Unusual intra-individual karyotypical variation and evidence of cryptic species in Amazonian populations of *Pristimantis* (Anura, Terrarana)

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We report a cytogenetic analysis of three *Pristimantis fenestratus* frog populations of the Amazon region in Brazil. The specimens were sampled in Borba and Manaus, Amazonas State, and in Rio Branco, Acre State. They were analyzed using Giemsa, silver staining, C-banding and FISH with rDNA probes. The karyotypes of the three populations revealed 2n=34 chromosomes, but they differed in the number and position of Ag-NORs and in the heterochromatin pattern as well. The NOR was located on the pairs 05 and 07 in the Rio Branco specimens, pair 10 in the Manaus specimens, and pair 1 in the Borba specimens. A small C-band was detected on the telomeric region of the pair 05 in the Borba population, while in the Manaus there was a heterochromatic block adjacent to the centromere of pair 9. An unusual intra-individual variation of chromosome number was observed in metaphases of Rio Branco specimens, comprising fundamental numbers of 33, 34 and 35. Additionally, interchromosome thread connections were detected between telomere–telomere, centromere–telomere and centromere–centromere regions, and among chromosomal heterochromatin-rich sites. The NOR sites were also involved in those connections. We hypothesize that this variation is due to chromosome missegregation during mitosis. The inter- and intraindividual variation in chromosome number suggests chromosomal instability in *P. fenestratus*, which has not been detected in any other anuran group. Since Borba is the type-locality of *P. fenestratus*, a taxonomic review of the Manaus and Rio Branco populations should be done, as indicated by the cytogenetic evidence that they could be new species of *Pristimantis*.

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The "eleutherodactyline" frogs (sensu Frost et al. 2006) are undergoing nominal restructuring and partitions into new taxa. Recently, Frost et al. (2006) divided the genus *Eleutherodactylus* into five genera named *Craugastor* (116 sp.), *Euhyas* (94 sp.), *Pelorius* (6 sp.), *Syrrhopus* (24 sp.) and *Eleutherodactylus* (492 sp.), and assigned them to the family Brachycephalidae. Afterwards, Heinicke et al. (2007) proposed about 400 species of *Eleutherodactylus* based on DNA sequences. They were grouped into a large clade, referred to as the South American clade, for which the new generic name *Pristimantis* was suggested. Recently, Hedges et al. (2008) allocated the 882 described "eleutherodactyline" species into a new taxon named Terrarana divided into four families, Eleutherodactylidae, Craugastoridae, Brachycephalidae and Strabomantidae.

Nearly 29 *Pristimantis* species are found, or presumably occur, in the north and central-western Brazil (Heinicke et al. 2007; Frost 2008). Twenty of the *Pristimantis* species have already been cytogenetically analyzed, including the five Brazilian representatives *P. conspicillatus* (Bogart 1970a, 1973a; DeWeese 1975), *P. lacrimosus* (DeWeese 1975) and *P. altamazonicus* (Bogart 1970a, 1970c; DeWeese e 1975), all with 2n=34 chromosomes, and *P. lanthanites* (Bogart 1973b) and *P. ventrimarmoratus* (Bogart 1970a, 1970b), both with 2n=36.

Cytogenetic analysis has been successfully applied to anuran groups as a valuable tool to investigate chromosome evolution, infer species relationships, corroborate suggestion of new species, and differentiate cryptic species (Giaretta and Aguiar 1998; Aguiar et al. 2002, Medeiros et al. 2003; Lourenço et al. 2006;

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Ananias et al. 2007). The understanding of chromosome evolution in "eleutherodactyline" frogs has been limited by a low number of cytogenetic studies, contrasting with the recent advances in molecular taxonomy.

We compared karyotypes of *Pristimantis fenestratus* specimens from its type-locality in the Amazon region of Brazil to other two populations attributed to the same taxon.

