

Cytogenetic study of *Anopheles albitarsis* (Diptera: Culicidae) by C-banding and in situ hybridization

M. S. RAFAEL¹, I. P. SANTOS Jr¹, W. P. TADEI¹, K. A. CARVALHO², S. M. RECCO-PIMENTE², M. A. M. SALLUM and O. P. FORATTINI³

¹Coordenação de Pesquisas em Ciências da Saúde, Instituto Nacional de Pesquisas da Amazônia (INPA), Manaus, AM, Brazil

²Departamento de Biologia Celular, Instituto de Biologia, Universidade Estadual de Campinas (UNICAMP), Campinas, SP, Brazil

³Departamento de Epidemiologia, Faculdade de Saúde Pública, Universidade de São Paulo (USP), São Paulo, SP, Brazil

Rafael, M. S., Santos-Jr, I. P., Tadei, W. P., Carvalho, K. A., Recco-Pimente, S. M., Sallum, M. A. M. and Forattini, O. P. 2006. Cytogenetic study of *Anopheles albitarsis* (Diptera: Culicidae) by C-banding and in situ hybridization. — *Hereditas* 143: 62–67. Lund, Sweden. eISSN 1601-5223. Received August 29, 2005. Accepted January 13, 2006

The C-banding pattern and the size and location of the nucleolar organizer regions (NORs) are described for the first time in Brazilian populations of *Anopheles* (*Nyssorhynchus*) *albitarsis sensu lato*. C-banding revealed variation in the size of the centromeric heterochromatic blocks in autosomal chromosomes and in the acrocentric (X) and punitiform (Y) sex chromosomes. Fluorescence in situ hybridization showed that the NORs were located in the pericentromeric region of the sex (XX/XY) chromosomes and that this coincided with the number and location of centromeric constitutive heterochromatin blocks previously revealed by C-banding. The NORs varied in size among the homologues of the three populations. These findings of the populations studied support the hypothesis that the stability of NORs in the *A. albitarsis* complex is characterized by the presence of clustered and conserved sites in a unique pair of chromosomes.

Miriam Silva Rafael, INPA – Instituto Nacional de Pesquisas da Amazônia, CPSC – Coordenação de Pesquisas em Ciências da Saúde, Lab. de Vetores da Malária/Dengue, Av. André Araújo, 2936 – Bairro Petrópolis, 69060-001, Manaus, Amazonas, Brazil. E-mail: msrafael@inpa.gov.br

Malaria in the Amazon region of Brazil accounts for more than 97% of all cases recorded in this country (FUNASA 2000), with *Anopheles* (*Nyssorhynchus*) *darlingi* Root 1926 being the main vector of human malaria in Brazil (TADEI and DUTARY-THATCHER 2000). However, *Anopheles* (*Nyssorhynchus*) *albitarsis* Lynch-Arribálzaga, 1878 has also been found naturally infected with *Plasmodium* species and has been implicated as a local malaria vector (PÓVOA et al. 2001; CONN et al. 2002; SILVA-VASCONCELOS et al. 2002). Previous cytogenetic studies of the *A. albitarsis* complex using populations from Brazil, Venezuela and Colombia identified the B1, B2 and C species complex based on the inversion of polytene chromosomes (KREUTZER et al. 1976). Chromosome preparations of fourth instar larvae of *A. albitarsis* from Iranduba and Coari (AM) and Ilha Comprida (SP) were analyzed for karyotype determination and to improve cytogenetic identification of this species. *Anopheles albitarsis* possesses $2n=6$ chromosomes, with two pairs (submetacentric and metacentric) of autosomes and one pair of sex chromosomes, with X-Y dimorphism. The sex pair is homomorphic and acrocentric in females and heteromorphic in males, with a punctiform Y chromosome (RAFAEL et al. 2005). However,

there have been insufficient cytogenetic studies of the *A. albitarsis* complex to allow an adequate assessment of their ability to transmit the parasites of human malaria. Brazilian *Anopheles* species, such as *Anopheles cruzii* (RAMÍREZ 1989) and *Anopheles darlingi* (RAFAEL and TADEI 1998), have a karyotype of $2n = 6$, with two pairs of metacentric and submetacentric autosomal chromosomes and one pair of sex chromosomes that shows X-Y heteromorphism.

