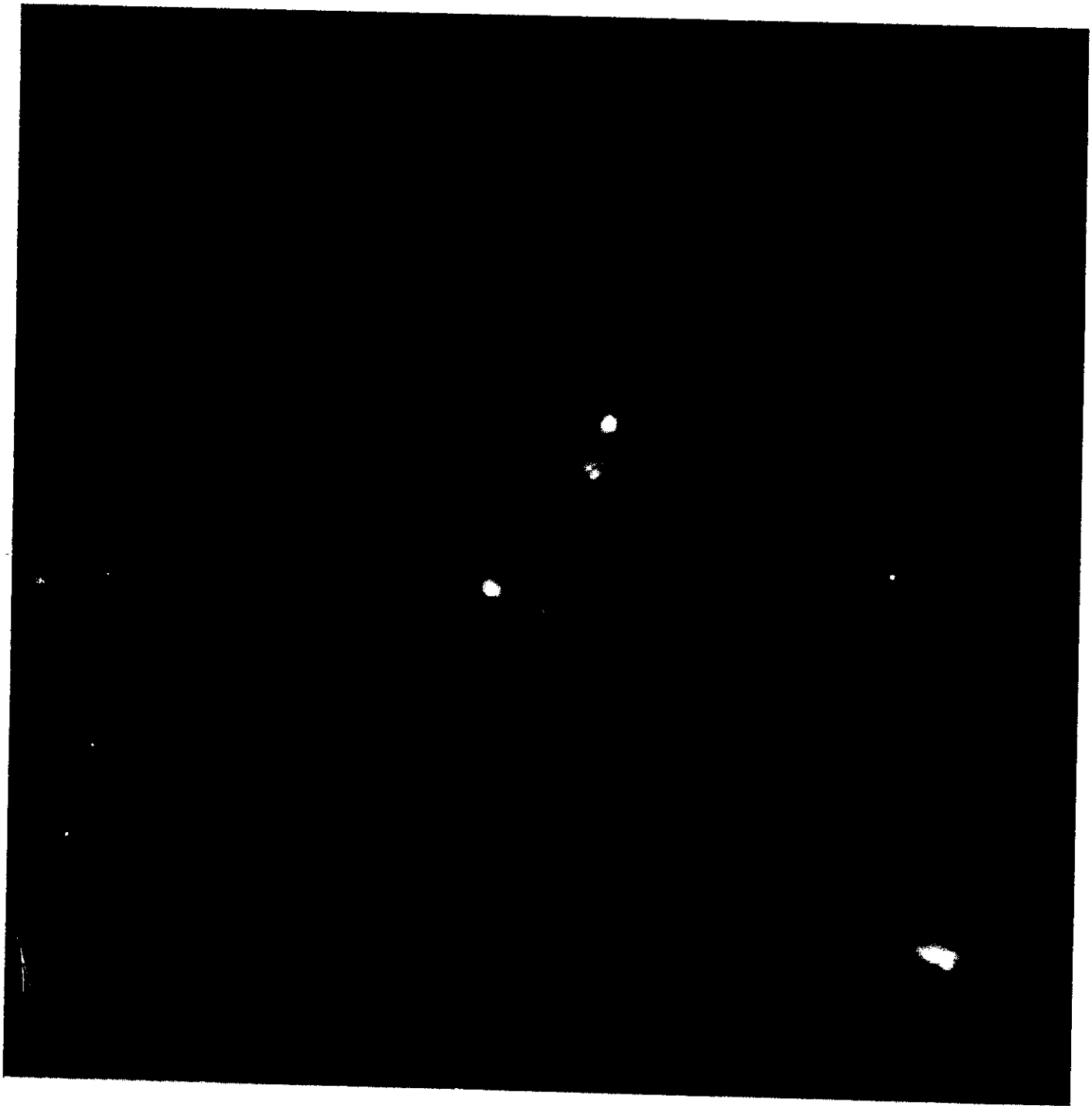
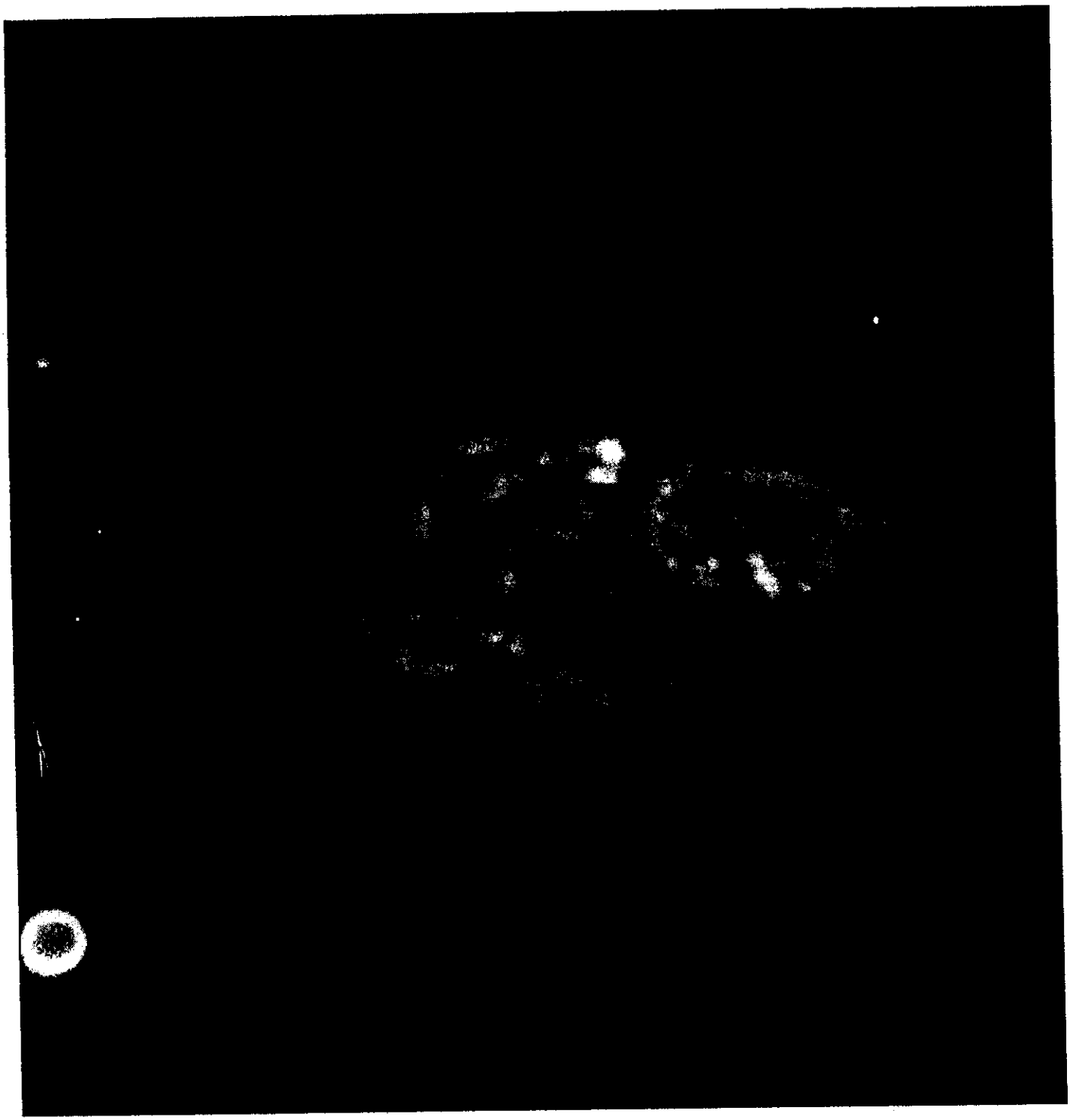


Figure (15 - a): Photomicrograph representing FISH with K20.1A5 / 2392 to *Ae. triseriatus* metaphase chromosomes stained by DAPI.



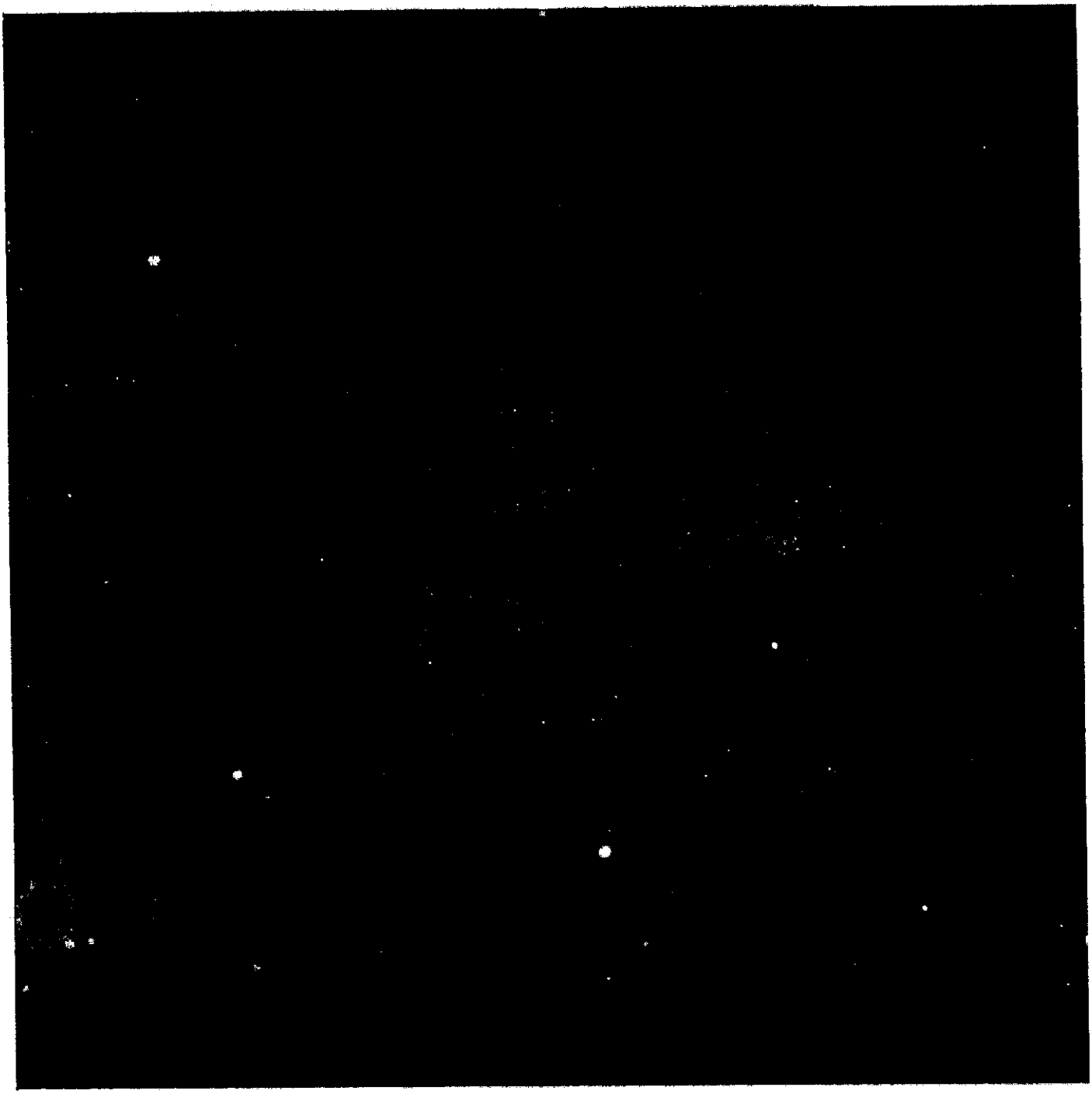


Figure (16 -a): Photomicrograph representing FISH with 1887/2392 to *Ae. albopictus* metaphase chromosomes stained by DAPI

Figure (16 - b): **Photomicrograph representing FISH with 1887 / 2392 to *Ae. albopictus* metaphase chromosomes detecting 1887 stained by FITC.**