MATERIAL AND METHODS

Specimens

The analyzed *Pristimantis fenestratus* populations were sampled in three Amazonian locations. The samples consisted of 12 specimens (five males and seven females) from Borba, Amazonas State, which is the species type-locality, plus 18 specimens (three males and 15 females) from the Reserva Florestal Adolpho Ducke, Manaus, Amazonas State, and 31 (12 males and 19 females) from the Parque Zoobotânico of the Universidade Federal do Acre (UFAC), Rio Branco, Acre State. Sampling was done under permission of the Instituto Brasileiro do Meio Ambiente e Recursos Naturais Renováveis (IBAMA - Proc. 02010.000025/2005-51). Voucher specimens were deposited in the Museu de Zoologia "Prof. Dr. Adão José Cardoso" (ZUEC), at the Universidade Estadual de Campinas (UNICAMP), São Paulo, Brazil, and in the Instituto Nacional de Pesquisa da Amazônia (INPA), Manaus, Amazonas, Brazil, under the accession numbers ZUEC 13300-13302, 13304, 13307-13309, 13311-13312, 13314-13317, 13320-13326, 14103-14107, 14109 (specimens from Rio Branco), INPA-H-20.926-20.937 (from Borba) and ZUEC 13.327–13.338 and INPA-H-20.920-20.9925 (from Manaus).

Chromosome preparation and techniques

Mitotic chromosomes were obtained from intestinal epithelium and testis cell suspensions of frogs previously treated with colchicine 2% for about 4 h, as described by King and Rofe (1976) and Schmid (1978). The slides were stained with 10% Giemsa solution and processed for C-banding technique (Sumner 1972), with a modification in the pretreatment as described by Siqueira et al. (2008). In addition, chromosomes were analyzed by Ag-NOR staining (Howell and Black 1980) and fluorescent in situ hybridization (FISH) (Viegas-Péquignot 1992) using the HM123 recombinant plasmid containing a rDNA fragment of *Xenopus laevis* (Meunier-Rotival et al. 1979) as a probe. Metaphases were examined with a BX60 Olympus microscope and the images captured using Image Pro-Plus 5.1 software. The chromosomes of 20 metaphases from each of the three populations were measured and classified according to Green and Sessions (1991).

RESULTS

Chromosome morphology, NOR and heterochromatin distribution

The karyotypes of the three *P. fenestratus* populations consisted of a diploid number of 34 chromosomes and all pairs were telocentric (Fig. 1–3). The fundamental number (FN) was also 34. The Ag-NOR sites were located on the telomeric region of pair 1 in the Borba population, adjacent to the centromere of the pair 10 in the Manaus individuals and on telomeric regions of pairs 5 and 7 in the Rio Branco specimens (Fig. 1B, 2B, 3B). In the three populations, small amounts of heterochromatin were detected at the centromeric region of almost all chromosomes (Fig. 1C, 2C, 3C). In the Borba specimens there was an interstitial band in pair 6 and a faint additional C-band on the telomeric region of pair 1, which was coincident with the NOR (Fig. 1C). In the Manaus population, blocks of heterochromatin were detected adjacent to the centromere in pairs 1 and 10, and interstitially in pair 9 (Fig. 2C). The heterochromatic block of pair 10 was coincident with the NOR. In the Rio Branco specimens, heterochromatic blocks were observed on the telomeric region of pairs 1 and interstitially in pairs 5 and 8 (Fig. 3C). The telomeric band of pair 5 was coincident with the NOR (Fig. 3B–C).

Karyotypical variations in the Rio Branco population

Intraindividual variation in the fundamental number (FN=34) was observed in 14 out of 27 specimens from the Rio Branco population. About 1.8% of the metaphases within nine specimens had FN=35 due to the presence of a small metacentric or an extra telocentric chromosome (Fig. 4A, B, 5B, Table 1). In 13 specimens, 39 (3,8%) of 1028 metaphases had FN=33 due to the absence of one chromosome (monosomy) (Fig. 4C, Table 1). The chromosomes were so similar in size and morphology that it was not feasible to identify the telocentric homologues forming metacentric chromosomes, or to determine if the metacentric chromosome arms were composed of homologues from the same pair and precisely recognize the monosomic chromosome.

