ELECTROPHORETIC METHOD FOR RECOGNITION OF SIBLING SPECIES OF ANOPHELINE MOSQUITOES A PRACTICAL APPROACH

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ABSTRACT

Laboratory hybridization tests, comparative study of the banding patterns of polytene chromosomes, and electrophroetic techniques for isozyme analysis are the methods that are usually employed for the recognition of sibling species of anopheline and some other mosquitoes. Hybridization tests under laboratory conditions are sufficient for measuring postmating reproductive isolation, but premating mechanisms are generally undetectable. Difficulty in obtaining good quality preparations tends to limit the usefulness of the chromosomal approach for the identification of sibling species. Generally, isozyme analysis is more accurate and reliable in uncovering cryptic variation, providing meaningful information on genetic relatedness, and in the identification of sibling species. We have presented a step-by-step analysis of electrophoretic data for the identification of both allopatric and sympatric sibling species.

RESUMEN

Pruebas de hibridación en el laboratorio, estudio comparativo de los patrones de las bandas de los cromosomas de polytene, y técnicas electroforéticas para el análisis de isoenzyma son los métodos que usualmente se emplean para reconocer especies hermanas de anopheline y algunos otros mosquitos. Pruebas de hibridación bajo condiciones de laboratorio son suficientes para medir la reproducción en aislamiento después del acoplamiento, pero mecanismos antes del acoplamiento no son generalmente detectables. Dificultad en obtener preparaciones de buena calidad tienden a limitar la utilidad del método usando los cromosomas para la identificación de especies hermanas. Generalmente el análisis de isoenzyma es más exacto y confiable en descubrir variaciones crípticas, en proveer información sobre la afinidad genética, y en la identificación de especies hermanas. Nosotros hemos presentado paso a paso un análisis de datos electroforéticos para la identificación de especies hermanas alopátricas y simpátricas.

Sibling species are genetically distinct, but these closely related types are quite often indistinguishable morphologically. Since speciation is a gradual process, sibling species are thought to represent an incipient level of evolutionary divergence in comparison to morphologically distinguishable species. The lack of clear morphological differences creates a serious problem for taxonomists and other scientists, especially control specialists who are attempting to exploit biological and genetic strategies for insect control. Before the development of electrophoretic methods that were suitable for analysis of isozymes (Hubby & Throckmorton 1965, 1968), sibling species were identified by laboratory hybridization tests and comparative study of karyotypes (especially the banding patterns of polytene chromosomes in species of Diptera). Both these approaches have serious limitations. For the most part, only postmating reproductive isolation mechanisms are detected in hybridization tests under laboratory conditions, because the abnormal situation of the laboratory can obscure premating isolation. For some species of anopheline mosquitoes, the polytene chromosomes are of good quality and

can be used for the purpose of correct identification of sibling forms, but there are problems in obtaining good preparations for some species, even to the extent that this technique can not be used on a routine basis. Such limitations do not exist in the electrophoretic analysis of gene-enzyme systems. A comprehensive discussion on the applications of hybridization, karyotypes, and electrophoretic studies for evolutionary and taxonomic inferences in the study of sibling species in mosquitoes can be found in review articles by Bullini & Coluzzi (1982) and Narang & Seawright (1988). Throughout the text, we have used three terms to designate a given sibling species in order to highlight the taxonomic tool used for its identification. A sibling species identified on the basis of diagnostic allozymes will be referred to as "allospecies", in contrast to "cytospecies" based on chromosomal identification, and "biological species" based on hybridization tests.

During the last few years, we have used electrophoretic methods for analysis of gene-enzyme systems to identify sibling species in natural populations of the *Anopheles quadrimaculatus* complex (Narang et al. 1988). A dichotomous electrophortic key was prepared (Narang et al. 1988) and can be used accurately for the identification of field-collected adults of the *A. quadrimaculatus* complex. In the early stages of this work, studies on ovarian nurse cell polytene chromosomes and laboratory hybridization tests were used to complement and correlate the identification of the members of the complex (Kaiser et al. 1988). In this paper, we present a step-by-step approach to the analysis of electrophoretic data for recognition of sibling species, both in sympatry and allopatry. Electrophoretic data of Narang et al. (1988) will be used to illustrate these steps.