The detection of constitutive heterochromatin (C-banding) has been useful in identifying intra- and interspecific variations in mitotic chromosomes of the *Anopheles* species complex, especially in the X and Y chromosomes (BAIMAI and HARRISON 1984; BAIMAI 1998). *Anopheles* species from the Brazilian Amazon show intraindividual variations in their C-banding pattern, mainly in the centromeric regions of the sex (XX/XY) and autosomal chromosomes (RAFAEL and TADEI 2000).

In situ hybridization techniques have mapped the polytene and metaphase chromosomes of mosquitoes using ribosomal 18S and 28S probes (MARCHI and PILI 1994). The rRNA genes are located in the nucleolar organizer regions (NORs) and coincide with the location of constitutive heterochromatin in

the sex chromosomes of 15 mosquitoes species from Italy, USA and Uganda (MARCHI and PILI 1994). *Anopheles darlingi* and *Anopheles nuneztovari* from the Brazilian Amazon have heteromorphic sex chromosomes with intraindividual variations in the size of the rRNA genes (a rDNA probe from *Drosophila melanogaster*) located in the pericentromeric region; this location coincides with that of constitutive heterochromatin (RAFAEL et al. 2003).

In the present study, we used C-banding and FISH techniques to examine the chromosomal organization and structure of mosquitoes of the *A. albittarsis* complex from Iranduba and Coari, in the State of Amazonas (AM), in the Brazilian Amazon, and Ilha Comprida, in the State of São Paulo (SP), south-eastern Brazil. This is the first cytogenetic analysis of this type in *A. albittarsis*, and the results described here certainly contribute to the chromosome characterization of these mosquitoes that transmit malaria in the Amazon region.

MATERIAL AND METHODS

Mosquitoes samples and chromosome preparations

Adult females of *A. albittarsis* were collected while they were feeding on cattle and resting on a trap in Ilha Comprida, SP, Iranduba and Coari, AM. Females were transported to the Malaria Vector Laboratory at the Instituto Nacional de Pesquisas da Amazônia, in Manaus, AM. Morphological characterization of the specimens was done according to FORATTINI (1962), GORHAM et al. (1967) and FARAN and LINTHICUM (1981). Brain ganglia from fourth instar larvae were treated with a hypotonic solution containing 0.005% colchicine and used to prepare chromosome slides, as described by IMAI et al. (1988). Fifty-seven slide preparations from Ilha Comprida, 79 from Iranduba and 46 from Coari were examined for C-banding and in situ hybridization.

C-banding

Sixty-two, 79 and 51 metaphase nuclei from Ilha Comprida, Iranduba and Coari, respectively, were processed for C-banding, as described by SUMNER (1972), with a slight modification to allow the localization of constitutive heterochromatin. The slide preparations were incubated in 0.2 N HCl at 37°C for 7 min followed by incubation in 5% Ba(OH)₂ at 57°C for 9 min, and in 2 × SSC, pH 7.0, at 57°C for 9 min. Samples were subsequently washed with distilled water and stained with Giemsa in 8% phosphate buffer, pH 6.8, for 20 min. The metaphases were then washed with distilled water and photographed using a

Axioplan Zeiss photomicroscope loaded with Kodak Imagelink HQ ISO 25 film.

