LOW COURTSHIP SONG VARIATION IN SOUTH AND SOUTHEASTERN BRAZILIAN POPULATIONS OF *Drosophila meridionalis* (DIPTERA, DROSOPHILIDAE)

COSTA, C. T. A., KUHN, G. C. S. and SENE, F. M.

Departamento de Genética, Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo, CEP 14049-900, Ribeirão Preto, SP, Brazil

Correspondence to: Fábio M. Sene, Departamento de Genética, Bloco A, Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo, Av. Bandeirantes, 3900, CEP 14049-900, Ribeirão Preto, SP, Brazil

Received January 11, 1999 – Accepted Juny 15, 1999 – Distributed February 28, 2000

(With 3 figures)

ABSTRACT

Drosophila meridionalis is a cactus-breeding species with a wide distribution in South America. Most populations of this species are geographically isolated, what provides a promising scenario for studying evolution. Former studies of this species revealed a remarkable karyotypic variation among its populations. Up to six distinct metaphase chromosomes were described, showing that this species is polymorphic at least at the chromosomal level. In order to elucidate the taxonomic status of populations showing different metaphase chromosomes, we analyzed the courtship song of five populations of *D. meridionalis* in South and Southeastern Brazil. In addition, we analyzed the metaphase chromossomes of each population. Our results show that, despite the two karyotype observed, most courtship song parameters did not vary among the populations. Altogether, our results suggest that *D. meridionalis* from South and Southeastern Brazil represents one species with an inter-population chromosomal variability.

Key words: *Drosophila meridionalis*, courtship song, karyotype differentiation.

RESUMO

Baixa variação da corte sonora em populações de *D. meridionalis* (Diptera, Drosophilidae) do Sul e Sudeste do Brasil

Drosophila meridionalis é uma espécie cactófila amplamente distribuída na América do Sul. A maioria de suas populações está geograficamente isolada, o que representa um ótimo cenário para estudos em evolução. Estudos anteriores mostraram uma variação cariotípica notável entre as populações dessa espécie. Foram descritos seis tipos distintos de cromossomos metafásicos, mostrando que essa espécie é politípica em relação a esse marcador. Com o objetivo de elucidar o status taxonômico de populações que mostram diferentes cromossomos metafásicos, analisamos a corte sonora de cinco populações de *D. meridionalis* no Sul e Sudeste do Brasil. Adicionalmente, analisamos os cromossomos metafásicos de cada população. Nossos resultados mostraram que, apesar dos dois cariótipos observados, a maioria dos parâmetros da corte sonora não variou entre as populações. Nossos resultados sugerem que populações de *D. meridionalis* do Sul e Sudeste do Brasil representam apenas uma espécie com uma variabilidade cromossômica interpopulacional.

Palavras-chave: *Drosophila meridionalis*, corte sonora, diferenciação cariotípica.

INTRODUCTION

Drosophila meridionalis is a cactus-breeding species endemic to South America. This species has a wide distribution and has been found in low frequency in most of the collection sites (Vilela *et al*., 1983). In spite of its wide range, it has never been collected in forests and cerrados. The breeding sites identified are the cactus species *Opuntia ficus-indica*, *Opuntia vulgaris*, *Cereus pernambucensis*, *Cereus peruvianus*, *Cereus hildmannianus*, *Pilosocereus machrisii* (Pereira *et al*., 1983). Former studies on this species revealed a remarkable karyotypic differentiation among populations from different areas which cover the geographical range of this species (Baimai *et al*., 1983). Based on this character, *D. meridionalis* has 6 distinct metaphase karyotypes mainly due to variation on the amount of heterocromatin present in the 6th chromosome (or microchromosome). In each case, the re-patterning of the microchromosomes seem to have occurred independently, which suggest independent allopatric origin for the various metaphase karyotypes present in this species (Baimai *et al*., 1983). In the genus *Drosophila*, allopatric speciation seems to be the most frequent mode of speciation. The main question in this context is to investigate whether these 6 different types of karyotype found in *D. meridionalis* represent 6 different populations or different species. Studies on other species of the *Drosophila repleta* group showed that small differences on the micro-chromosomes combined with other genetic, ecological, morphological and behavior data could lead to a good picture of the taxonomic status of different populations.