Materials and Methods

The following text describes the materials and methods used for identification of sibling species of *A. quadrimaculatus* complex. This general approach should be useful for recognizing sibling species in other species complexes of mosquitoes.

Adult mosquitoes of *A. quadrimaculatus* were collected from about 30 localities throughout the distribution range of this species in the eastern half of the U.S. Wild-caught adults were stored frozen at –70° C until used for electrophoresis. Horizontal starch gel electrophoresis was performed according to Steiner & Joslyn with a few modifications (Narang et al. 1988). Gel trays measuring 13 X 20 cm were used. Individual mosquitoes were homogenized in 30ul of grinding buffer (10mM Tris, 1mM EDTA, 1mM 2-mercaptoethanol, pH 7.0) in a multiple sample grinding block. Homogenate of each adult was absorbed with three 3 X 10 mm wicks (Whatman 3 mm paper) and analyzed on three 10 mm thick starch gels (one wick from each mosquito per gel). Homogenates of thirty-two adults including equal numbers of mosquitoes from two localities and three samples of a standard reference strain were run on each gel as described by Narang et al (1988). After electrophoresis, each gel was cut into six 1.5 mm thick slices. These 18 slices from three gels were stained for different enzymes. Samples of 30-50 adults from each locality were analyzed for preliminary electrophoretic test for recognition of genetic substructuring. Electromorphs (enzyme bands) at thirty four presumptive loci of 16 enzyme systems were scored using bands of the reference strain as controls as described earlier (Narang et al 1988).

Statistical analysis of electrophoretic variability data was performed by using the computer program, Biosys 1 (Swofford & Selander 1981). The tests included: (1) chi-square test for conformance of observed electromorph frequencies to those expected under Hardy-Weinberg equilibrium; (2) Selander's (1970) coefficient for deficiency or excess of heterozygotes for each polymorphic locus; (3) genetic identity and genetic distance (Nei 1978); and (4) the diagnostic value of a locus (Ayala & Powell 1972).

Results and Discussion

The following text, divided into 5 steps, describes a sequential approach to the analysis of electrophoretic data for recognition of sibling species in natural populations of *A. quadrimacultus. We have used some data from previously reported studies on A. quadrimaculatus* (Narang et al. 1988) to serve as an example for this paper. Electrophoretic data on populations from four localities, Shell Mound, Levy Co. FL (LEV), Bear Bay Swamp, Dixie Co. FL (BBS), Noxubee, Noxubee Co. MS (NOX) and Panasoffkee, Sumter Co. FL (PAN), will be used for illustrations.

Step I. Recognition of genotypes with allelic clusters:

The first level of evidence for the occurrence of presumed sympatric sibling species (allospecies) can be obtained from visual observation of zymograms of polymorphic loci as shown in Figures 1-5. For example, in the LEV samples (lanes 5-8 in each figure), adults can be grouped into two classes. In the first (lanes 5 & 6 in each figure), there is a cluster of alleles such as: Had-1 (92), Had-3 (45), Got-2 (38), Pep-2 (110), Pgi (95), Idh-2 (162), Idh-1 (100). The second, (lanes 7 & 8 in each figure) lacks these alleles at the respective loci. These results give the erroneous indication of strong linkage among these loci. In addition, without formal statistical analysis, there are obvious deficiencies of heterozygotes for alleles at these loci. These results suggest that the two classes of adults are reproductively isolated from each other. Similarly, clustering of Idh-1 (86) and Idh-2 (162) electromorphs to certain individuals is indicative of sympatric sibling species in NOX. On the contrary, no allelic clustering is apparent in samples from PAN and BBS; therefore, presumably at this point it appears that pure populations were collected at those locations.

Step II. Chi-square test for Hardy-Weinberg equilibrium and Selander's D coefficient:

The chi-square test helps to define statistically the presence of sympatric sibling species. This test is based on the assumption that a population is in Hardy-Weinberg equilibrium, i.e., the observed frequencies of alleles can be used to calculate expected genotypic frequencies that are not significantly different from the observed genotypic frequencies. A significant chi-square value is indicative of genetic substructuring, i.e., sibling species. As shown in Table 1, there is a lack or significant deficiency of heterozygotes for alleles at certain loci in LEV (Had-1, Had-3, Got-2, Pep-2, Pgi, and Idh-2), and in NOX (Idh-1, Idh-2 and Had-1). Therefore, these results indicate that at both localities, there are sympatric allospecies. Furthermore, on the basis of the number and types of loci for which there a deficiency of heterozygotes, one of the species in LEV is different from the two species in NOX. The chi-square values for the BBS and PAN populations were not significant. Selander's D coefficient also indicates whether a population is in equilibrium, but this statistic also provides information on the proportion and direction (either positive or negative) of an unusual frequency of heterozygotes at each locus. For the data in Table 1, there was general agreement, as expected, between the values for Selander's D coefficient and chi-square. By Selander's D coefficient, there was no evidence for the occurrence of sympatric allospecies in the populations from BBS and PAN.

Step III. Significant differences in allelic frequencies:

Comparison of allelic frequencies at various loci will help recognize presumed allospecies in allopatry. As shown in Table 2, samples from PAN and BBS are either fixed

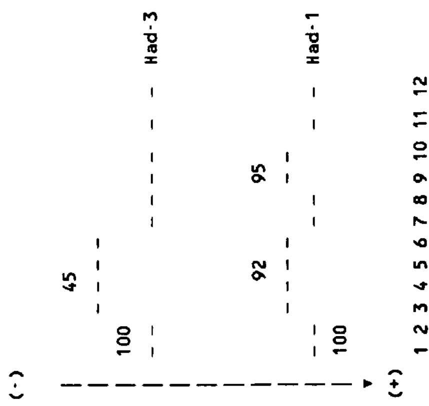


FIGURE 1
(-)
(+)
100
45
Had-3
92
95
Had-1
100
1 2 3 4 5 6 7 8 9 10 11 12

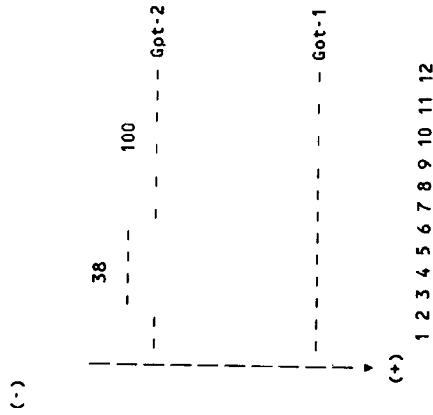


FIGURE 2
(-)
(+)
38
100
Gpt-2
Got-1
1 2 3 4 5 6 7 8 9 10 11 12

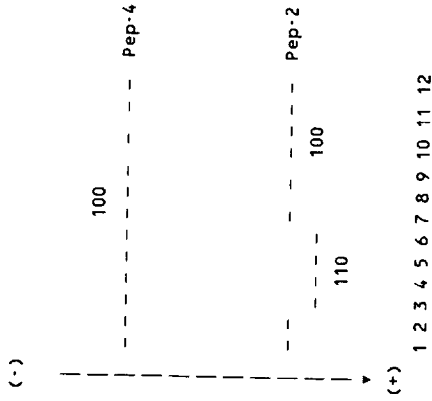


FIGURE 3
(-)
(+)
100
Pep-4
110
100
Pep-2
1 2 3 4 5 6 7 8 9 10 11 12

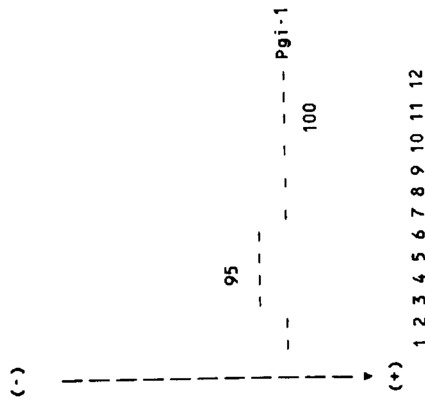


FIGURE 4
(-)
(+)
95
100
Pgi-1
1 2 3 4 5 6 7 8 9 10 11 12

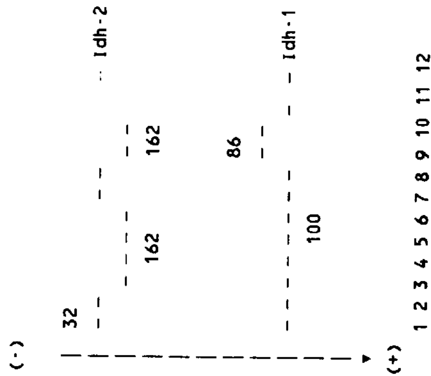


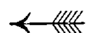
FIGURE 5
(-)
(+)
32
162
162
Idh-2
86
100
Idh-1
1 2 3 4 5 6 7 8 9 10 11 12

TABLE 1. COEFFICIENTS FOR HETEROZYGOTE DEFICIENCY AT CERTAIN LOCI IN SAMPLES OF *ANOPHELES QUADRIMACULATUS* FROM FOUR LOCALITIES (PAN, BBS, NOX AND LEV).