Fluorescence in situ hybridization (FISH)

FISH was done according to VIEGAS-PEQUIGNOT (1992), modified by RAFAEL et al. (2003), using the recombinant pDm 238 from *D. melanogaster* (45S rDNA) as the probe. The rDNA probe was labeled with biotin-14-dATP by a standard nick translation (GIBCO BRL) procedure. Sixty-five, 59 and 48 metaphases nuclei from Ilha Comprida, Iranduba and Coari, respectively, were processed for FISH. The slides were immersed in RNase (100 µg ml⁻¹) at 37°C for 1 h, dehydrated in an ethanol series, and denatured for 2 min in 70% formamide in 10 × SSC, followed by dehydration in a cold ethanol series. The pDm 238 probe was denatured at 100°C and added to the slides that were then incubated for 36 h at 37°C to allow in situ hybridization.

The slides were subsequently washed in 50% formamide (in 2 × SSC) and twice in 2 × SSC. The biotin-labeled probe was detected by incubation with anti-biotin antibody diluted in a solution containing bovine serum albumin at 37°C for 45 min. After washing in PBT buffer (30% BSA, 0.1% Tween 20 and phosphate-buffered saline 1 ×), the slides were incubated with the second antibody (IgG-FITC, 1:100, v/v, in PBT). All of the preparations were counterstained with propidium iodide (2 µg ml⁻¹) and mounted with anti-fading (Vectashield). Photographs were taken on 400-ASA color negative Kodak film and the images were optimized for contrast and brightness using Adobe Photoshop 6.0.

RESULTS

C-banding showed heterochromatic blocks in the pericentromeric regions of the X, II and III metaphase chromosomes of the brain ganglia of fourth instar larvae (Fig. 1A-D). In some metaphases, the intensities of the heterochromatic band in the X chromosomes varied with the degree of chromosome condensation (high in Fig. 1A, 1B and low in Fig. 1C, 1D); all of the Y chromosomes were entirely heterochromatic (Fig. 1A, 1C). Autosome III from Iranduba contained a large amount of centromeric heterochromatin (Fig. 1B), whereas the C-positive segments in autosome II showed little or no staining (Fig. 1A-C). Interstitial marks were observed on the autosomes of *A. albittarsis* in 1 out of 10 slides from each locality.

FISH with a 45S rDNA probe revealed nucleolar organizer regions (NORs) of variable size on the XX and XY chromosomes in all three populations of *A. albittarsis*. All of the signals coincided with the location