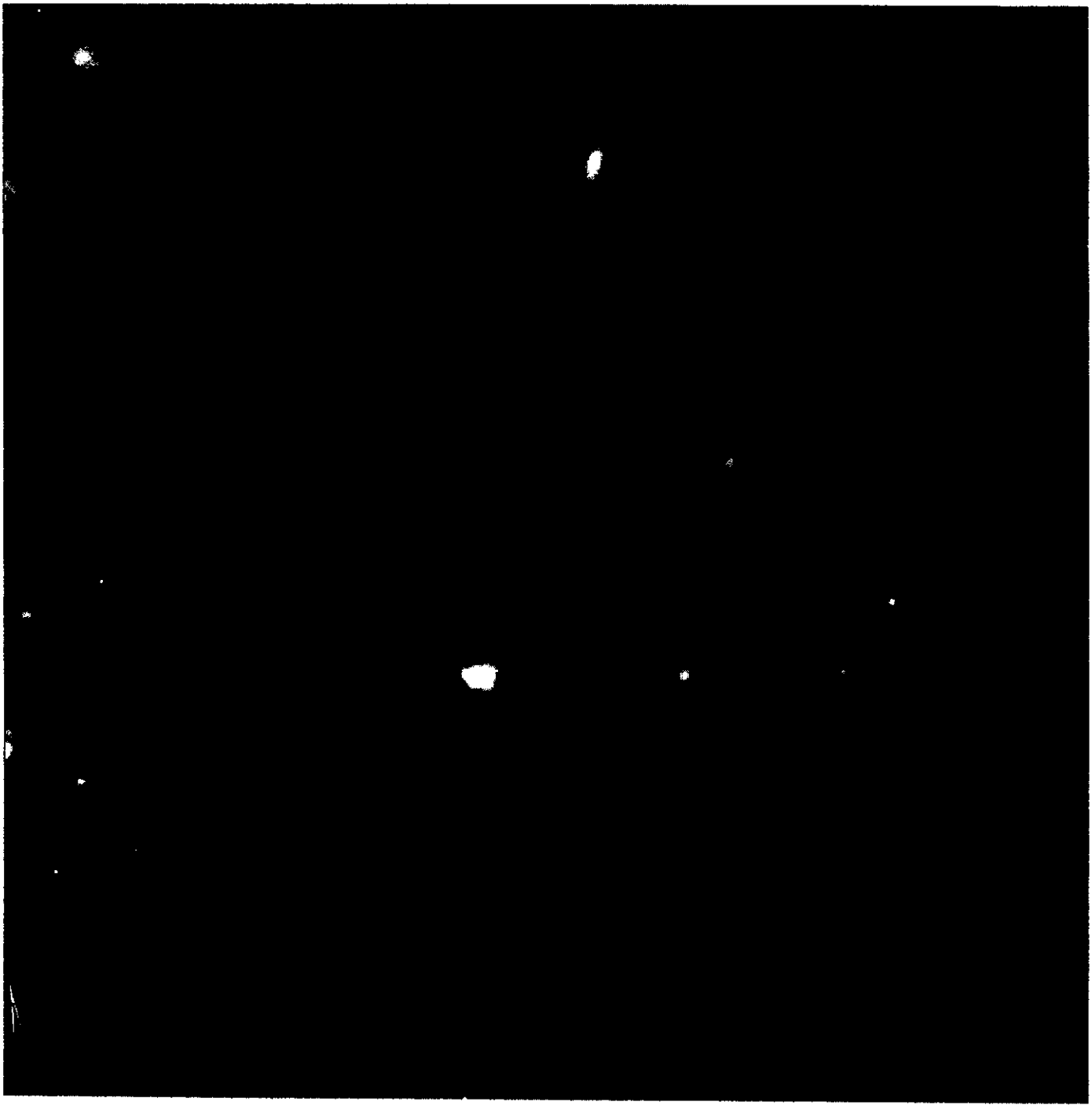


Figure (16 - c): **Photomicrograph representing FISH with 1887 / 2392 to *Ae. albopictus* metaphase chromosomes detecting 2392 stained by rhodamine.**

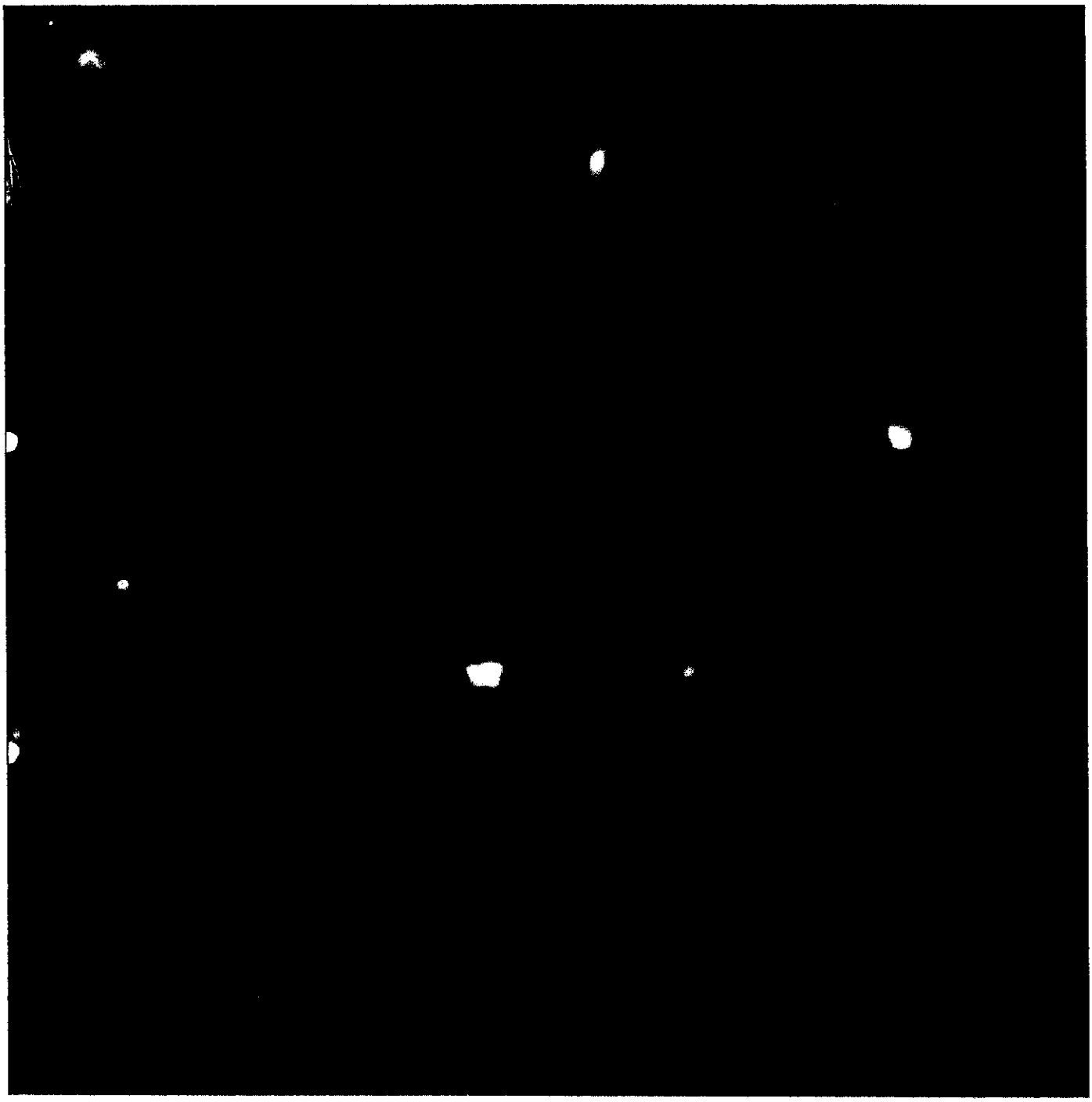
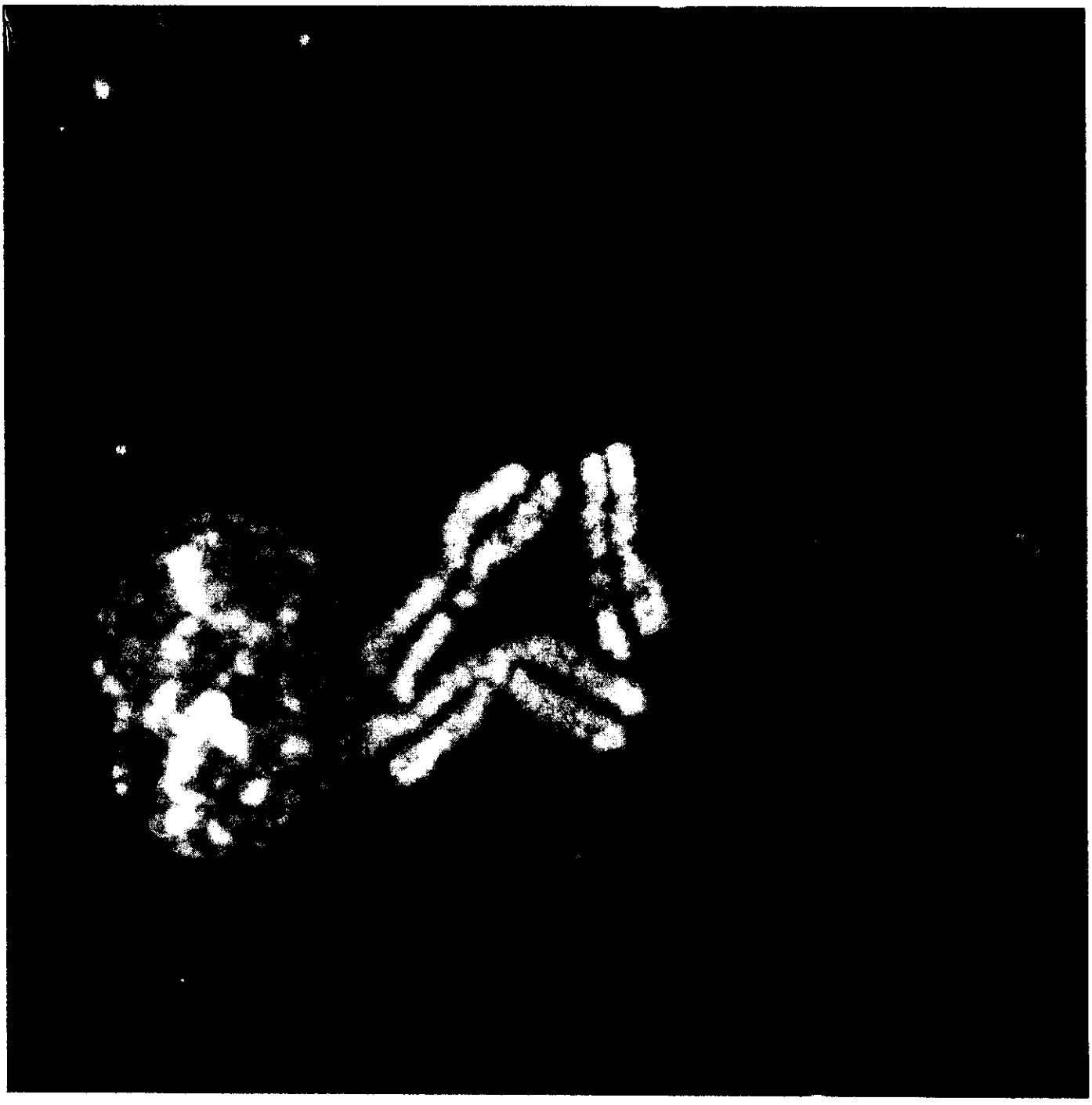
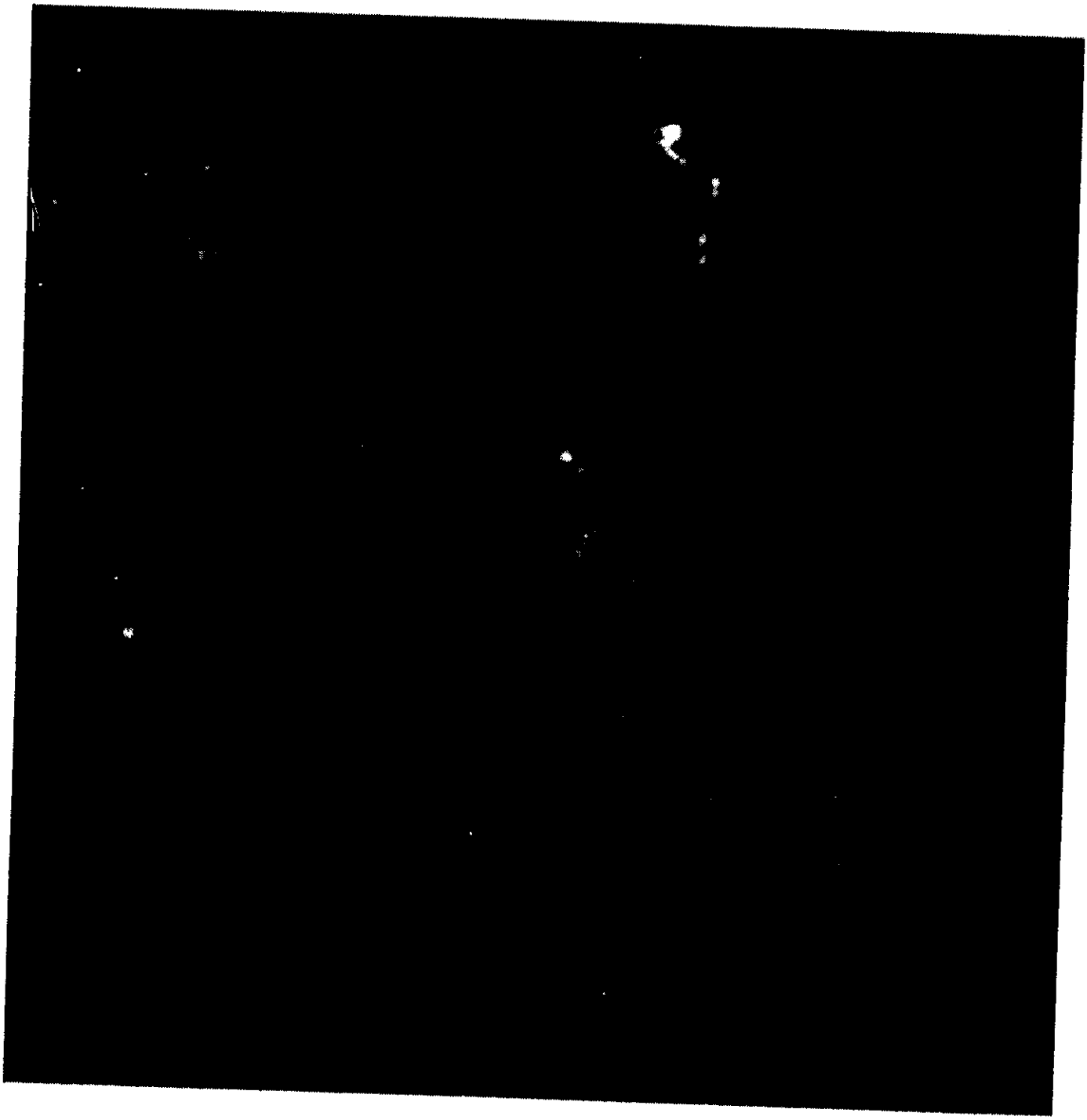