All *P. fenestratus* male and female specimens showed several metaphases with interchromosome thread connections, which were observed as centromeric–telomeric, telomeric–telomeric and centromeric–centromeric connections between few chromosomes (Fig. 5A–H). These connections were obvious in Giemsa stained (Fig. 5A–F) and C-banded (Fig. 5G–H) metaphases as well as in the Ag-NOR and in situ hybridization (Fig. 6D–E, 6M–N). Thread connections of sister chromatids were observed between Ag-NOR and rDNA fluorescent sites (Fig. 6B, 6L). Additionally, an intriguing intra-individual variation in

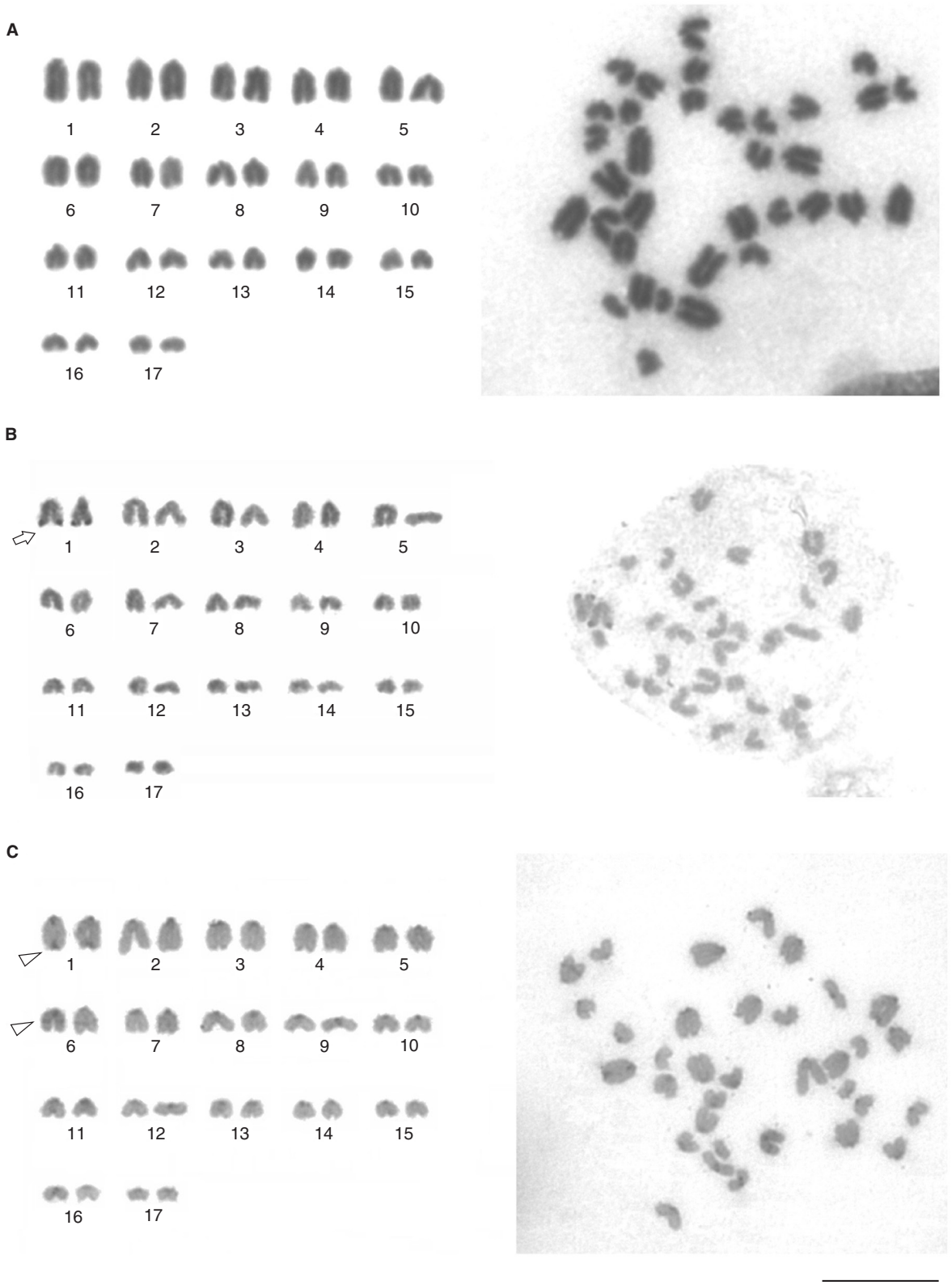

Fig. 1A–C. Karyotypes of *Pristimantis fenestratus* from Borba: (**A**) Giemsa staining; (**B**) Ag-NOR staining; (**C**) C-banding. The arrow indicates the NOR. The arrowheads indicate the interstitial and telomerical heterochromatins. Note the complete metaphase plates beside the karyogram. Bar=10 μm.