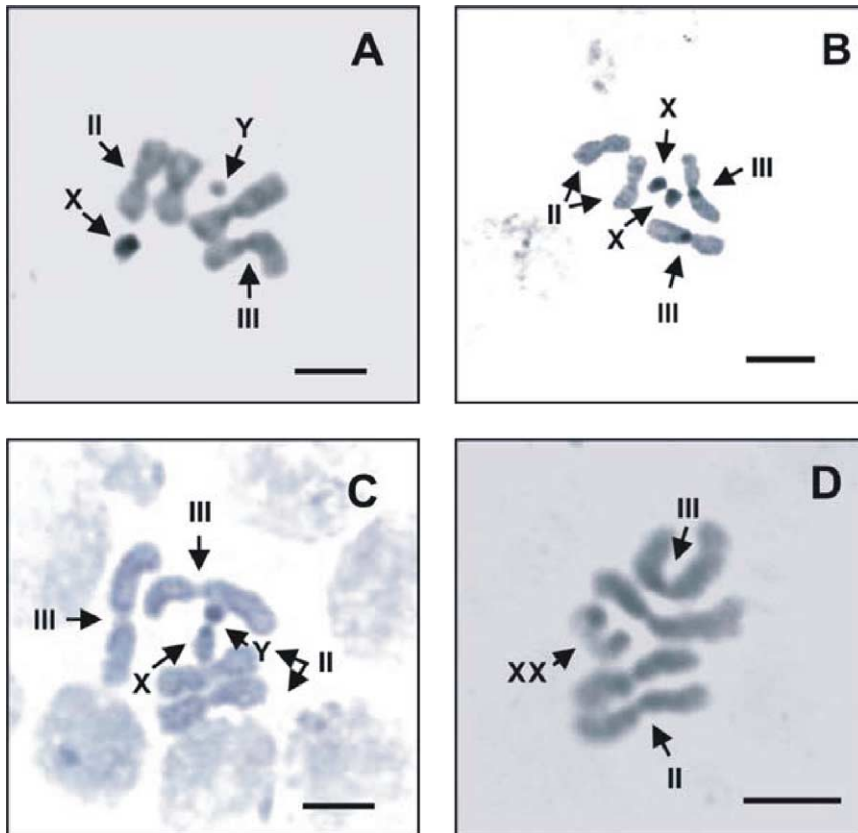


Fig. 1A–D. C-banding in metaphases of *A. albitarsis*. (A) male and (D) female from Ilha Comprida (SP); (B) female from Iranduba (AM); (C) male from Coari (AM). (A–D) Centromeric C-bands on the autosomes and X chromosomes (arrows); the Y chromosomes were totally heterochromatic (arrowheads); (B) Note the strong centromeric spot on autosome III. Scale = 10 µm.

of the constitutive heterochromatin blocks detected by C-banding. Fluorescent signals were observed in the centromeric and pericentromeric regions of the XX and XY chromosomes, although the intensity varied among these chromosomes. The X and Y chromosomes of *A. albitarsis* specimens from Ilha Comprida, Iranduba and Coari showed both strong (Fig. 2B, 3A, 4B–C) and weak (Fig. 2A, 3B, 4A) labeling. Metaphase

nuclei had conspicuous somatic ectoparing (Fig. 2B, 4D). All of the interphase nuclei had fluorescent nucleoli.

DISCUSSION

The C-banding of metaphase chromosomes of *Anopheles* can help to identify cryptic species based on

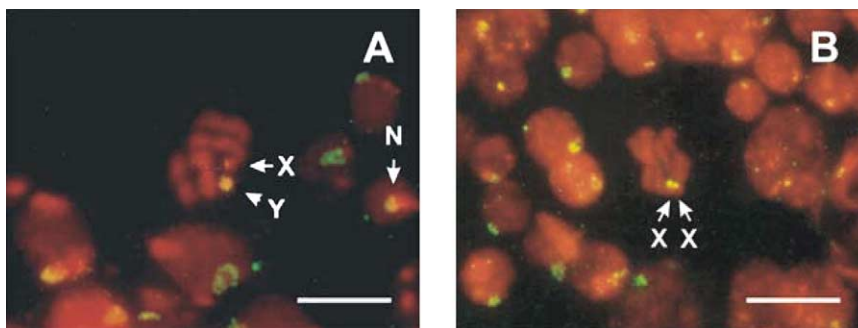


Fig. 2A–B. FISH in *A. albitarsis* from Ilha Comprida (SP). (A) The Y chromosome was totally mapped (arrowhead); (A, B) X chromosomes showing weak and strong fluorescent spots (arrows), respectively. Scale = 10 µm.

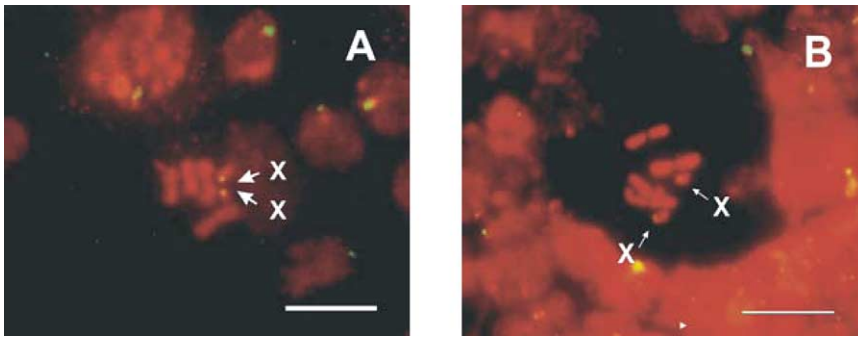


Fig. 3A–B. FISH in females of *A. albitarsis* from Iranduba (AM). (A) XX chromosomes and nucleoli show clear fluorescent signals (arrows); (B) XX chromosomes of another female showing weak rRNA cistrons (arrows). Scale = 10 μ m.