Figure (17 - a): Photomicrograph representing FISH with K20.1A5 / 2392 to *Ae. albopictus* metaphase chromosomes stained by DAPI.





stained with FITC, in this figure there are lighter signals on chromosome 1, but figure (18-c) represents FISH with 1887/2392 to *Cx. pipiens* metaphase chromosomes detecting the position of 2392 which was stained by anti-dioxigenin-rhodamine. Figure (19- a) represents FISH with K20.1A5/2392 to *Cx. pipiens* metaphase chromosomes stained with DAPI counterstain, but figure (19 - b) shows the results of hybridization of K20.1A5 probe for *Cx. pipiens* chromosomes, stained with FITC, where no signals were found in any chromosomes.

Hence FISH technique was used to identify the three chromosomes of *Ae. aegypti*, *Ae. albopictus*, *Ae. triseriatus* and *Cx. pipiens*, and visualize hybridized probes to target chromosome, using fluorescence digital imaging microscopy using camera (CCD) which is ideally suited for this purpose. A cooled-CCD (charge coupled device) camera allows detection of signals too faint to be seen by eye, the digital formatted image can stored magnetically and displayed on a monitors. Also it is easily manipulated the digital files so that the different wavelength images from the same slide can be pseudo colored and superimposed in software. The image which resulting displays the genomic DNA counterstain and all the probe signal simultaneously observed. On the metaphase chromosomes, the digoxigenin visualizes in red and the biotin in green (FITC). The FITC signal is very intense, and also some minor binding sites are visible. The fact that the green and red fluorescence signals are not exactly on the same position, it is needed to change filter block for FITC and digoxigenin, thereby slightly changing the orientation of the dichronic mirror. By applying these double labeled probes in combination with only two single labeled probes, it is possible to perform triple hybridization using only two fluorochromes (FITC : green, and rhodamine : red).

Figure (18 - c): **Photomicrograph representing FISH with 1887 / 2392 to *Cx. pipiens* metaphase chromosomes detecting 2392 stained by rhodamine.**

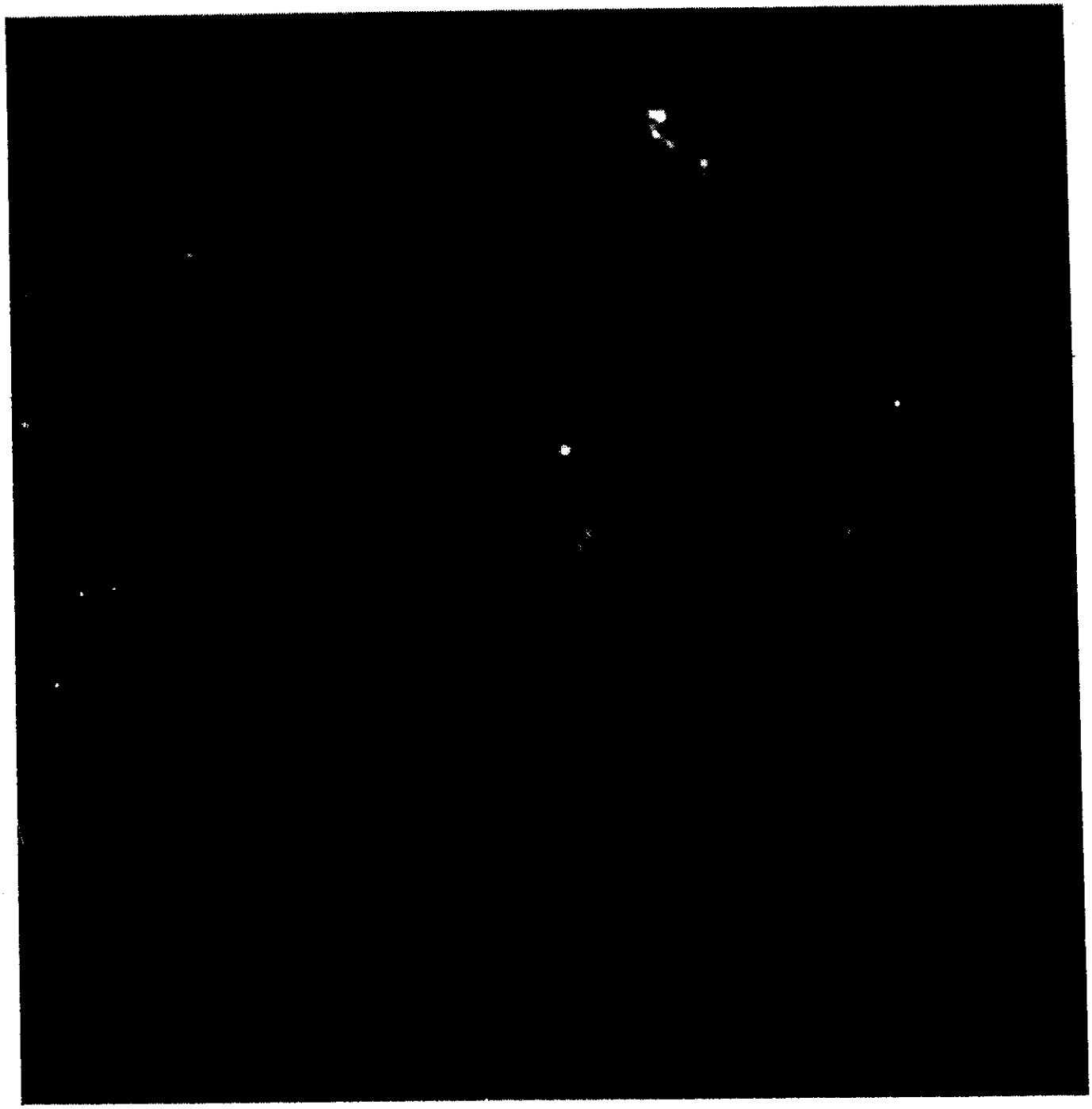
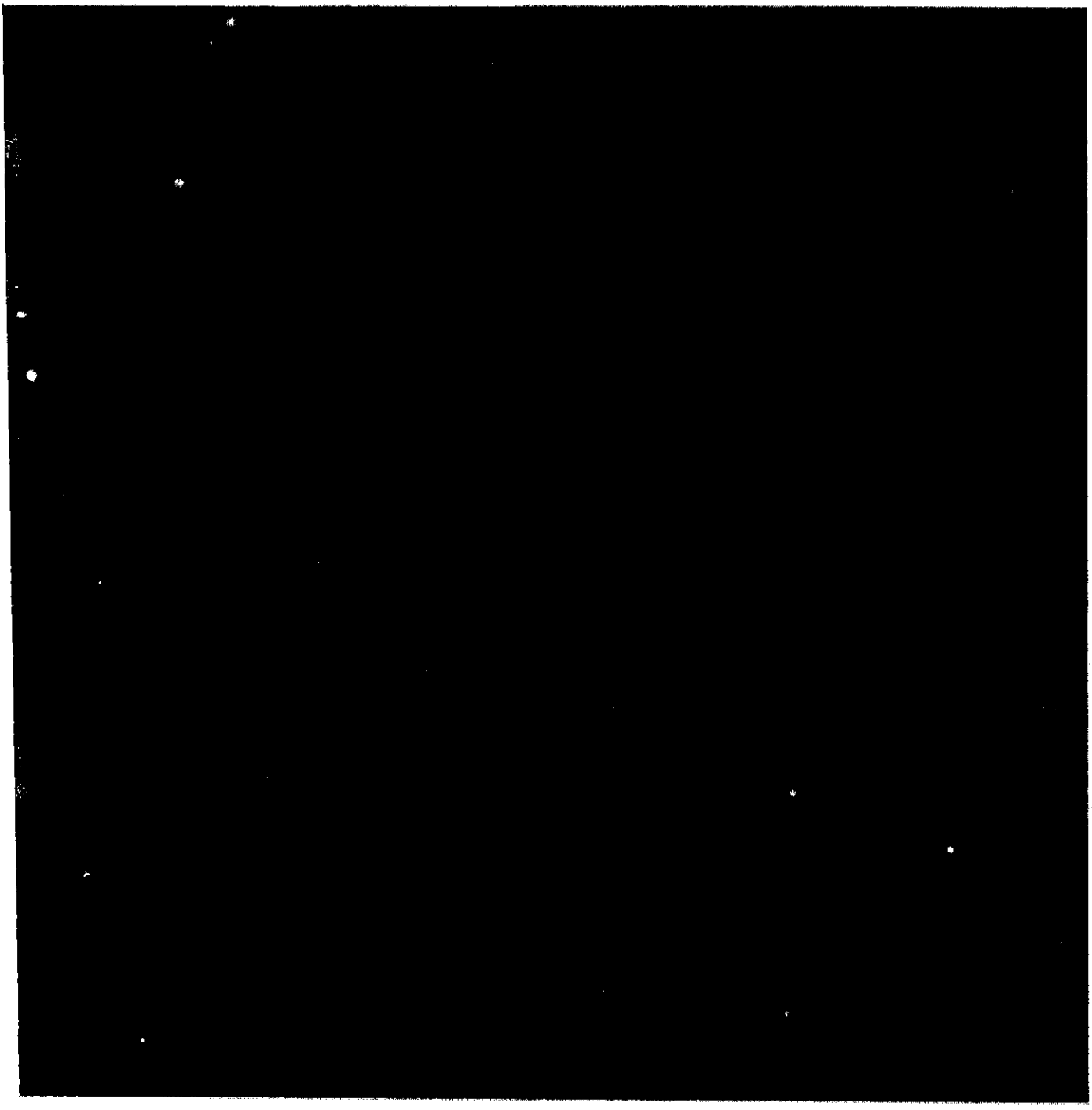


Figure (19 - a): **Photomicrograph representing FISH with K20.1A5/2392 to *Cx. pipiens* metaphase chromosomes stained by DAPI.**



This has the advantage that DAPI (blue) can be used as total DNA counterstain. The major advantage of the fluorescent detection of *in situ* hybrids over autoradiography is its spatial resolving power which is only limited by the optical system used to observe it. Another advantage is that the results of *in situ* hybridization experiments can be available within 1 day. The preparation appear to be stable over several weeks when stored dry at 4°C. Lastly the location of signals were measured electronically on the final merged image using segmented ruler software instead of using rulers on paper prints or projected slides.