The analysis of the courtship behavior has proved to be a powerful marker for discriminating related species of *Drosophila*, especially because males of most species emit elaborate and specific sounds when they vibrate their wings during sexual courtship (Ewing, 1983; Manfrin *et al*., 1997). Comparative studies of the courtship song have been carried out in many species groups. The songs have been shown to vary significantly among species of the *affinis* group (Chang & Miller, 1978), the *melanogaster* group (Cowling & Burnet, 1981), the *virilis* group (Hoikkala *et al*., 1982), the *repleta* group (Ewing & Miyan, 1986), the *auraria* group (Tomaru & Oguma, 1994) and the *willistoni* group (Ritchie & Gleason, 1995). Reproductive isolation among closely related species of insects often seems to be primarily determined by mating signals, so that speciation will be influenced by changes in signals (Ritchie & Kyriacou, 1994). If male sound characteristics are indistinguishable among species, sexual isolation which is caused by female choice would be ineffective (von Schilder & Dow, 1977). The courtship song in *Drosophila* usually consists of a series of low-frequency pulses produced by short wing vibrations. The species differences are characterized by one or more sound parameters such as interpulse interval (IPI), intrapulse interval (PI), intrapulse frequency or fundamental (FFT), number of pulses per burst and burst length (Hoikkala & Lumme, 1987; Wheller *et al*., 1988).

In this paper, we report our results on the metaphase chromosome and courtship behavior from 5 laboratory stocks of *D. meridionalis* which represent 5 localities in South and Southern Brazil, a previously poorly known area.

MATERIAL AND METHODS

Samples of *D. meridionalis* were collected with banana-orange baits from five different localities in South and Southeastern Brazil. The collection sites are indicated in Fig. 1.

All localities studied here have in common the presence of one or more cactus species, which provide the feeding, and breeding substrate for *D. meridionalis*. Otherwise, they belong to quite different habitats. The locality H80 (Altinópolis, SP) is a rocky field on the top of an isolated hill and have been considered as a relic area originated from climatic changes during the Quaternary period. A single cactus species is present in this place, *Pilosocereus machrisii* (Zappi & Taylor, 1993). At the locality H45 (Guaritas, RS) the dominant cactus is *Cereus hildmannianus* which is oft associated to relic vegetation originated at the Chaco. However, *Opuntia monacantha* is also occasionally present. The locality H49 (Bertioga, SP) is a narrow stripe of rocky shore separating the Forest (“Mata Atlântica”) from the Ocean where a single non-epifitic cactus, *Cereus pernambucensis*, could be seen.