differences in the distribution of constitutive heterochromatin on the X and Y chromosomes. This approach has been used to distinguish the species in the *A. dirus* complex of southern Asia (BAIMAI et al. 1996) and *Anopheles maculatus* from Indonesia and Malasia (BAIMAI 1998). As shown here, the C-banding of three populations of the *A. albitarsis* complex revealed constitutive heterochromatin on the sex (XX) and autosomal (II and III) chromosomes, whereas the Y chromosome was totally heterochromatic. In addition, there was considerable intraindividual variation

in the size of the bands in the centromeric and pericentromeric regions. In dipteran insects, constitutive heterochromatin may account for more than 60% of the length of the X chromosome (BAIMAI 1998).

A criterion to identify *A. albitarsis* as a species complex, would be that populations must exhibit interspecific variation based on differences in the amount and distribution of constitutive heterochromatin in the sex chromosomes and/or in the centromeric regions of the autosome (s), such as it were observed in other *Anopheles* malaria vectors in Thai-

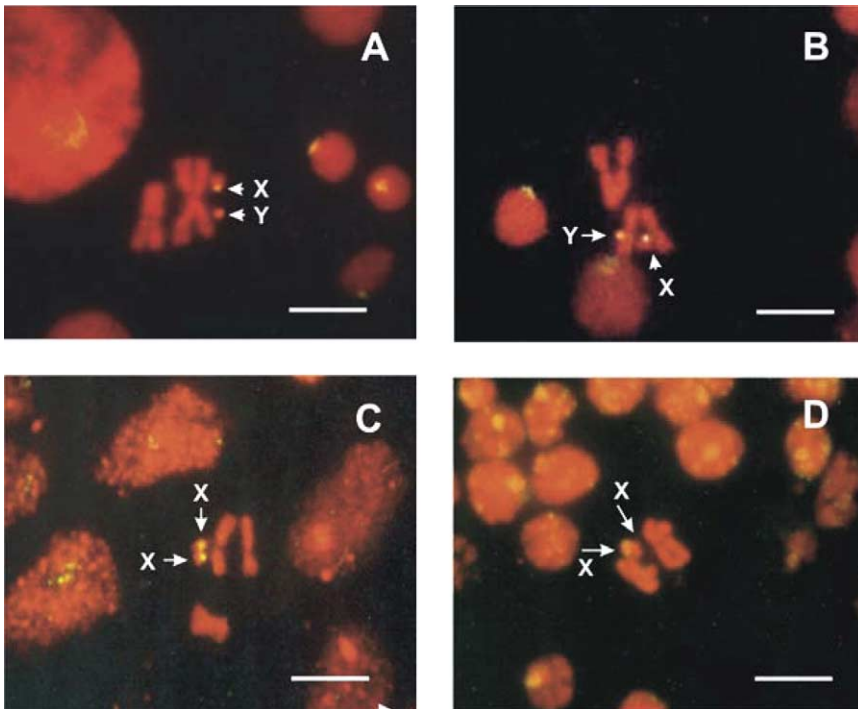


Fig. 4A–D. FISH in males (A, B) and females (C, D) of *A. albitarsis* from Coari (AM). (A, B, C) X chromosomes showing weak (arrowheads) and strong (arrow) rRNA cistrons, respectively; In (A) and (B), the Y chromosomes were either weakly (arrowhead) or totally (arrow) mapped; (D) Nuclei with conspicuous somatic ectypairing. Scale = 10 μ m.

land and southeast Asia (BAIMAI and HARRISON 1984; BAIMAI and TRAIKVASIN 1987; BAIMAI et al. 1993, 1996; BAIMAI 1998).