4- Cytogenetic studies

The morphology of pupal testes chromosomes has been studied in four species of mosquitoes, namely *Ae. aegypti, Ae. triseriatus, Ae. albopictus* and *Cx. pipiens.* The diploid chromosome number of all species examined is 2n = 6. This result agree with what have been reached by previous studies which indicated that the number of chromosome in salivary gland ***(Sutton 1942),*** in larvae ***(Risler 1959)*** and prepupal brain cells ***(Breland and Gassner 1961)*** of *Ae. aegypti,* is six. ***Rai (1963)*** studied the karyotype of 12 species of mosquitoes, included *Corethra* species, *Anopheles quadrimaculatus, Wycomyia smithii, Cx. pipiens, Cx. restuans, Cx. territans, Ae. togoi, Ae. vexans, Ae. albopictus, Ae. aegypti, Ae. atropalpus* and *Ae. stimulans*, and found that the diploid chromosome number of all species examined, except *Corethra* (2n = 8), is six. ***Akstein (1962),*** mentioned that the chromosome number of *Ae. aegypti, Ae. albopictus, Cx. laticinctus, Cx. theileri* and *Theobaldia longiareolata* is six. ***Kitzmiller (1963)*** stated that the karyotypes of several species of *Anopheles, Aedes* and *Culex* conform to the general pattern 2n=6. ***Baker and Aslamkhan (1969)*** found that the diploid chromosome number of 15 species of Culicine mosquitoes is six. Also

Breland (1961), Rao & Rai (1987), Baker et al., (1971), Munstermann and Marchi (1986) found that the chromosome number of mosquito species is three pairs.

The position of the centromere of each chromosome was measured from the terminus of the short arm (p-arm) of the chromosome (pter) relative to the total length of the chromosome and reported as a percent fractional length or % FLpter. These FLpter measurements were made directly on the computer monitor using a computer -generated segmented ruler. The length of the chromosomes and chromosome's arms were measured by microns. The three pairs of chromosomes of the four studied mosquito species can be distinguished by their relative length as well as the position of their centromeres.

(A) ***Ae. aegypti***

One pair of chromosomes is small and the other two pairs are relatively larger. Chromosome 1 is the smallest pair and it is metacentric, the length of each arm is 4.7 microns, the total length of chromosome 1 is 9.4 microns, and the position of the centromere is 50% FLpter. The longer pair (chromosome2) is metacentric, the length of the arm is 6.3 microns, the total length of the chromosome 2 is 12.6 microns, and the position of the centromere is 50%FLpter.The other larger pair (chromosome3) is submetacentric, the length of the longer arm of chromosome 3 is about 6.2 microns, and the length of the shorter arm is about 5.3 microns, the total length of chromosome 3 is 11.5 microns, and the position of the centromere is 46.1% FLpter. (Table 4 and fig. 10 - a).

Our results in table (4) indicate that total lengths of the first, second and third metaphase chromosomes in *Ae. aegypti* are 9.4, 12.6 and 11.5 microns, respectively. Whereas the data of ***Baker and Aslamkhan (1969)*** in table (5) indicated that the lengths of I, II and III metaphase

Table (4) Measurements of metaphase chromosomes in microns and the position of centromere reported as % FL pter

Species	Chromosome I				Chromosome II				Chromosome III				Ratio
	Length in microns		Total length in microns	Position of centromere % FL pter	Length in microns		Total length in microns	Position of centromere % FL pter	Length in microns		Total length in microns	Position of centromere %FL pter	I / 2&3
	P	q			P	q			P	q			
Ae. aegypti	4.7	4.7	9.4	50%	6.3	6.3	12.6	50%	5.3	6.2	11.5	46.1%	0.39
Ae. albopictus	5.2	6.2	11.4	45.6%	8.7	9.0	17.7	49.2%	7.8	7.8	15.6	50%	0.34
Ae. triseriatus	4.7	4.9	9.6	49%	5.2	6.2	11.4	45.6	4.7	5.6	10.3	45.6%	0.44
Culex pipiens	3.1	3.1	6.2	50%	4.8	5.2	10.0	48%	3.9	3.9	7.8	50%	0.35

Table (5): **Measurements of metaphase chromosomes in microns. after (*Baker & Aslamkhan 1969*).**

Species	Tissue	Chromosome 1			Chromosome 2			Chromosome 3		
		Arm A	Arm B	Total length in μ	Arm A	Ar m B	Total length in μ	Arm A	Arm B	Total length in μ
Ae. thomsoni	testes	4.1	4.1	8.2	5.0	5.0	10.0	6.0	5.0	11.0
Ae. albopictus	ovary	6.5	6.5	13.0	8.6	8.2	16.8	9.6	9.4	19.0
Ae. aegypti	ovary	5.6	5.2	10.8	7.2	6.2	13.4	7.3	7.0	14.3
C. p. fatigans	testes	6.7	6.5	13.2	8.0	7.6	15.6	8.4	8.2	16.6
	ovary	2.3	2.3	4.6	3.4	2.9	6.3	3.4	3.2	6.6
	testes	3.3	3.3	6.6	4.5	4.0	8.5	5.0	4.5	9.5

chromosomes are 13.2, 15.6 and 16.6 microns respectively. On the other hand, ***Rai's (1963)*** measurements of brain chromosomes of *Ae. aegypti* are much shorter than our measurements of chromosomes from testes. ***Rai*** found that the length of chromosome 1 , 2 and 3 are 5.4, 6.9 and 7.6 microns respectively.

(B) Ae. triseriatus

Fig. (14 - a), shows that chromosome 1 is the smallest pair and it is slightly submetacentric, the length of the longer arm is about 4.9 microns, and the length of the shorter arm is about 4.7 microns, the total length of chromosome 1, is 9.6 microns, and the position of the centromere is 49% FLpter. Chromosome 2 is the longest chromosome, it is submetacentric, the length of the longer arm is about 6.2 microns, but the length of the shorter arm is about 5.2 microns, the total length of chromosome 2 is 11.4 microns, and the position of centromere is 45.6% FLpter. The other equally long chromosomal pair (chromosome 3) is submetacentric, the length of longer arm is 5.6 microns and the length of the other arm is 4.7 microns. The total length of chromosome 3 is about 10.3 microns, and the position of the centromere is 45.6% FLpter. (Table 4 and fig. 14 - a).

(C) Ae. albopictus

Figure (17-a), shows that chromosome 1(the smallest pair) is submetacentric, the length of the longer arm is about 6.2 microns, the length of the shorter arm is about 5.2 microns, the total length of the chromosome 1 is 11.4 microns, and the position of the centromere is 45.6% FLpter. The longest chromosomal pair (chromosome 2) is slightly submetacentric, the length of the longer arms is about 9.0 microns, but the length of the shorter arm is about 8.7 microns, the total length of the chromosome 2 is 17.7 microns and the position of centromere is 49.2% FLpter. Chromosome 3 is metacentric, the length of each arm is 7.8

microns, the total length of chromosome 3 is about 15.6 microns, and the position of centromere is 50% FLpter. (Table 4 and figure 17 - a).

The results in table (4) also indicate that the total length of metaphase chromosome in *Ae. albopictus* are 11.4, 17.7 and 15.6 microns for the first, second and third chromosomes respectively. ***Baker and Aslamkhan (1969)*** found that the length of I, II and III metaphase chromosomes are 13.0, 16.8 and 19.0 microns respectively (table 5), while ***Rai's (1963)*** measurements of brain chromosomes of *Ae. albopictus* are about half as long as our measurements of chromosomes from testes at metaphase. ***Rai*** found that the length of the three chromosomes are 6.2, 7.6 and 9.2 microns for chromosome I, II and III respectively.

(D) *Cx. pipiens*

Chromosome I is the shortest pair, it is metacentric, the length of each arm is 3.1 microns, the total length of chromosome 1 is 6.2 microns, and the position of centromere is 50% FLpter. The chromosome 3 is considerably shorter than chromosome 2 , it is metacentric, the length of each arm is 3.9 microns, the total length of chromosome 3 is about 7.8 microns, and the position of centromere 50% FLpter. The chromosome 2 is slightly submetacentric, the length of the longer arm is about 5.2 microns, but the length of the shorter arm is about 4.8 microns, the total length of chromosome 2 is 10.0 microns and the position of centromere is 48% FLpter (Table 4 and figure 18 - a).

The total length of metaphase chromosomes in *Cx. pipiens* (table, 4) are 6.2, 10.0 and 7.8 microns for the first, second and third chromosome respectively. Whereas the results of ***Baker and Aslamkhan (1969),*** in table (5) indicated that the length of first, second and third metaphase chromosomes are 6.6, 8.5 and 9.5 microns respectively.

triseriatus, whereas it is metacentric in *Ae. albopictus* and *Cx. pipiens*. Our results agree with the definitions of some authors who stated that the slightly off-center centromeres in *Ae. albopictus* and *Ae.aegypti* should be considered metacentric. However in all species of culicine mosquito that have been studied cytologically, the centromeres are either median or submedian in position. This situation causes the chromosome arms to be equal or nearly equal in length; no species of culicine mosquito has been reported in which the chromosome arms are greatly unequal in length ***(Breland and Gassner 1961).*** Our results agree with that of ***Breland and Gassner*** who found that in some well-spread *Ae. aegypti* metaphase stages, three sizes of chromosomes can be distinguished, the position of the centromeres are slightly off-center in each pair of chromosomes,the large and medium pairs of chromosomes are similar in size. Also ***McDonald and Rai (1970)*** stated that *Ae. aegypti* is characterized by the presence of three pairs of chromosomes, the shortest pair is metacentric; the medium-length pair is submetacentric and the longest pair is metacentric. Our results also agree with that of ***Baker et al., (1971)*** who reported that in *Cx. tritaeniorhynchus*, there are three sizes of chromosomes, the smallest pair, chromosome 1, is always metacentric. The other two pairs are larger, and generally are described as being equal in total length. The two longer pairs of chromosomes can usually be distinguished by the position of the centromeres. One pair is slightly submetacentric; one arm being longer than the other arm. The other equally long chromosomal pair is nearly always more metacentric, with both arms of equal length (but the authors stated that chromosome 3 is submetacentric whereas chromosome 2 is metacentric). Also ***Baker et al., 1977)*** stated that in metaphase chromosomes from the ovaries of *Cx. tritaeniorhynchus*, all chromosome pairs can be distinguished by their relative lengths and the position of their centromeres. The smallest

chromosome is metacentric and the two longer chromosomes are approximately equal in length and can be distinguished by the position of centromeres. The results also agree with that of ***Rao and Rai (1987)*** who reported that the individual chromosome pairs were distinguished by the total length, either submetacentric or metacentric depending on genus and species. Our results agree with that of ***Munstermann and Marchi (1986)*** who studied the culicine mosquito *Stabethes cyaneus* species and demonstrated that the three pairs of chromosomes are slightly submetacentric, one pair (chromosome 1) was shorter, chromosome 2 was the longest chromosome but the length of chromosome 3 was between the other chromosomes.