Locus	Locality	N	Observed heterozygotes	Exptected heterozygotes[1]	X^2	Selander's D
Had-1	PAN	50	6	6	0	0
	BBS	42	0	0	—	0
	NOX	52	7	26	13.88*	–0.73
	LEV	78	1	18	16.06*	–0.94
Had-3	PAN	50	6	7	0.14	–0.14
	BBS	50	3	3	0	0
	NOX	260	40	41	0.02	–0.02
	LEV	243	25	73	31.56*	–0.66
Got-2	PAN	48	18	21	0.43	–0.14
	BBS	50	5	5	0	0
	NOX	154	26	26	0	0
	LEV	171	28	49	9*	–0.43
Pep-2	PAN	41	6	6	0	0
	BBS	44	8	9	0.11	–0.11
	NOX	63	0	0	—	0
	LEV	136	37	58	7.60*	–0.36
Pgi-1	PAN	50	3	3	0	0
	BBS	50	5	6	0.17	0
	NOX	119	5	5	0	0
	LEV	241	42	75	14.52*	–0.44
Idh-1	PAN	50	12	14	0.28	–0.14
	BBS	50	5	6	0.17	–0.16
	NOX	323	37	120	57.41*	–0.69
	LEV	171	22	25	0.36	–0.12
Idh-2	PAN	47	24	27	0.33	–0.11
	BBS	42	1	1	0	0
	NOX	227	33	80	27.61*	–0.59
	LEV	143	28	32	0.5	–0.13

*Significant chi-square values ($p < 0.05$).
[1]Based on Hardy-Weinberg equilibrium.

for different alleles or show significant differences in allelic frequencies (most common allele in PAN:BBS shown in parenthesis) at loci : Had-1 (100:92), Had-3 (100:45), Got-2 (100:38), Pep-2 (100:110), Pgi-2 (100:95) and Idh-2 loci (132:162). It is also clear that alleles at these loci in BBS are similar to alleles in some of the adults of LEV, indicating



Figs. 1-5. Schematic representations of zymograms of adult samples from PAN, BBS, LEV and NOX. Individual adults were run on one gel and gel slices stained for various enzymes. The sample order is same in all zymograms. From left to right samples no. 1-2 (PAN), 3-4 (BBS), 5-8 (LEV), 9-12 (NOX). Notice that samples no. 3, 4, 5 and 6 have different bands for most loci (species C specific cluster of alleles). Sample 9, 10 (species B) differs from others at Had-1, Idh-1 and Idh-2.

TABLE 2. FREQUENCIES OF ELECTROMORPHS AT SEVEN LOCI IN 3 SIBLING SPECIES OF THE *ANOPHELES QUADRIMACULATUS* COMPLEX. DIAGNOSTIC ELECTROMORPHS (RELATIVE TO SPECIES A) ARE UNDERLINED