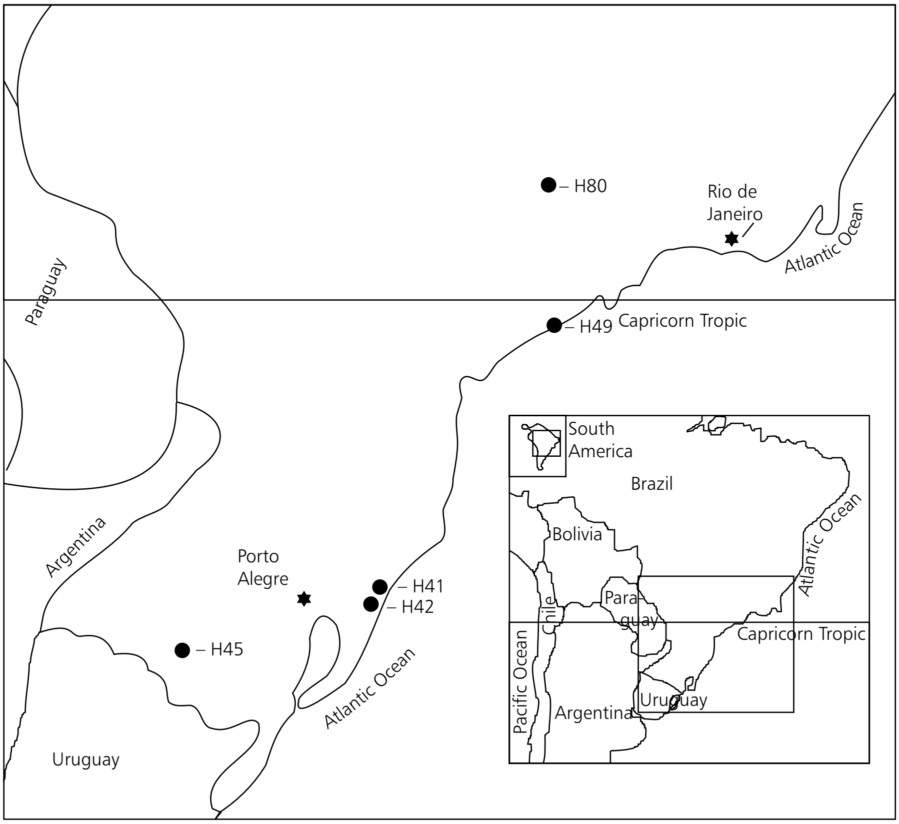

Fig. 1 — Geographical map of Central-Southern Brazil showing the positions of the 5 localities where *D. meridionalis* was collected. H80: Altinópolis, SP; H49: Bertioga, SP; H41: Arroio Teixeira, RS; H42: Capão da Canoa, RS; H45: Guaritas, RS.

The localities H41 (Arroio Teixeira, RS) and H42 (Capão da Canoa, RS) are sand dunes on the Atlantic Litoral of Rio Grande do Sul. Here *C. hildmannianus* as well as *Opuntia monacantha* are present.

The five laboratory stocks analyzed (H80E4-Altinópolis, SP, H49C24-Bertioga, SP, H42M1 Capão da Canoa, RS, H41F1-Arroio Teixeira, RS and H45F1-Guaritas, RS) were derived from single females collected in the wild.

Courtship song

Courtship songs were examined from 5 virgin males from each isofemale line. The analyses of each male were performed by using 10 measures from the 7 different parameters showed in Table 1.

Newly emerged flies were separated according to sex. For sound recording, a single pair of 7 day old flies of the same strain was introduced into an acrylic recording chamber as described by Sene & Manfrin (1998). This chamber containing one couple of flies was placed on the diaphragm of an ultra-sensitive microphone (Bennet-Clark, 1984) set up by Dr. Alan T. Ohta, Department of Entomology, University of Hawaii, HI. The song was recorded inside an insulating box in a room kept at 25^{o}C.

The acoustic signals produced by wing vibrating males were monitored with an earphone and recorded on a cassette tape. The pairs of flies were observed for a maximum period of 10 minutes or until copulation.

The courtship sounds that resulted in copulation were computed at 5 kHz using a digital analog converter. The digitized data and the PI and IPI values were obtained using the AqDADOS 4 software (Lynx – 1993).

TABLE 1

Song characters used as parameters to describe the *D. meridionalis* courtship song.

IPI	Interpulse interval (ms)
PI	Total length of song pulse (ms)
PI-A	Length of song pulse A (ms)
PI-B	Length of song pulse B (ms)
FFT/PI	Intrapulse frequency of PI (Hz)
FFT/PI-A	Intrapulse frequency of PI-A (Hz)
FFT/PI-B	Intrapulse frequency of PI-B (Hz)

The fundamental frequency was discriminated in the frequency spectrum with the aid of the MATLAB software (MathWorks, Inc., 1993).

The values for each parameter were tested separately for analysis of variance (ONE-WAY ANOVA) with the level of significance set (a) at 0.05. The principal components analyses (PCA) were also applied in order to reduce the 7 former parameters (Table 1) in two principal components (PC1 and PC2) which were responsible for most observed variation. To accomplish this, the statistic program SYSTAT 5.01 for WINDOWS (Copyright 1990-1992 by SYSTAT, Inc.) was used, where all the original variables were turned into a logarithm scale before the PCA. An elipses graphic was obtained including 80% of the values from PC1 and PC2 for each population of *D. meridionalis*.