In our results, we adopted the new system proposed by ***Mc Donald & Rai (1970). Rai (1963)*** numbered the chromosomes from I to III in increasing order of their length with chromosome I being the shortest and chromosome III being the longest one, and indicated that the chromosome I is metacentric, the next largest chromosome (chromosome II) is submetacentric in *Ae. aegypti*, *Ae. atropalpus* and *Ae. stimulans* but it is metacentric in all other species, and chromosome III which is the longest one is submetacentric in *Ae. stimulans* and metacentric in other species examined. In *Cx. pipiens* the smallest chromosome I may be submetacentric , and it is shorter than chromosome II and III.

System	Chromosome length		
	shortest	**Median**	**Longest.**
Old system ***(Rai 1963)***	I	II	III
New system proposed by ***McDonald & Rai (1970)***	1	3	2

The comparison between metaphase chromosomes of *Cx. pipiens*, *Ae. aegypti*, *Ae. albopictus* and *Ae. triseriatus*, indicates that in general, the *Cx. pipiens* chromosomes 1, 2 and 3 are smaller in length than the chromosomes in the other species respectively.

5 - FISH landmarks for mosquito species chromosomes

FISH mapping to metaphase chromosomes requires high-quality chromosome preparations with high mitotic indices. Pupal testes represent an excellent source of chromosomal material because mitotic and meiotic characters are present in the organ, and the chromosomal material may be found in various stages of condensation. It also requires tagged probes which contain sufficient single copy sequence such that a fluorescent signal can be detected (***Brown et al 1995)***. In this work it was found that chromosome preparations made from the pupal testes of *Ae. aegypti*, *Ae. albopictus*, *Ae. triseriatus* and *Cx. pipiens*, are well suited to FISH analysis because the chromosome morphology in preparation was consistent and reproducible.

The combination of FISH and digital imaging has provided a direct method to map a specific nucleic acid probe onto the total genomic DNA of an organism ***(Ferguson et al., 1996).*** The signal position was then measured as a fractional length from the p-terminus (FLpter) along the chromosome. The pter was assigned to the smallest arm of the metacentric to submetacentric chromosomes.

A single plasmid, p2392, which contained the three chromosome tag sequences was constructed to yield an *Ae. aegypti* chromosome tagging reagent which has been used to simplify FISH physical mapping of unknown probes ***(Brown & Knudson, 1997)***. *Ae. aegypti* chromosome specific tags (plasmids p2405 , p2056 and p1887) and a centric heterochromatin probe (BAC K20.1A5)were identified, each of which

produced a strong signal on paired metaphase chromosomes. ***Brown & Knudson, (1997)*** mentioned that each chromosome of the haploid genome can be unambiguously identified and oriented by fluorescence *in situ* hybridization (FISH) and digital imaging microscopy, the FISH tags were derived from *Ae. aegypti.*

6 - Characteristics of chromosome landmarks

The metaphase chromosomes of *Ae. aegypti, Ae. albopictus, Ae. triseriatus* and *Cx. pipiens* were used as the DNA target in FISH hybridization with the individual components of p2392 tagging reagent. The plasmid p2392 has been constructed that contains the three chromosome tag sequences (p1887, p2405 and p2056), the orientation of the three inserts in p2392 was determined. Now the p2392 tagging reagent used in all hybridization reactions when unknown probes are placed to metaphase chromosome and the position of the probes was measured and reported as the fractional length from the p-terminus (% FLpter), ***Brown & Knudson (1997)***. Hence, chromosome tags have been developed to identify each chromosome and unambiguously oriented. The three prominent signals, one for each chromosome, are located in the q-arm or longer arm of the chromosome. Plasmid p1887 was chosen to label a broad region in chromosome 1 q-arm, and it was derived from cosmid K2.9, plasmid p2405 hybridized chromosome 2 on the q-arm, and it was derived from cosmid K2.40.2, and plasmid p2056 exhibited a prominent signal on chromosome 3, and it was derived from cosmid K2.61.

(A) Characteristics of *Ae. aegypti* chromosome landmarks

When p1887 was used as a FISH probe to metaphase chromosomes, the signal produced broad bands where the midpoint of the intense signals

was located at ~70% fractional length from the p-terminus (FLpter) or the smaller arm of chromosome 1 (figure 20 and table 6).

When p2405 was used as a FISH probe, detectable signals were seen on all three chromosomes with the prominent signal on the q-terminus of chromosome 2 located at ~ 96% FLpter (figure 21). As was reported previously, lighter signals were also seen subtelomeric on the p-terminus of chromosome 2, midway on the q-arm of chromosome 1, and subtelomeric on the p-arm of chromosome 3 (figure 21 and table 6). When p2056 was used as a FISH probe, the signals were located on chromosome 3 at ~ 85.7% FLpter (figure 22 and table 6).

The plasmid p2392 tagging reagent probe yielded three prominent signals indicating the presence of the three sequences in whole insect genome. The signals were located at chromosome 1, 2 and 3 of *Ae. aegypti* (figures 20, 21, 22).

*** The centric heterochromatin probe of *Ae. aegypti:***

The centric heterochromatin probe (K20.1A5), exhibited strong hybridization signals by FISH to chromosome 1 and 3 with a faint signal on chromosome 2 (figure 23). These results indicate that the centromeric region of chromosome 1 and 3 are similar in sequence with chromosome 2 exhibiting only a minor sequence similarity.

(B) Other mosquito species chromosome landmarks

In *Ae. albopictus* chromosomes, an 18S ribosomal cistron probe (p1887) labeled a broad region in chromosome 1, q-arm located at 71.4% FLpter, (fig. 24 and table 6). When chromosome 2 and chromosome 3 specific probes, p2405 and p2056, were used in FISH to pupal testes metaphase chromosome spreads, signals were not seen. Thus, the p2392 tagging reagent would not work as chromosome landmark in this species.

Table (6). Characteristics of chromosome landmarks.

Species	Chromosome landmark	Plasmid	Chromosome location % FLpter
Ae. aegypti	Chromosome 1 tag Chromosome 2 lag Chromosome 3 lag	p1887 p2405 p2056	~ 70 ~ 96 ~85.7
Ae. albopiculs	Chromosome 1 tag Chromosome 2 lag Chromosome 3 lag	p1887 p2405 p2056	~71.4 - -
Ae. triseriatus	Chromosome 1 tag Chromosome 2 lag Chromosome 3 lag	p1887 p2405 p2056	~70.8 (chromosome 1) ~71.4 (chromosome 2) - -
Cx. pipiens	Chromosome 1 tag Chromosome 2 lag Chromosome 3 lag	p1887 p2405 p2056	~ 69.5 - -

Figure (21): **FISH of p2405 and p2392 to an *Ae. aegypti* metaphase chromosomes. The plasmid p2405 (green) labels all three chromosomes with prominent signal on the q-terminus of chromosome 2, lighter signals were also seen on the p-terminus of chromosome 2, midway on the q-arm of chromosome 1, and subtelomeric on the p-arm of chromosome 3. The p2392 (red) labels a region of all three chromosomes. The DAPI counterstain (blue) delineates the individual chromatids.**

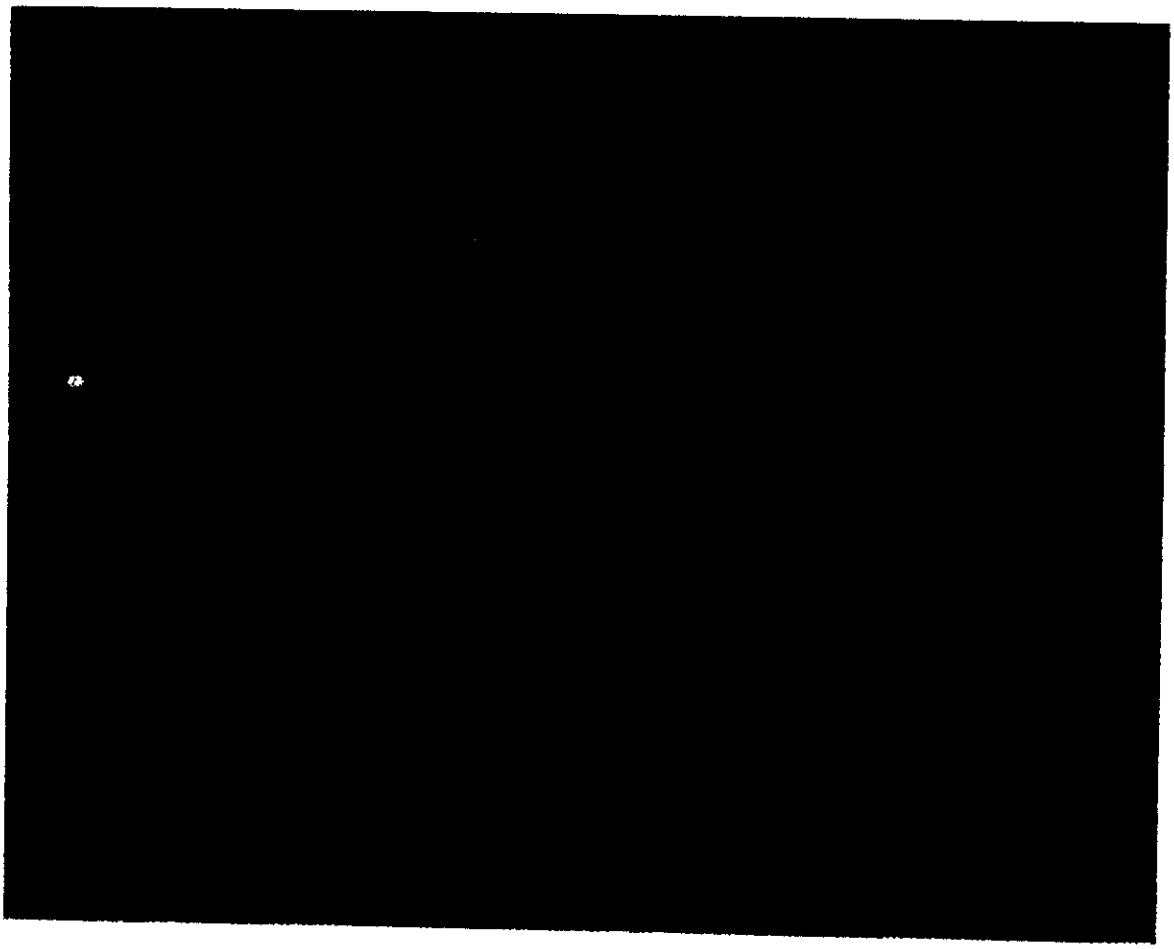


Figure (22):FISH of p2056 and p2392 to an *Ae. aegypti* metaphase spread. Plasmid p2056 (green) labels a region of chromosome 3. The p2392 (red) labels a region of chromsome 1,2 and 3. The DAPI counterstain (blue) delineates the individual chromatids.