Locus	A (NOX)	A (LEV)	A (PAN)	B (NOX)	C (LEV)	C (BBS)
Had-1						
(N)	17	10	50	35	68	42
104	0	0	0.05	0	0	0
100	1.00	1.00	0.93	0.12	0	0
95	0	0	0	0.88	0.02	0
92	0	0	0.02	0	0.98	1.00
Had-3						
(N)	80	39	50	180	204	50
156	0.07	0.05	0.09	0.05	0	0
100	0.91	0.87	0.89	0.93	0.03	0.01
45	0.02	0.08	0.02	0.02	0.96	0.99
25	0	0	0	0	0.01	0
Got-2						
(N)	57	28	48	97	143	50
170	0.02	0	0.03	0.01	0	0
138	0	0.04	0	0	0	0
115	0.02	0	0	0.02	0	0
100	0.86	0.67	0.69	0.92	0.06	0.05
80	0	0	0	0.01	0	0
38	0.10	0.29	0.28	0.04	0.94	0.95
Pep-2						
(N)	29	26	41	34	110	44
115	0	0.06	0	0	0.04	0.03
110	0	0.17	0.07	0	0.85	0.88
100	1.00	0.77	0.92	1.00	0.11	0.09
89	0	0	0.01	0	0	0
Pgi-1						
(N)	42	41	50	77	206	50
105	0.02	0	0	0	0	0
103	0	0	0	0	0	0
100	0.95	0.79	0.97	0.98	0.08	0.06
95	0	0.21	0.02	0.02	0.91	0.94
80	0.03	0	0.01	0	0.01	0
Idh-1						
(N)	100	23	50	223	148	50
110	0.03	0.02	0.02	0	0.01	0.05
100	0.75	0.88	0.83	0	0.97	0.94
86	0.17	0.10	0.15	1.00	0.02	0.01
83	0.05	0	0	0	0	0
Idh-2						
(N)	74	19	47	153	124	42
209	0.02	0.02	0.05	0	0.02	0.01
181	0.02	0	0	0	0	0
162	0.19	0.54	0.32	1.00	0.97	0.99
132	0.66	0.42	0.56	0	0.01	0
100	0.11	0.02	0.07	0	0	0

that the species in BBS is most likely the same that occurs in sympatry in LEV. Table 3 shows the diagnostic values (Ayala & Powell 1972) for these loci, which can be used for taxonomic identification of sibling species.

Step IV. Partitioning of genotypic frequency data of samples containing allospecies in sympatry into data corresponding to respective presumed allospecies (using information from step I and II). Hardy-Weinberg equilibrium test on partitioned data set:

The results in Table 1 and 2 (steps I and II) indicated that the NOX population was composed of two presumed allospecies which differ at the loci for Idh-1, Idh-2, and Had-1. Adults with the allelic cluster at these three loci, Idh-1 (86), Idh-2 (162) and Had-1 (95) can be tentatively assigned to one allospecies (species B). Adults of NOX lacking this cluster can be assigned to a distinct allospecies A (see alleles of A at three loci in Table 3). At this point the chi-square test for conformance of each data subset (of allospecies A and B) to Hardy-Weinberg equilibrium should be done. If the partitioned genotypic frequency data of each subset is in equilibrium (i.e.. the chi-square value is not significant), this can be considered adequate to identify and characterize sibling species A and B. When partitioned, the LEV population was found to be composed of two presumed species, (1) allospecies A similar in genotypes at Idh-1, Idh-2 and Had-1 loci to that of A in NOX and allospecies C characterized by a cluster of alleles such as: Had-1 (92), Had-3 (45), Got-2 (38), Pep-2 (110) Pgi (95) and a characteristic two-locus genotype, Idh-2:Idh-1 (162/100).

Step V. Genetic Identity (I) and genetic distance (D).

The coefficients of genetic relationships (Nei 1978). i.e. genetic identity (I) and genetic distance (D, –log I), can be very useful indicators for recognition of allopatric sibling species. As shown in Table 4 the intra-specific I is 0.95 to 0.97 in species A, 0.98 in species B and 0.98 in species C. The interspecific I ranged from 0.80 to 0.85 between A and B, 0.54 to 0.64 between A and C, and 0.54 between B and C. Allopatric populations of PAN and BBS, which were both in Hardy-Weinberg equilibrium for genotypes at all loci showed I value of 0.61 (N in each locality was 50) confirming the two populations were composed of two distinct allopatric allospecies.

TABLE 3. DIAGNOSTIC VALUES[1,2] OF SOME ENZYME LOCI, WHICH CAN BE USED TO IDENTIFY SYMPATRIC SIBLING SPECIES OF *ANOPHELES QUADRIMACULATUS* COMPLEX.

Locus	Species		
	A:B	A:C	B:C
Idh-1	98.82	—	99.97
Idh-2	95.03*	95.01*	—
Had-1	97.66*	99.98	99.97
Had-3	—	99.92	99.92
Got-2	—	99.19	99.75
Pep-2	—	99.07	99.36
Pgi-1	—	99.99	99.65

[1]Diagnostic values were calculated by the method of Ayala and Powell (1972). Frequencies of electromorphs of species A from pooled samples of PAN and NOX. B was from NOX and C from Levy Co. and BBS were used.