In Brazilian *Anopheles* species, C-banding has revealed intraspecific variation in the size of the centromeric and pericentromeric regions of autosomal and sex chromosomes of *A. darlingi* and *A. nuneztovari* (RAFAEL and TADEI 2000) and *A. (Kerteszia) cruzii* (RAMÍREZ and DESSEN 1994). Similarly, intraspecific variation in the size of the constitutive heterochromatin blocks in the sex and autosomal pairs of the *A. albitarsis* complex may have occurred through the partial gain or loss of heterochromatin by these chromosomes. The gain or loss of heterochromatin blocks in *Anopheles*, such as seen in *A. gambiae* and *A. arabiensis* (GATTI et al. 1982) and in *A. darlingi* and *A. nuneztovari* (RAFAEL and TADEI 2000; RAFAEL et al. 2003), reflects a continuous dynamic process throughout the evolution of this genus (VASANTHA et al. 1982; BAIMAI et al. 1996). Although the functional role and implications of heterochromatin in species differentiation is an unsolved problem, the presence of heterochromatin in eukaryotic chromosomes suggests its significant role in the regulatory function and concerted evolution of the genome.

In *Anopheles*, the rDNA units are repeated in tandem 440–500 times per haploid genome (GALE and CRAMPTON 1989). As shown here, the rDNA cistrons were located on the sex chromosomes and coincided with heterochromatin in the *A. albitarsis* samples from Ilha Comprida, Iranduba and Coari. Similar results were obtained for the intensity of the hybridization signal and the number of cistrons in the sex chromosomes of *A. petragani* and *A. hispaniola* from Italy and Uganda using 18S and 28S rRNA probes (MARCHI and PILI 1994). In *A. darlingi* and *A. nuneztovari* from the Brazilian Amazon (RAFAEL et al. 2003), the heteromorphic sex chromosomes showed variation in the size of the 45S rDNA sites detected in the pericentromeric region and these sites coincided with those of constitutive heterochromatin. The presence of rRNA genes in mosquitoes has also been reported (KUMAR and RAI 1990), with the sequences involved being highly conserved (MUNSTERMANN and CONN 1997). The location of these conserved rRNA genes reveals a constant association with constitutive heterochromatin located on the two sex chromosomes of the genome of these mosquitoes (RAO and RAI 1987; MARCHI and PILI 1994; RAFAEL et al. 2003).

In *Anopheles*, the partially homologous X and Y chromosomes undergo recombination, with unequal crossing-over leading to differences in arm length and heterochromatin content (MARCHI and MEZZANOTE

1990; BAIMAI et al. 1996). The association between the NOR and heterochromatin is well known in several organisms (JAMILÉNA et al. 1990), but its precise role is unclear. In *A. albitarsis*, although FISH revealed no inter- or intraindividual variations in the number and location of the rRNA loci, there was variation in the size of the fluorescent signal. We suggest that the cistrons detected by FISH were indicative of a conserved genome in *A. albitarsis* because of the constancy of the NOR configuration in the corresponding nucleoli organizer.

The NOR characters seem to be a good tool for establishing physical gene maps of chromosomes in *Anopheles* genera (MARCHI and PILI 1994) and *Anopheles* species from Amazon region (RAFAEL et al. 2003). The variation in the size of the rDNA cistrons of *A. albitarsis* suggested intraindividual difference in the distribution of rDNA copies associated with constitutive heterochromatin. Our results provide important genetic information for understanding the chromosomal structure of *A. albitarsis sensu lato* and of other Anopheline species from the Amazon basin and southeastern Brazil.

Acknowledgements – This work was supported by a grant and MSc studentship from the Fundação de Amparo Pesquisa do Estado do Amazonas (FAPEAM)/PIPT, and by the Ministério da Ciência e Tecnologia (MCT) (grant PPI 3680 and the project PIATAM/CTPETRO).