Figure (22):**FISH of p2056 and p2392 to an *Ae. aegypti* metaphase spread. Plasmid p2056 (green) labels a region of chromosome 3. The p2392 (red) labels a region of chromsome 1,2 and 3. The DAPI counterstain (blue) delineates the individual chromatids.**

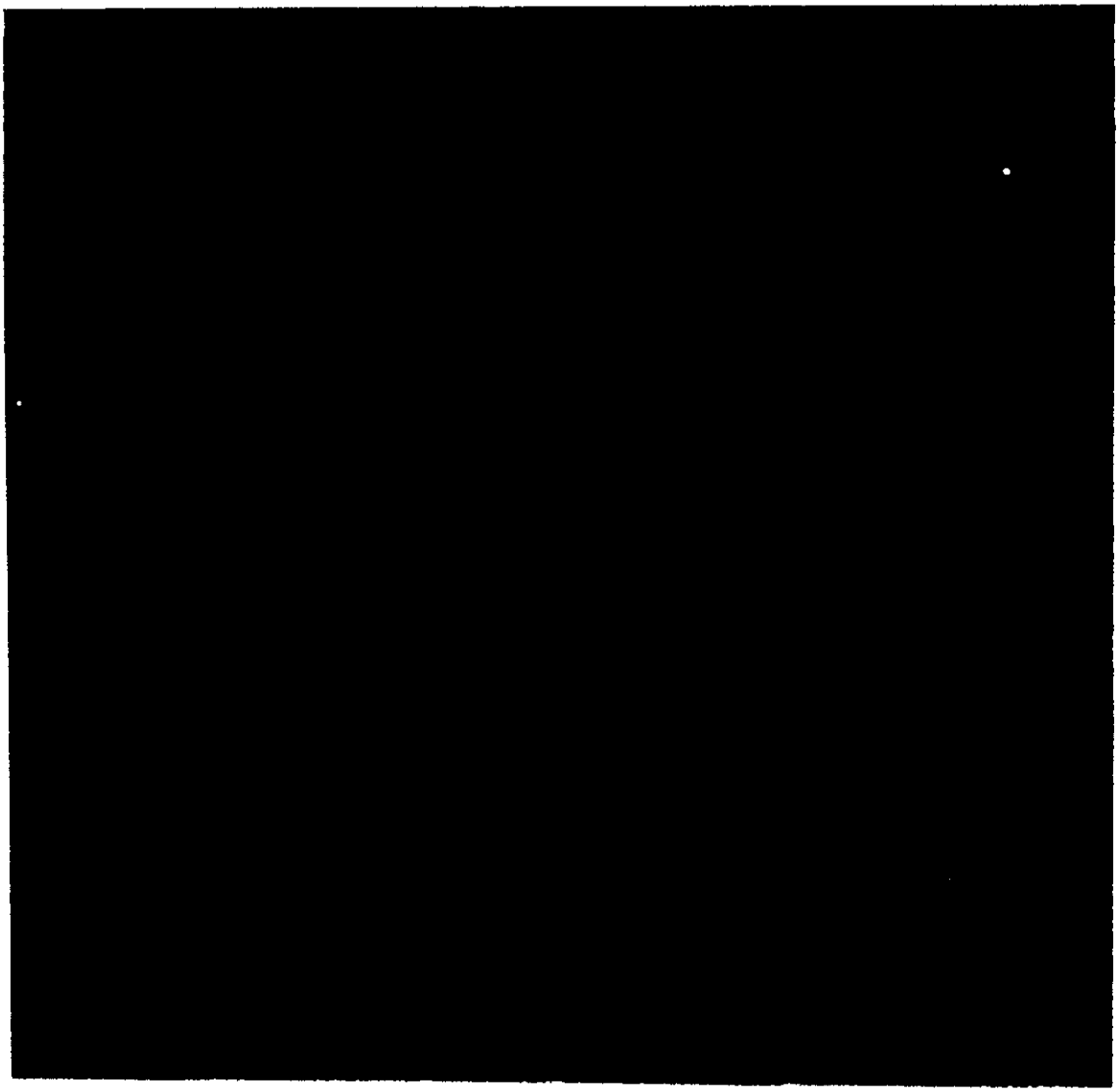
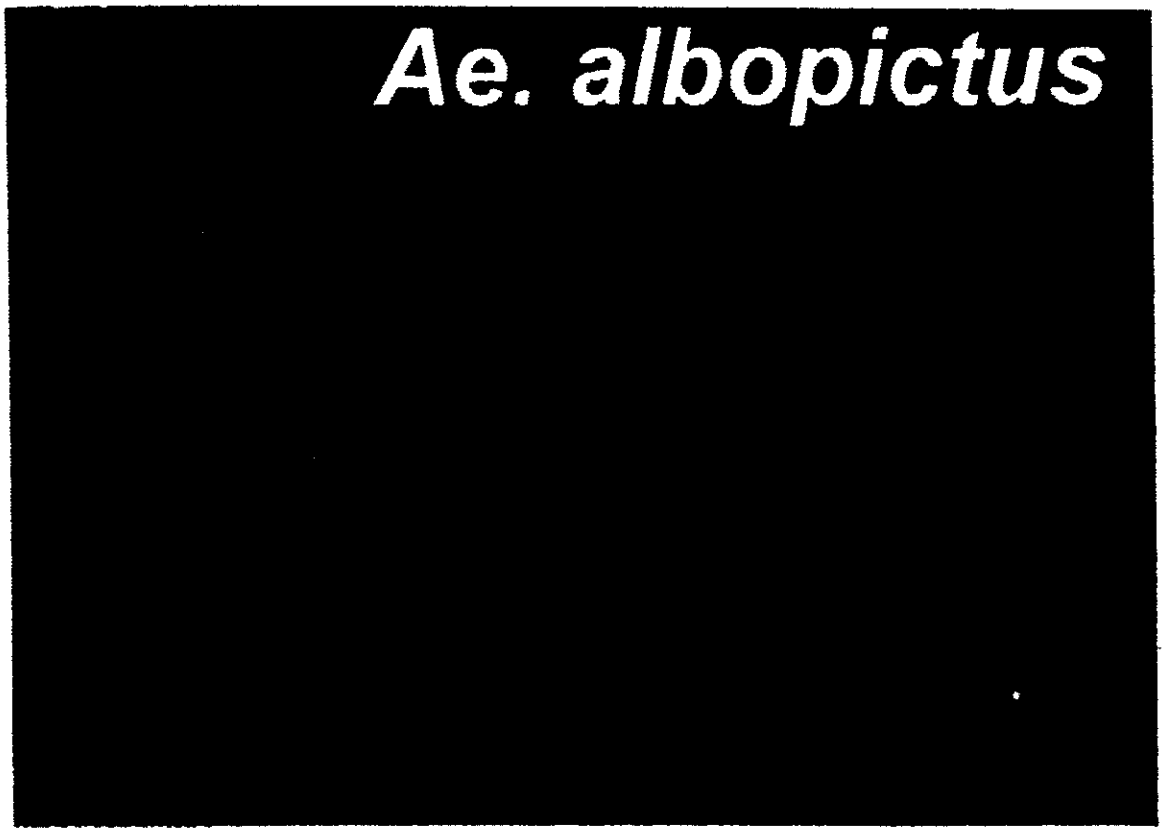


Figure (23): **FISH of a centric heterochromatin probe K20.1A5 and p2392 to an *Ae. aegypti* metaphase chromosomes. The clone K20.1A5 (green) labels a region of chromosome 1, chromosome 2 and chromosome 3. The p2392 (red) labels a region of all six chromosomes. The DAPI counterstain (blue) delineate the chromosomes.**


Ae. aegypti


Ae. albopictus

When plasmid p1887 was used as a FISH probe to metaphase chromosomes of *Ae. triseriatus*, it produced a detectable FISH signal on the q-arm of chromosome 1 located at 70.8% FLpter, it also hybridized chromosome 2 at 71.4 % FLpter (fig. 25 and table 6). When the chromosome 2 specific probe (p2405) was used as a FISH probe, signal was not seen, also when the chromosome 3 specific probe (p2056) was used, the signals were not observed in the chromosomes. Then the tagging reagent p2392 did not work as chromosome landmarks in *Ae. triseriatus.*

When plasmid p1887 was used in FISH to *Cx. pipiens* pupal testes metaphase chromosome spreads, signal was seen on the q-arm of chromosome 1 located at 69.5% FLpter, (fig. 26 and table 6). When chromosome 2 and chromosome 3 specific probes p2405 and p2056, were used as a FISH probes, signals were not seen in any chromosome. Thus the tagging reagent p2392 did not work as chromosome landmarks in *Cx. pipiens.*

*** The centric heterochromatin probe of other mosquito species**

When the centeric heterochromatin probe (K20.1A5) was used in FISH to pupal testes metaphase chromosomes from *Ae. albopictus, Ae. triseriatus* and *Culex pipiens,* the FISH signals were not observed in pupal testes material derived from *Ae. albopictus* (fig. 27), *Ae. triseriatus* (fig. 28) and *Cx. pipiens* (fig. 29).

Figure (25): **FISH of p1887 and p2392 to an *Ae. triseriatus* metaphase chromosome spread. Plasmid p1887 (green) labels two regions on two different chromosomes. The p2392 (red) labels a region of two different chromosome. The DAPI counterstain (blue) delineates the individual chromatids.**


Ae. triseriatus

Figure (26): **FISH of p1887 and p2392 to *Cx. pipiens* metaphase chromosome spreads. plasmid p1887 (green) labels a region of chromosome1. The p2392 (red) labels a region of chromosome 1. The DAPI counterstain (blue) delineates the individual chromatids.**

Figure (27): **FISH of a centric heterochromatin probe K20.1A5 and p2392 to *Ae. albopictus* metaphase chromosome spreads. The clone K20.1A5 (green) signals were not observed in any chromosome. The p2392 (red) labels a region of chromosome 1. The DAPI counterstain (blue) delineates the individual chromatids.**

Figure (28): **FISH of a centric heterochromatin probe K20.1A5 and p2392 to *Ae. triseriatus* metaphase chromosome spreads. The clone K20.1A5 (green) signals were not observed in any chromosome. The p2392 (red) labels two regions on two different chromosomes.. The DAPI counterstain (blue) delineates the individual chromatids.**