Metaphase Chromosomes

Metaphase plates were prepared from larval brain ganglia using the conventional dry-slide method, as described in Baimai (1977). The dried slides were stained with 2% Giemsa in phosphate buffer solution. Seven larvae were analyzed per stock.

RESULTS

Courtship song

The oscillogram of the *D. meridionalis* courtship sound is illustrated in the Fig. 2a and was visually similar in all analyzed populations. Fig. 2b shows how the measurements of IPI, PI, PI-A and PI-B were obtained. This courtship song pattern of *D. meridionalis* (here for the first time described) is composed of "doublet pulses" which was also observed by Ewing & Miyan (1986) in others *Drosophila* species like *D. canapalpa*, *D. paranaensis*, *D. buzzatii* and *D. meridiana*, being the last species a sister species of *D. meridionalis*. These authors also showed that the intervals between the doublets tend to be multiples of the intervals between individual pulses of doublets, which also seems to be the case for the *D. meridionalis* courtship song (Fig. 2b).

The statistical analyses showed that the songs parameters PI-A ($F_{4,20} = 0.72$, $p > 0.25$), PI-B ($F_{4,20} = 1.61$, $p > 0.10$), FFT/PI ($F_{4,20} = 2.48$, $p > 0.05$) and FFT/PI-A ($F_{4,20} = 0.95$, $p > 0.25$) were not significantly different among the populations. However, IPI ($F_{4,20} = 7.42$, $p < 0.001$), PI ($F_{4,20} = 4.52$, $p < 0.01$) and FFT/PI-B ($F_{4,20} = 3.09$, $p < 0.05$) varied significantly among the five populations (Table 2).

Table 3 shows how these parameters vary among the populations in all combinations of two populations. The populations H42 and H49 differ from each other in relation to all the three parameters: IPI ($F_{1,8} = 10.55$, $p < 0.05$), PI ($F_{1,8} = 6.52$, $p < 0.05$) and FFT/PI-B ($F_{1,8} = 10.17$, $p < 0.05$). H49 and H80 show significant differences concerning the IPI ($F_{1,8} = 5.45$, $p < 0.05$) and FFT/PI-B ($F_{1,8} = 7.95$, $p < 0.05$). The combinations between H42 and H45, H42 and H80, H41 and H49 and H49 and H45 showed significant variation in just one parameter. The other populations did not show significant differences from each other.

The principal components analyses (PCA) using all 7 parameters plotted the populations on the basis of their song characteristics. The plot of the first two principal components PC1 and PC2 (Fig. 3) showed that there was a considerable overlap of the courtship sound among the five analyzed populations of the *D. meridionalis* species.