REFERENCES

- Baimai, V. 1998. Heterochromatin accumulation and karyotypic evolution in some dipteran insects. – *Zool. Stud.* 37: 75–88.
- Baimai, V. and Harrison, B.A. 1984. Heterochromatin variation in the sex chromosomes in Thailand populations of *Anopheles dirus* A (Diptera: Culicidae). – *Can. J. Genet. Cytol.* 26: 633–636.
- Baimai, V. and Traipakvasin, A. 1987. Intraspecific variation in sex heterochromatin of species B of the *Anopheles dirus* complex in Thailand. – *Genome* 29: 401–404.
- Baimai, V., Kijchalao, U., Rattanarithikul, R. et al. 1993. Metaphase karyotypes of *Anopheles* of Thailand and southeast Asia: II. *maculatus* group, *Neocellia* series, subgenus *Cellia*. – *Mosq. Syst.* 25: 116–123.
- Baimai, V., Treesucon, A. and Kijchalao, U. 1996. Heterochromatin variation in chromosome X in a natural population of *Anopheles willmori* (Diptera, Culicidae) of Thailand. – *Genetica* 97: 235–239.
- Conn, J. E., Wilkerson, R. C., Segura, M. N. O. et al. 2002. Emergence of a new Neotropical malaria vector facilitated by human migration and changes in land use. – *Am. J. Trop. Med. Hyg.* 66: 18–22.
- Faran, M. E. and Linthicum, K. J. 1981. A handbook of the Amazonian species of *Anopheles* (*Nyssorhynchus*) (Diptera: Culicidae). – *Mosq. Syst.* 13: 1–91.
- Forattini, O. P. 1962. *Entomologia Médica*. Faculdade de Higiene e Saúde Pública da USP. – Editora da USP.