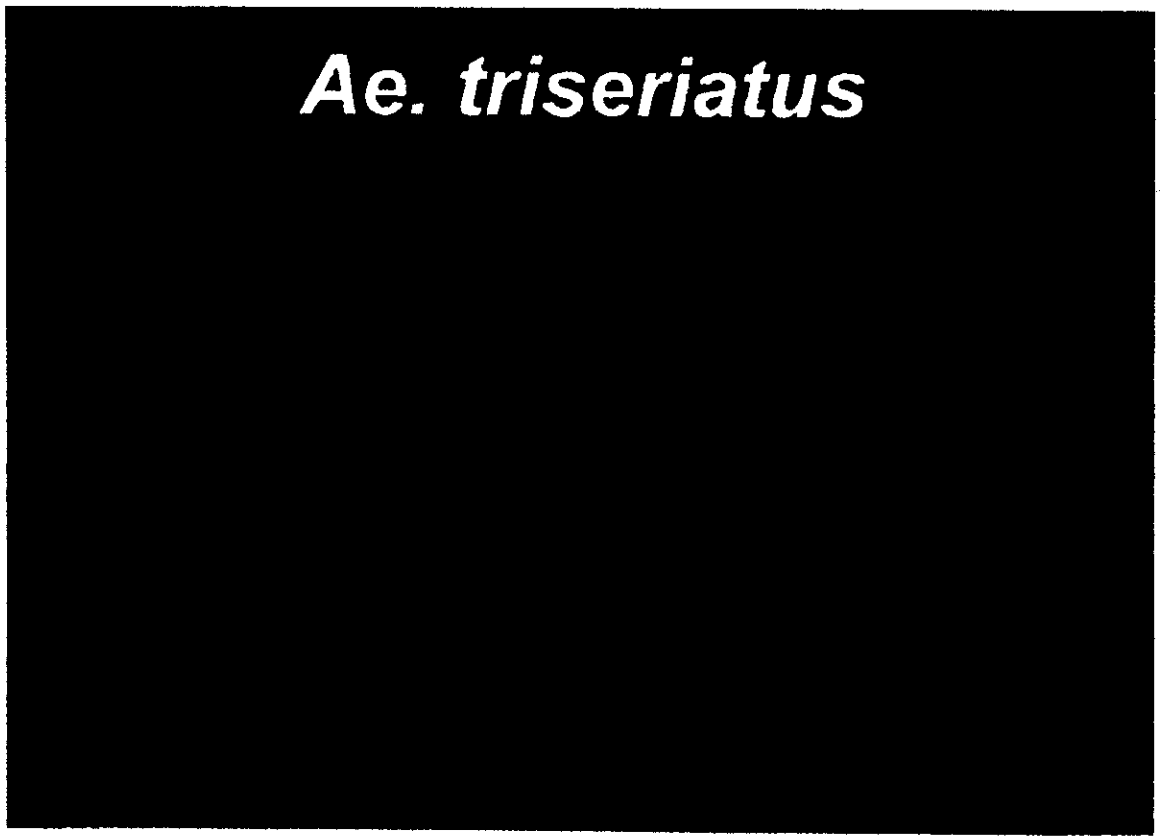
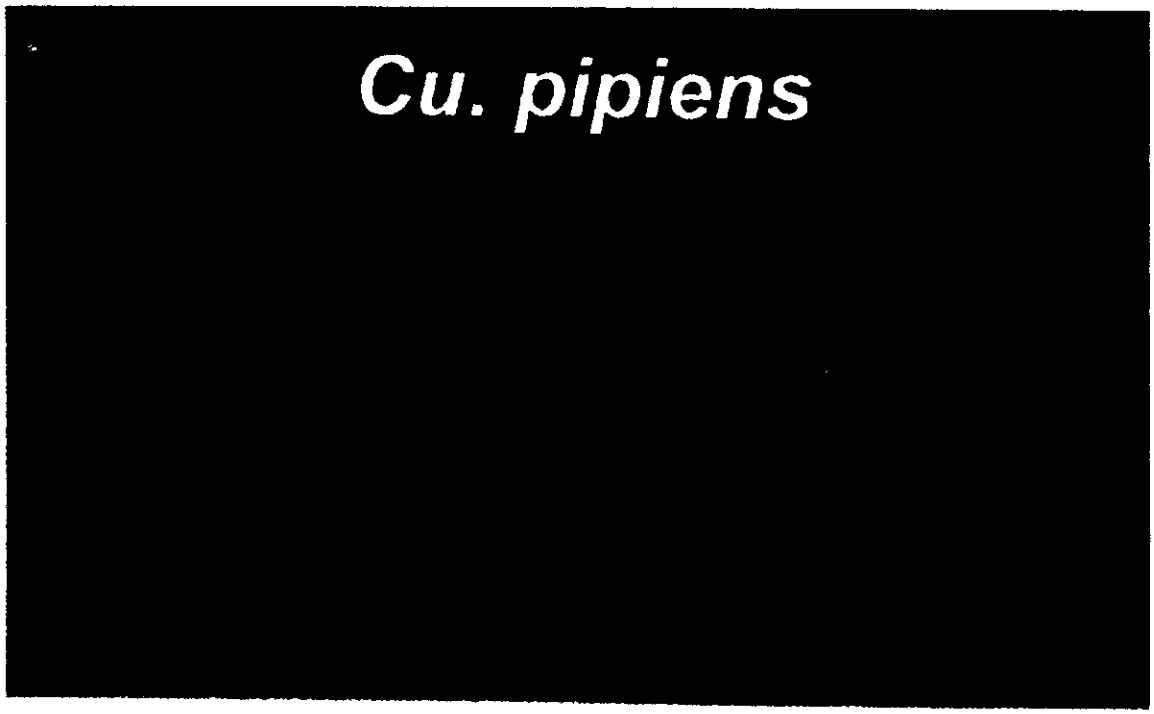

Ae. triseriatus

Figure (29): **FISH of a centric heterochromatin probe K20.1A5 and p2392 to *Cx. pipiens* metaphase chromosome spreads. The clone K20.1A5 (green) signals were not observed in any chromosome. The p2392 (red) labels a region of chromosome 1. The DAPI counterstain (blue) delineates the individual chromatids.**

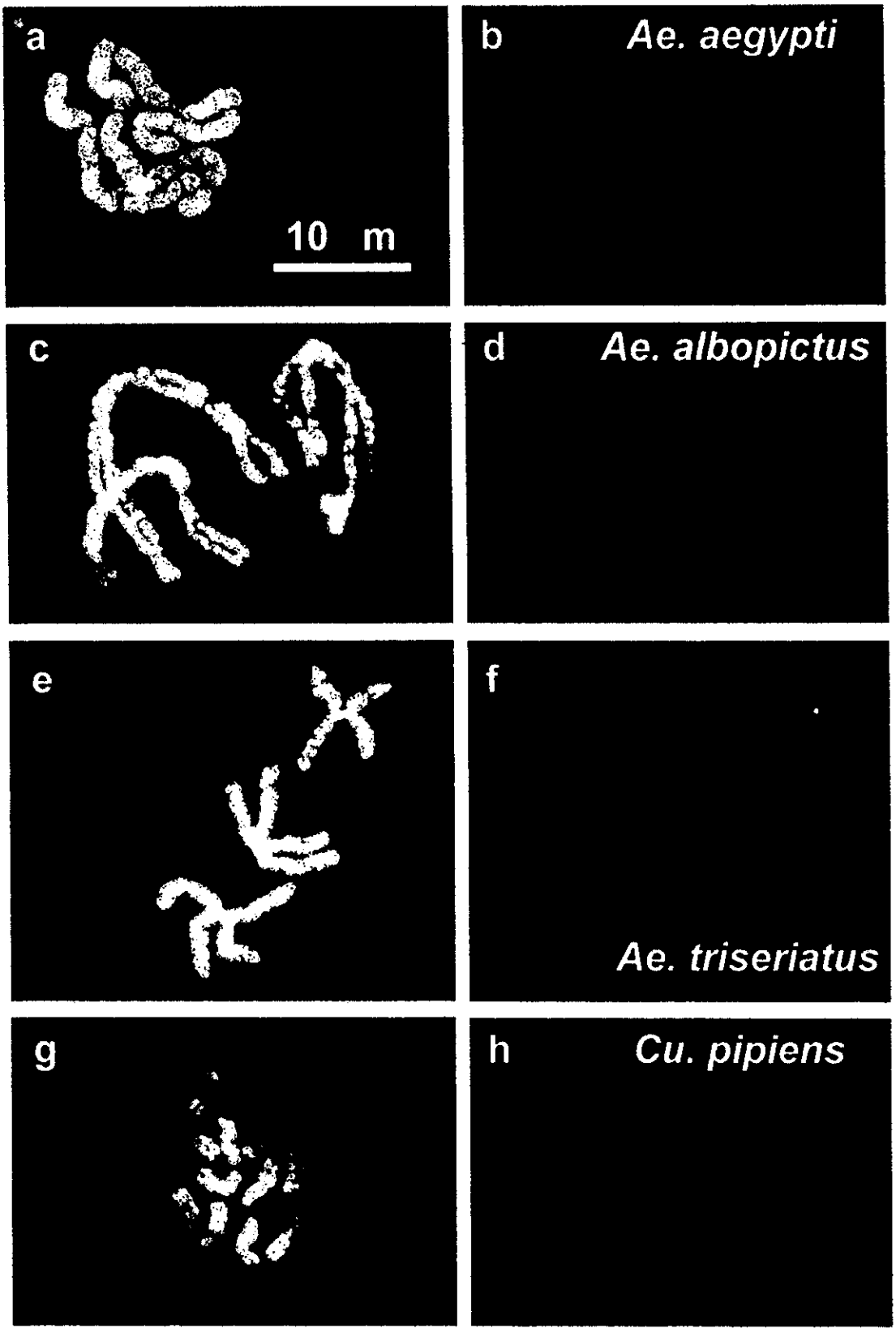

Cu. pipiens

7 - Comparison between the four mosquito species

When the plasmid p1887 was used as a FISH probe to pupal testes metaphase chromosome spreads from *Ae. aegypti, Ae. albopictus, Ae. triseriatus* and *Cx. pipiens,* the signals were observed as a broad bands with the signal midpoint located at ~ 70% fractional length from the p-terminus (FLpter) or smaller arm of chromosome 1 of *Ae. aegypti* (fig. 30 b). Also *Ae. albopictus* and *Cx. pipiens* exhibited one ribosomal locus per haploid genome at chromosome 1, located at 71.4 % FLpter from the p-terminus of chromosome 1 in *Ae. albopictus,* and located, at 70% FLpter from the p-terminus of chromosome 1 in *Cx. pipiens* (fig. 30 d and h). Similar result was obtained by ***Brown & Knudson (1997)*** who stated that when p1887 was used as a FISH probe to ATC-10 cell line metaphase chromosomes of *Ae. aegypti,* the signals were characterized as broad band with the signal midpoint located at 70.6% fractional length from the p-terminus or smaller arm of chromosome 1. The result also agrees with that of ***Ferguson et al (1996)*** who stated that the 18S ribosomal cistron probe (p1887) labels a broad region in the middle of chromosome 1 at the q-arm of *Ae. aegypti* metaphase chromosomes which prepared from ATC - 10 cell line.

Ae. triseriatus had signals on two different chromosomes indicating that two ribosomal loci per haploid genome were present (fig. 30 f). This result agrees with ***Kumer and Rai (1990)*** who demonstrated that only *Ae. triseriatus* had two ribosomal loci in the 20 mosquito species belonging to 8 genera of subfamilies Culicinae and Anopheline examined in radioactive *in situ* hybridization studies. The two ribosomal loci were present in chromosome 1 and chromosome 3. On the other hand, the ribosomal loci were located on one arm of chromosome 1 in *Ae. albopictus, Ae. aegypti, Cx. pipiens* and all other species of Aedes examined, except

Figure (30): **FISH Landmark probes to an *Ae aegypti, Ae. albopictus, Ae. triseriatus* and *Cx. pipiens* metaphase chromosome spreads. FISH of the tagging clone (p1887) is depicted in raw grayscale digital images and in merged, colourized images that include FISH hybridization using the p2392 probe. The grayscale images were collected using a narrow bandwidth filter specific for FITC (fig. a, c, e, g) and the p2392 image was collected through a narrow bandwidth filter specific for the rhodamine. The two digital images for chromosome 1 were colourized and merged in software to yield the composite final image (fig. b, d, f, h).**


a
10 m
b
Ae. aegypti
c
d
Ae. albopictus
e
f
Ae. triseriatus
g
h
Cu. pipiens

in *Ae. mediovittatus* and *Haemagogus equinus*, where the rDNA was proved to be located at chromosome 2, but in *Armigeres subalbatus* and *Tripteroides bambusa*, the rDNA was hybridized on chromosome 3. The conservation of the ribosomal RNA sequence in mosquitoes have also been confirmed by FISH ***(Marchi and Pili, 1994,)*** who demonstrated that the ribosomal RNA gene are localized on the heterochromatic arm of both sex chromosome in Anopheles genus examined. In Culicinae subfamily, the rRNA genes map was on the sex chromosomes (chromosome 1). In *Cx. pipiens*, the rRNA probe hybridized to the short arm of chromosome 1 in a region proximal to the centromere. Also ***Schafer and Schafer (1980)*** found that the rDNA hybridized on sex chromosomes of *D. simulans* strains.

When the chromosome 2 specific probe, p2405, was used as FISH probe to pupal testes metaphase chromosomes of *Ae. aegypti*, *Ae. albopictus*, *Ae. triseriatus* and *Cx. pipiens*, detectable signals were seen on all chromosomes of *Ae. aegypti*, with prominent signal on the q-arm of chromosome 2 located at ~96% FLpter, lighter signals were also seen subtelomeric on the p-terminus of chromosome 2, midway on the q-arm of chromosome 1, and subtelomeric on the p-arm of chromosome 3 (fig. 31 f). Similar results were reported previously by ***Brown & Knudson (1997)*** who used ATC-10 cell line metaphase chromosome of *Ae. aegypti* as a target genome, and demonstrated that when p2405 was used as a FISH probe, all chromosomes exhibited detectable signals.