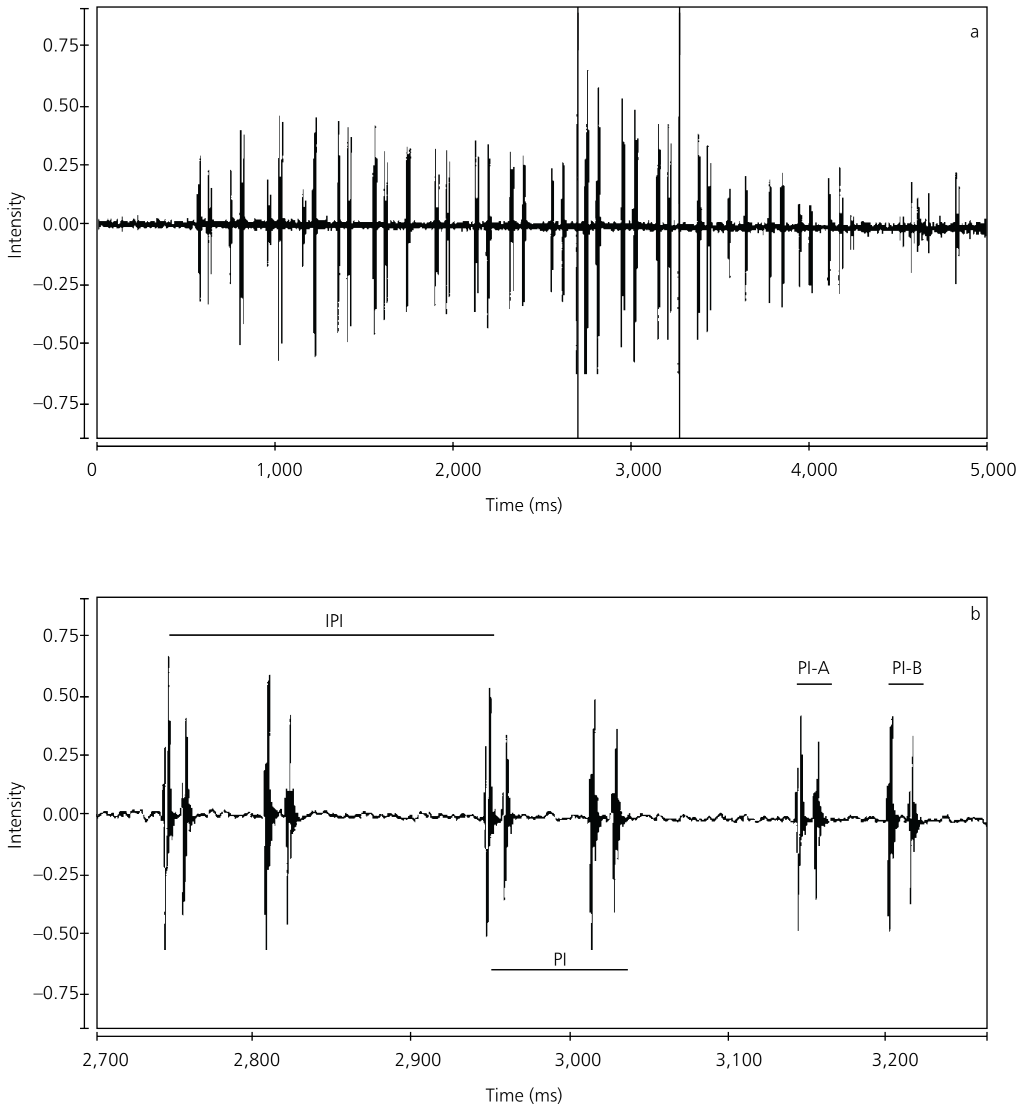

Fig. 2 — (a) Oscillogram of the courtship song of *D. meridionalis*; (b) Oscillogram showing how the measurements of IPI, PI, PI-A and PI-B were obtained from *D. meridionalis* courtship song. The Y-axis has arbitrary intensity units.

Metaphase chromosomes

Two different karyotypes were observed in the 5 populations analyzed. The first one, (type IV in Baimai *et al*., 1983) was found in 4 localities (H41, H42, H49 and H80) and consists of 1 pair of metacentric, 2 pairs of acrocentic, 1 pair of medium-sized dot (microchromosome) submetacentric and 1 pair of acrocentric sex chromosomes. The other one (type V in Baimai *et al*., 1983) was found in the locality H45 and differs from the first one by having an acrocentric microchromosome.

DISCUSSION

The analyses of the metaphase chromosomes from 5 stocks of *D. meridionalis* representing 5 distinct localities splits these populations in two groups: Four populations, (H41, H42, H49 and H80) showed the type IV karyotype while the H45 population showed the karyotype named type V. These karyotypes have been already described by Baimai *et al*. (1983) and they differ from each other by the amount of heterocromatin present on the microchromosomes.

TABLE 2

Summary of the data obtained from the statiscal analyses for the five populations of *D. meridionalis* species (5 individuals from each population and 10 measures from each individual). F values and P produced by ONE-WAY ANOVA are also indicated.