[2]A locus is considered diagnostic, if the probability of correct diagnosis of an individual to a given taxon is 99% or higher. Loci marked with asterisk (*) can be used only as two-loci diagnostics.

TABLE 4. ESTIMATES OF NEI'S (1978) UNBIASED GENETIC IDENTITY (ABOVE DIAGONAL) AND GENETIC DISTANCE (BELOW DIAGONAL) FOR NATURAL POPULATIONS OF SIBLING SPECIES OF *ANOPHELES QUADRIMACULATUS* COMPLEX.

Populations	1	2	3	4	5	6
1. PAN species A	—	0.973	0.948	0.825	0.578	0.539
2. NOX species A	0.027	—	0.924	0.818	0.541	0.563
3. LEV species A	0.053	0.079	—	0.799	0.642	0.610
4. NOX species B	0.193	0.201	0.442	—	0.544	0.587
5. LEV species C	0.548	0.614	0.442	0.610	—	0.964
6. BBS species C	0.618	0.574	0.494	0.533	0.037	—

An alternative approach to identify sympatric sibling species directly from step I consists of examining chromosomal banding patterns in adults showing allelic clusters. Ovaries from individual wild gravid females are removed for study of ovarian nurse cell polytene chromosome banding pattern and the remaining part of each mosquito is used for electrophoretic analysis of selected gene.enzyme systems (steps I). This simultaneous chromosome-electrophoretic analysis is helpful to correlate the identity of allospecies with that of cytospecies. Our results (Narang et al. 1988, Kaiser et al. 1988) showed that each of the three allospecies, A, B and C had indeed diagnostic chromosomal complements. Chromosomes of gravid females of allospecies A from NOX and LEV were similar to those of the pure population of allospecies A from PAN. Similarly, females of allospecies C from LEV were similar to those of pure population of species from BBS. In order to designate these species as true biological species (more so for species in allopatry), hybrid breakdown among these taxa must be established by hybridization crosses in laboratory condition. Crosses among A, B and C showed hybrid sterility to different degrees (Kaiser 1988).

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CYTOTAXONOMY AS A TOOL FOR IDENTIFICATION OF SIBLINGS OF THE *ANOPHELES QUADRIMACULATUS* COMPLEX

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ABSTRACT

Wild populations of *Anopheles quadrimaculatus* were collected in the southeastern United States for cytogenetic studies. Two approaches, i.e., polytene chromosome comparisons and hybridization studies were used. Observations on the ovarian nurse cell polytene chromosomes indicated two distinct X chromosomes, one with a standard banding arrangement and the other with a fixed inversion. Three polymorphic inversions on the right arm of chromosome 3 were associated with the normal X (cytotype A). Individuals with the inverted X contained a polymorphic and a fixed inversion on both 3R and the left arm of chromosome 2 (cytotype B). The two types shared a small inversion on 2L and a complex arrangement on the left arm of chromosome 3 that included two distinct homokarotypes. The ovarian polytene chromosomes of the F_1 hybrids (A x B and reciprocal) demonstrated complete asynapsis in the X chromosome and 3L, and extensive asynapsis in the other arms. Differential mortality was expressed in F_1 hybrids depending on the cross; some crosses produced only females, some produced only males, and others resulted in normal sex ratios. The reproductive organs of F_1 adults were always abnormal, ranging from atrophied to complete absence. These aberrations were expressed in backcrosses where very high embryonic and larval mortality were observed. These studies proved that the chromosomally differentiated types A and B are distinct sibling species of the *Anopheles quadrimaculatus* complex.

RESUMEN

Se colectaron poblaciones salvajes de *Anopheles quadrimaculatus* en el sudeste de los Estados Unidos para estudios citogenéticos. Se usaron dos aproches, comparaciones de cromosomas de politeno, y estudios de hibridación. Observaciones de los cromosomas de politeno de la célula nodriza del ovario indicó dos cromosomas X distintos, uno con unas bandas patrones, y otro con una inversión fija. Tres inversiones polimórficas en el brazo derecho del cromosoma 3 fueron asociadas con la X normal (citotipo A). Individuos con la X invertida contenían una inversión polimórfica y fija en ambos 3R y en el brazo