When the plasmid p2056 was used as a FISH probe, to the four mosquito species, the FISH signals were located on chromosome 3 of *Ae aegypti* at ~ 85.7% FLpter (fig. 31d). Similar results were obtained by ***Brown & Knudson (1997),*** when they used plasmid p2056 as a FISH

Figure (31): **FISH landmark probes to *Ae. aegypti* metaphase chromosome spreads. FISH of the three tagging clones in raw grayscale digital images and in merged, colourized images that include FISH hybridization using the p2392 probe. The grayscale images were collected using a narrow bandwidth filter specific for FITC (fig. a, e, c) and the p2392 image was collected through a narrow bandwidth filter specific for the rhodamine. The two digital images for each chromosome were colourized and merged in software to yield the composite final image (fig. b, f, d).**

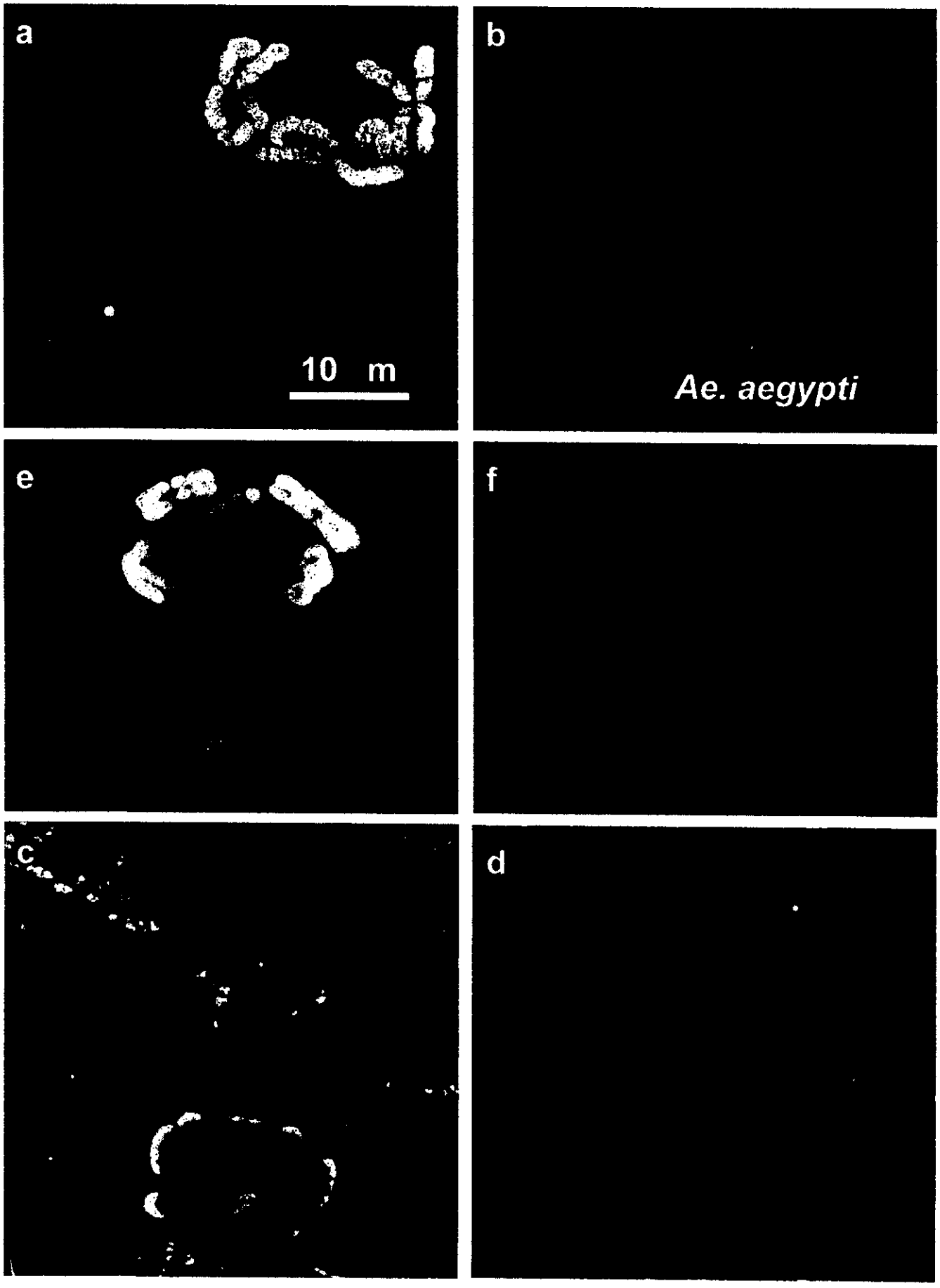
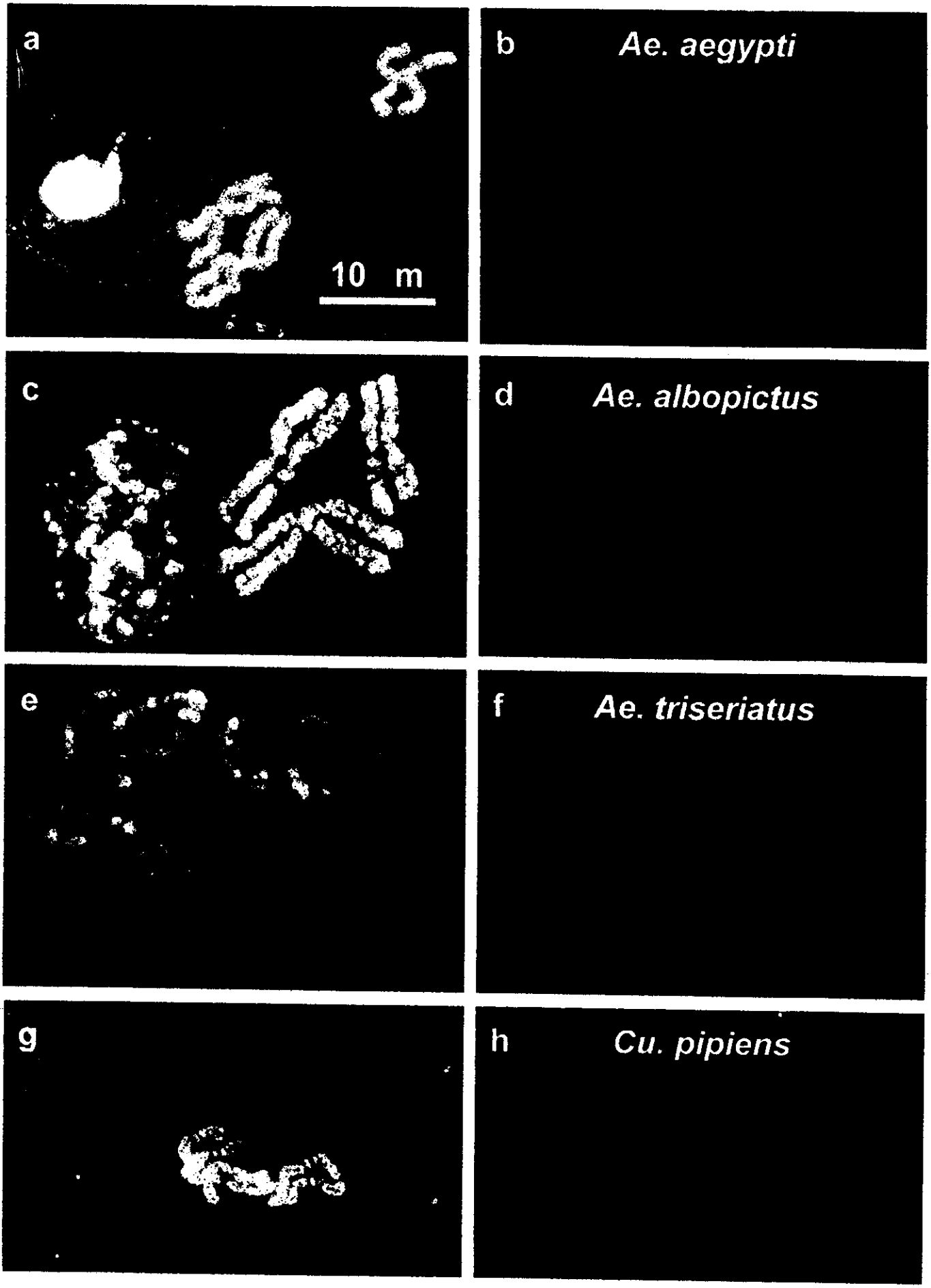
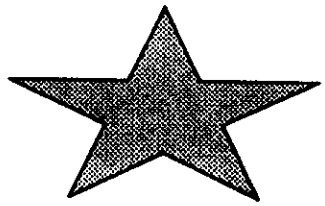
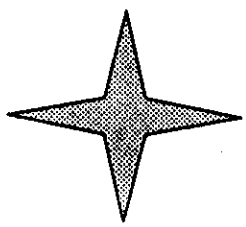

a
10 m
b
Ae. aegypti
e
f
c
d

Figure (32): **FISH landmark probes to *Ae. aegypti, Ae. albopictus, Ae. triseriatus* and *Cx. pipiens* metaphase chromosome spreads. FISH of the BAC K20.1A5 probe is depicted in raw grayscale digital images and in merged, colourized images that include FISH hybridization using the p2392 probe. The grayscale images were collected using a narrow bandwidth filter specific for FITC (fig. a, c, e, g) and the p2392 image was collected through a narrow bandwidth filter specific for the rhodamine. The two digital images for chromosome 1 were colourized and merged in software to yield the composite final image (fig. b, d, f, h).**


a
10 m
b
Ae. aegypti
c
d
Ae. albopictus
e
f
Ae. triseriatus
g
h
Cu. pipiens



Summary



(V) SUMMARY

The present work covered the following points

1) Obtaining metaphase chromosomes from pupal testes

In this work, metaphase chromosomes from *Ae. aegypti*, *Ae. albopictus*, *Ae. triseriatus* and *Cx. pipiens* were prepared following the standard procedures of ***French et al.,(1962)*** without pretreatment with colchicine. Good results were obtained from individuals, which were collected after 10-12 hours post pupation. Also good preparation of metaphase chromosomes were obtained when dissection was made in the morning and at spring and summer seasons as compared to the preparations made in the afternoon and at winter season.

2) Estimation and cutting DNA with restriction endonucleases

The concentration of the DNA sample was estimated by spectrophotomeric analysis at260 to280 nm using spectrophotometer. The best results are obtained when the ratio between 1.7 – 2.0.

3) Microscopy and digital imaging

By probe combination, pairs (or more) are mapped at the same time on the same slide. This is accomplished by labeling the probes with fluorochrome which emit light at different wavelengths. The *Ae. aegypti* chromosome- specific tags (plasmids p1887, p2405, and p2056) and a centric heterochromatin probe K20.1A5 were labeled with biotin-fluoroscein, the tagging plasmid p2392 which containing the chromosomal tags or landmarks was detected using anti-digoxigenin-rhodamine, and the chromosomes identification was generally done by DAPI counterstain. This allows three different probes to be hybridized and detected on one slide.

5) Characteristics of chromosome landmarks

Ae. aegypti pupal testes derived, ,metaphase chromosomes are used as DNA target in FISH hybridization with the individual components of the p2392 tagging reagent. The p1887 signal produced broad bands with the signal midpoint located at ~ 70% FLpter. When p2405 was used as a FISH probe, detectable signals were seen on all three chromosomes with the prominent signal on the q-terminus of chromosome 2 at ~ 96% FLpter, lighter signals were also seen subtelomeric on the p-terminus of chromosome 2, midway on the q-arm of chromosome 1, and subtelomeric on the p-arm of chromosome 3. The p2056 FISH signals were located on chromosome 3 at ~ 85.7% FLpter.

When these probes are used in hybridization with other aedine metaphase chromosomes, only the p1887 sequence produced a detectable FISH signal in *Ae. albopictus*, *Ae. triseriatus* or *Cx. pipiens* metaphase chromosomes. When chromosomes 2 and 3 specific probes, p2405 and p2056, were used in FISH to pupal testes metaphase chromosome spreads, signals were not seen, thus, the p2392 tagging reagent would not work as chromosome landmarks in these other aedine species . Although *Ae. albopictus* and *Cx. pipiens* exhibited one ribosomal cistron (p1887) locus per haploid genome, *Ae. triseriatus* had signals on two different chromosomes.

When the centeric heterochromatin probe (K20.1A5) is used in FISH to *Ae. aegypti* pupal testes, strong hybridization signals to chromosome 1 and 3 with a faint signal on chromosome 2 were seen. In contrast, FISH signals were not observed in pupal testes material derived from *Ae. albopictus*, *Ae. triseriatus* and *Cx. pipiens*