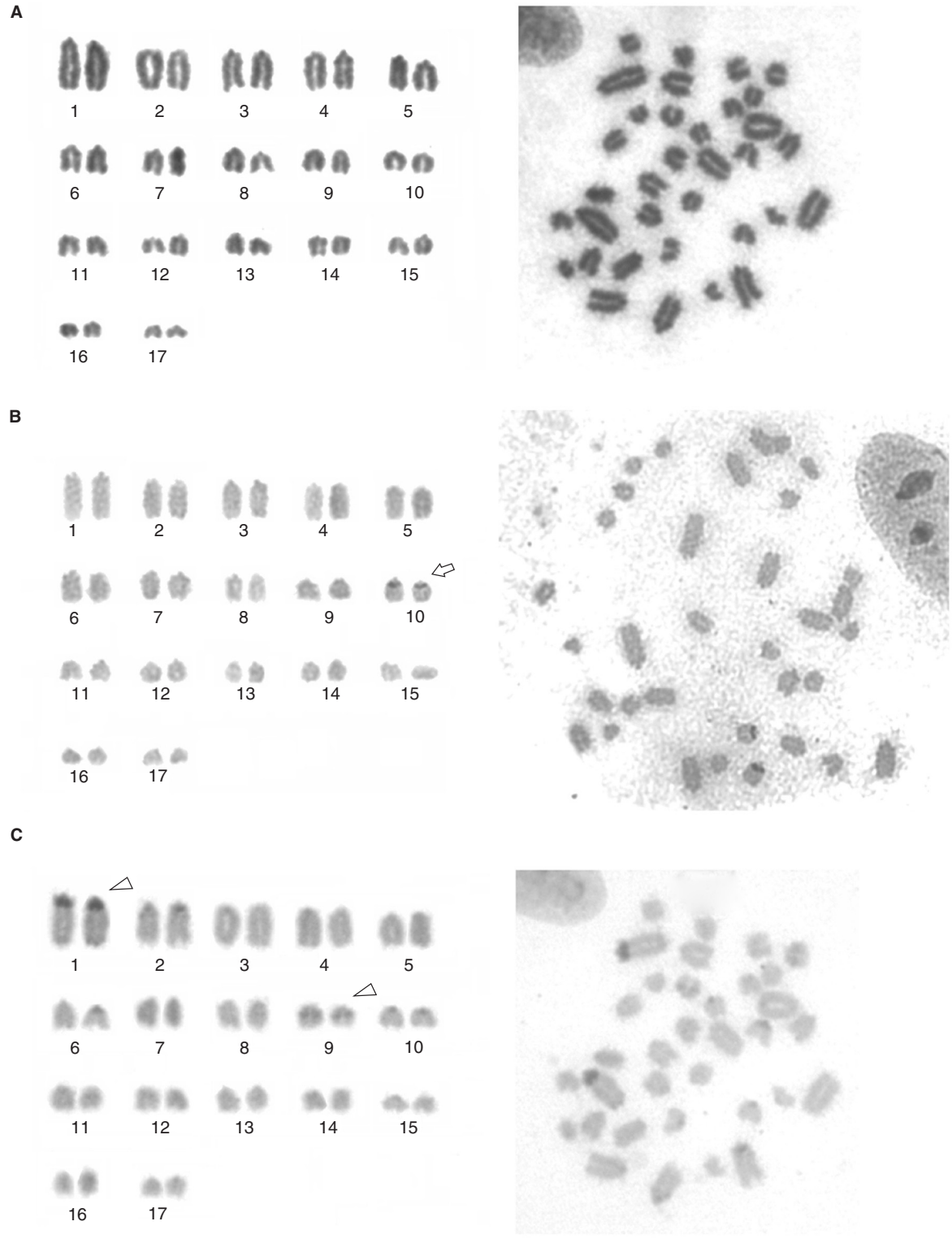

Fig. 2A–C. Karyotypes of *Pristimantis fenestratus* from Manaus: (**A**) Giemsa staining; (**B**) Ag-NOR staining; (**C**) C-banding. The arrow indicates the NOR. The arrowheads indicate the interstitial and pericentromerical heterochromatins. Note the complete metaphase plates beside the karyogram. Bar=10 μm.