Songs characters	H80-Altinópolis, SP				H49-Bertioga, SP				H41-ArroioTeixeira, RS				H42-Capão da Canoa, RS				H45-Guaritas, RS				$F_{4,20}$	P
	Mean	SD	Min	Max	Mean	SD	Min	Max	Mean	SD	Min	Max	Mean	SD	Min	Max	Mean	SD	Min	Max		
IPI (ms)	192.30	16.06	168.52	229.72	169.80	12.61	145.70	196.06	178.64	18.24	139.89	231.24	197.46	13.61	168.58	254.92	200.84	19.84	167.44	257.60	7.42	< 0.001
PI (ms)	76.42	6.14	64.15	87.19	65.07	4.38	56.50	73.05	69.30	6.07	58.63	84.73	77.61	6.23	68.69	91.41	79.34	9.19	65.72	103.97	4.52	< 0.01
PI-A (ms)	19.41	2.07	14.95	26.01	18.14	1.68	15.81	22.44	17.88	2.10	13.51	23.70	17.97	1.47	15.14	21.38	18.81	2.11	15.02	23.89	0.72	> 0.25
PI-B (ms)	21.53	1.61	19.11	25.71	19.30	1.64	17.37	22.99	20.52	1.66	17.75	24.03	19.96	1.35	17.10	22.77	21.24	2.25	18.02	25.93	1.61	> 0.10
FFT/PI (Hz)	433.21	48.49	293.00	517.60	503.91	44.81	400.40	595.70	461.52	55.08	322.30	585.90	473.26	38.22	361.30	566.40	454.30	68.83	302.70	556.60	2.48	> 0.05
FFT/PI-A (Hz)	419.53	36.02	351.60	507.80	471.87	62.56	312.50	585.90	453.92	68.74	312.50	546.90	453.93	54.04	351.60	585.90	448.45	78.63	234.40	625.00	0.95	> 0.25
FFT/PI-B (Hz)	436.72	46.49	312.50	546.90	508.60	42.12	429.70	625.00	490.65	45.42	429.70	585.90	471.14	31.98	390.60	546.90	478.13	62.43	312.50	585.90	3.09	< 0.05

TABLE 3

Statistical comparisons of IPI, PI and FFT/PI-B in all combinations of two populations.

Populations	IPI (ms)	PI (ms)	FFT/PI-B (Hz)
H80 - H49	$F_{1,8} = 5.45$, $p < 0.05$	ns	$F_{1,8} = 7.95$, $p < 0.05$
H80 - H41	Ns	ns	ns
H80 - H42	Ns	ns	$F_{1,8} = 5.61$, $p < 0.05$
H80 - H45	Ns	ns	ns
H49 - H41	Ns	$F_{1,8} = 6.83$, $p < 0.05$	ns
H49 - H42	$F_{1,8} = 10.55$, $p < 0.05$	$F_{1,8} = 6.52$, $p < 0.05$	$F_{1,8} = 10.17$, $p < 0.05$
H49 - H45	$F_{1,8} = 8.06$, $p < 0.05$	ns	ns
H41 - H42	Ns	ns	ns
H41 - H45	Ns	ns	ns
H42 - H45	$F_{1,8} = 7.35$, $p < 0.05$	ns	ns

ns: not significantly different among the populations ($F_{1,8} < 5.32$, $p > 0.05$), H80: Altinópolis, SP; H49: Bertioga, SP; H41: Arroio Teixeira, RS; H42: Capão da Canoa, RS; and H45: Guaritas, RS.

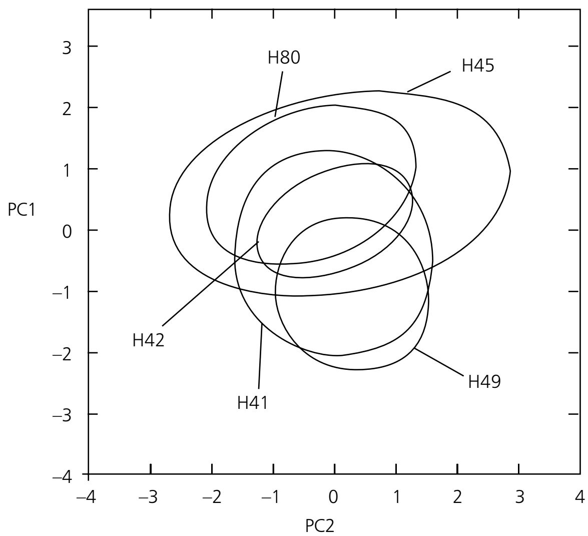

Fig. 3 — Scatterplot of the principal components (PC1), which explain 35% of the variation among populations against PC2, which explain 28% of the variation. The ellipses enclose 80% of the total data.