- FUNASA – Fundação Nacional de Saúde. 2000. http://www.funasa.gov.br/epi/malaria/pdfs/plano_estaduais.pdf.
- Gale, K. R. and Crampton, J. M. 1989. The ribosomal genes of the mosquito, *Aedes aegypti*. – *Eur. J. Biochem.* 185: 311–317.
- Gatti, M., Bonaccorsi, S., Pimpinelli, S. et al. 1982. Polymorphism of sex chromosome heterochromatin in the *Anopheles gambiae* complex. – In: Steiner, W. W. M., Tabachnick, W. J., Rai, K. S. et al. (eds), Recent developments in the genetics of insect disease vectors. Stipes Publ. Co, Champaign, IL, p. 32–48.
- Gorham, J. R., Stojanovich, C. J. and Scott, H. G. 1967. Chave ilustrada para los mosquitos anofelinos de Sudamerica Oriental. – Dept of Health, Education and Welfare, Public Health Service.
- Imai, H. T., Taylor, R. W., Crosdland, W. J. et al. 1988. Modes of spontaneous chromosomal mutation and karyotype evolution in ants with reference to the minimum interaction hypothesis. – *Jap. J. Genet.* 63: 159–185.
- Jamilena, M., Ruiz Rejón, C. and Ruiz Rejón, M. 1990. Variation in the heterochromatin and nucleolar organizing regions of *Allium subvillosum* L. (Liliaceae). – *Genome* 33: 779–784.
- Kreutzer, R. D., Kitzmiller, J. B. and Rabbani, M. G. 1976. Cytogenetically distinguishable sympatric and allopatric populations of the mosquito *Anopheles albitarsis*. – *Acta Amazonica* 6: 473–481.
- Kumar, A. and Rai, K. S. 1990. Chromosomal localization and copy number of 18S + 28S ribosomal RNA genes in evolutionary diverse mosquitoes (Diptera: Culicidae). – *Hereditas* 113: 277–289.
- Marchi, A. and Mezzanotte, R. 1990. Inter- and intraspecific heterochromatin variation detected by restriction endonuclease digestion in two sibling species of the *Anopheles maculipennis* complex. – *Heredity* 65: 135–142.
- Marchi, A. and Pili, E. 1994. Ribosomal RNA genes in mosquitoes: localization by fluorescence in situ hybridization (FISH). – *Heredity* 72: 599–605.
- Munstermann, L. E. and Conn, J. E. 1997. Systematics of mosquito disease vectors (Diptera, Culicidae): impact of molecular biology and cladistic analysis. – *Annu. Rev. Entomol.* 42: 351–369.
- Póvoa, M., Wirtz, R., Lacerda, R. et al. 2001. Malaria vectors in the municipality of Serra do Navio, State of Amapá, Amazon Region, Brazil. – *Mem Inst Oswaldo Cruz* 96: 179–184.
- Rafael, M. S. and Tadei, W. P. 1998. Metaphase karyotypes of *Anopheles (Nyssorhynchus) darlingi* Root and *A. (N.) nuneztovari* Gabaldón (Diptera; Culicidae). – *Genet. Mol. Biol.* 21: 351–354.
- Rafael, M. S. and Tadei, W. P. 2000. Heterochromatin variation in chromosomes of *Anopheles (Nyssorhynchus) darlingi* Root and *A. (N.) nuneztovari* Gabaldón (Diptera: Culicidae). – *Genet. Mol. Biol.* 23: 67–70.
- Rafael, M. S., Tadei, W. P. and Recco-Pimentel, S. M. 2003. Location of ribosomal genes in the chromosomes of *Anopheles darlingi* and *Anopheles nuneztovari* (Diptera, Culicidae) from the Brazilian Amazon. – *Mem. Inst. Oswaldo Cruz* 98: 629–635.
- Rafael, M. S., Santos-Junior, I. P., Tadei, W. P. et al. 2005. Karyotype of Brazilian *Anopheles albitarsis sensu lato* (Diptera: Culicidae). – *Genet. Mol. Res.* 4: 684–690.
- Ramírez, C. C. L. 1989. Estudo cromossômico em uma população de *Anopheles (Kerteszia) cruzii* Dyar & Knab, 1909. Tese de Mestrado. – Inst de Biociências, USP, São Paulo.
- Ramírez, C. C. L. and Dessen, E. M. B. 1994. Cytogenetic analysis of natural population of *Anopheles cruzii*. – *Rev. Bras. Genet.* 17: 41–46.
- Rao, P. N. and Rai, K. S. 1987. Comparative karyotypes and chromosomal evolution in some genera of *Nematoceros* (Diptera, Nematocera) families. – *Ann. Entomol. Soc. Am.* 80: 321–332.
- Silva-Vasconcelos, A., Kato, M. Y., Mourão, E. M. et al. 2002. Biting indices, host-seeking activity and natural infection rates of anopheline species in Boa Vista, Roraima, Brazil from 1996 to 1998. – *Mem. Inst. Oswaldo Cruz* 97: 151–161.
- Sumner, A. T. 1972. A simple technique for demonstrating centromeric heterochromatin. – *Exp. Cell Res.* 75: 304–305.
- Tadei, W. P. and Dutary-Thatcher, B. 2000. Malaria vectors in the Brazilian Amazon: *Anopheles* of the subgenus *Nyssorhynchus*. – *Rev. Inst. Med. Trop. S. Paulo* 42: 87–94.
- Vasanth, K., Subbarao, S. K., Adak, T. et al. 1982. Karyotypic variations in *Anopheles culifacies* complex. – *Ind. J. Malariol.* 19: 27–32.
- Viegas-Pequignot, E. 1992. In situ hybridization to chromosomes with biotinylated probes. – In: Willman, D. (ed.), In situ hybridization: practical approach. Oxford Univ. Press: IRL Press, pp. 137–158.