Former studies on type IV karyotype were restricted to just 2 localities, Serra do Cipó, MG and Itu, SP. Here, we expanded its geographical distribution eastwards, including the Atlantic Coast. This distribution pattern is particular curious, since the H80 and H49 populations are ecologically separated by the "Serra do Mar", a mountain range that runs parallel to the Atlantic Coast from Northeast to South Brazil. The predominant vegetation in this formation is called "Mata Atlântica". It is generally postulated that the present geographical distribution of the *D. meridionalis* populations is due to paleoclimatic changes which occurred during the Quaternary period (Sene *et al.*, 1982). Since the beginning of the Quaternay period, for 1,6 mya ago, there were at least 4 recurrent cycles of cold-dry and hot-wet weather, which caused expansions and contractions, respectively, of the xerophitic distribution. Consequently, given that the cactacea provide the feeding and breeding substrate for *D. meridionalis*, it seems probable that these flies followed the expansion and contraction of the *cactaceae* geographical distribution. The available data suggests that the last cold-dry weather occurred between 18,000 to 13,000 years ago. At this time, the "Mata Atlântica" was reduced to small islands, and the open formations showed a large expansion along the Atlantic Coast, providing a possible link between the H80 and H49 localities, what may explain the fact that H80 and H49 share the same karyotype. The type V karyotype was previously restricted to two localities in the Argentinean Chaco (La Vina and Puerto Tirol). We have expanded its geographical distribution towards South Brazil.

The analyses of variances (ANOVA) of all 7 song courtship parameters studied showed, in general, a very low courtship song variability among the five stocks analyzed. The interpulse interval (IPI) showed the highest variability ($p < 0.001$). The interpulse interval of courtship song is believed to be the main character responsible for the mating success and reproductive isolation (Ritchie *et al.*, 1994; Tomaru & Oguma, 1994; Ritchie & Gleason, 1995). If variation regarding this parameter is present, it may represent the beginning of a process of interpopulation differentiation that could eventually result in speciation. Concerning this parameter, the H49 population is different from the other populations, apart from the H41 population. Moreover, the H49 population shows significant differences between the populations H41 and H42 concerning the PI and between the populations H41 and H80 concerning the FFT/PI-B (Table 3). We do not know at this moment what effect these variations has although the data suggests that at least the H49 population might be undergoing a process of courtship song differentiation.

It is also interesting to mention that the *D. meridionalis* courtship song described in this paper, resemble the courtship of *D. meridiana* (Ewing & Miyan, 1986) since both species are closely related and show the same pattern of doublet pulses.

Putting our data together, there was no overlap between our chromosomal and behavior data. The statistical analyses showed that the sound parameters PI-A, PI-B, FFT/PI and FFT/PI-A were not significantly different among the populations. However, IPI, PI and FFT/PI-B varied significantly among the five populations (Table 1), despite their karyotypes types. The H42 and H49 populations showed the same karyotype IV but they are significantly different concerning all these 3 parameters. On the other hand, the H45 and H80 populations showed karyotype V and IV, respectively, and they were not different in all the 7 parameters analyzed, just to mention two examples. The low variability of the courtship song of the 5 populations of *D. meridionalis* studied here could be explained by strong selection or gene flow. Since we do not believe that the variation on the amount of heterocromatin present in the microchromosomes can, *per si*, result in speciation, we suggest that these 5 populations of *D. meridionalis* represent, in fact, just one species with a interpopulational chromosomal variability.

Acknowledgments — We are indebted to Denise Kuhn for reviewing the english version and Maura Manfrin for a critical reading of the manuscript. Financial support was furnished by CNPq and CAPES.

REFERENCES

BAIMAI, V., 1977, Chromosomal polymorphisms of constitutive heterochromatin and inversion in *Drosophila*. *Genetics, 85*: 85-93

BAIMAI, V., SENE, F. M. & PEREIRA, M. A. Q. R., 1983, Heterochromatin and kariotipic differentiation of some neotropical cactus-breeding species of the *D. repleta* species group. *Genetica, 60*: 81-92.

BENNET-CLARK, H. C., 1984, A particle velocity microphone for the song of small insects and other accustic measurements. *J. Exp. Biol., 108*: 459-463.

CHANG, H. C. & MILLER, D. D., 1978, Courtship and mating sounds in species of the *Drosophila affinis* subgroup. *Evolution, 32*: 540-550

COWLING, D. E. & BURNET, B., 1981, Courtship song and genetic control of the acoustic characteristics in sibling species of the *Drosophila melanogaster* subgroup. *Anim. Behav., 29*: 924-935.

EWING, A. W., 1983, Functional aspects of *Drosophila* courtship. *Rev. Brasil. Biol., 58*: 275-292.

EWING, A. W. & MIYAN, J. A., 1986, Sexual selection, sexual isolation and the evolution of song in the *Drosophila repleta* group of species. *Anim. Behav., 34*: 421-429.

HOIKKALA, A. & LUMME, H., 1987, The genetic basis of the evolution of the male courtship sounds of the *Drosophila virilis* group. *Evolution, 41*: 827-845.

HOIKKALA, A., LAKOVAARA, S. & ROMPPAINEN, E., 1982, Mating behaviour and male courtship sounds in the *Drosophila virilis* group. *In*: S. Lakovaara (ed.), *Advances in Genetics*. Development and Evolution of *Drosophila*, Plenum, New York.

MANFRIN, M. H., PRADO, P. R. R. & SENE, F. M., 1997, Analysis of sound components of sexual courtship of two subspecies of *Drosophila mercatorum* (Diptera, Drosophilidae). *Rev. Brasil. Biol., 57*: 349-355.

PEREIRA, M. A. Q. R., VILELA, C. R. & SENE, F. M., 1983, Notes on breeding and feeding sites of some species of the *repleta* group of the Genus *Drosophila* (Diptera, Drosophilidae). *Cienc. Cult., 35*: 1313-1319.

RITCHIE, M. G., YATE, V. H. & KYRIACOU, C. P., 1994, Genetic variability of the interpulse interval of courtship song among some European populations of *Drosophila melanogaster*. *Heredity, 72*: 459-464.

RITCHIE, M. G. & KYRIACOU, C. P., 1994, Genetic variability of coutship song in a population of *Drosophila melanogaster*. *Anim. Behav., 48*: 425-434.

RITCHIE, M. G. & GLEASON, J. M., 1995, Rapid evolution of courtship song pattern in *Drosophila willistoni* species. *J. Evol. Biol., 8*: 463-479.

SCHILDER, F. & DOW, M., 1977, Courtship behaviour in *Drosophila*: Sexual isolation or sexual selection? *Z. Tierpsychol., 43*: 304-310.

SENE, F. M. & MANFRIN, M. H., 1998, A chamber to record the courtship sound in *Drosophila*. *D.I.S., 81*.

SENE, F. M., PEREIRA, M. A. Q. R. & VILELA, C. A., 1982, Evolutionary aspects of cactus breeding *Drosophila* species in South America. pp. 97-106. *In*: J. S. F. Barker & W. T. Starmer (eds.), *Ecological Genetics and Evolution*. Academic Press, Sydney, Australia.

TOMARU, M. & OGUMA, Y., 1994, Differences in courtship song in the species of the *Drosophila auraria* complex. *Anim. Behav., 47*: 133-140.

VILELA, C. R., PEREIRA, M. A. Q. R. & SENE, F. M., 1983, Preliminary data on the geographical distribuition of *Drosophila* species within morphoclimatic domains of Brazil. II. The *repleta* group. *Cienc. Cult., 35*: 66-70.

ZAPPI, D. & TAYLOR, N. P., 1993, Eastern Brazilian cacti. *Piante Grasse*, *13*(4 suppl.): 65-78.

WHELLER, D. A., FIELDS, W. L. & HALL, J. C., 1988, Spectral analysis of *Drosophila* courtship songs: *D. melanogaster*, *D. simulans*, and their interspecific hybrid. *Behav. Genet., 18*: 675-703.