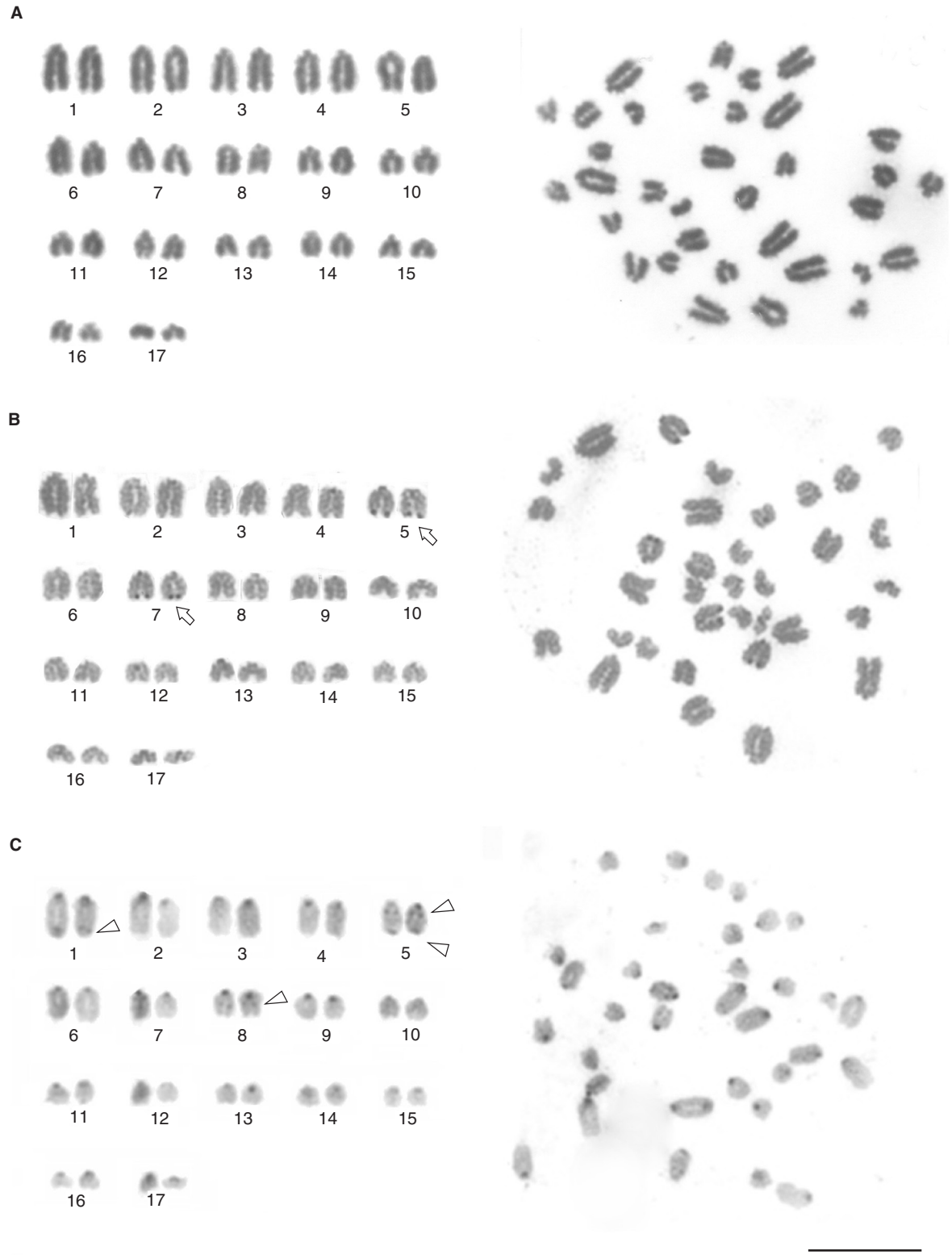

Fig. 3A–C. Karyotypes of *Pristimantis fenestratus* from Rio Branco: (**A**) Giemsa staining; (**B**) Ag-NOR staining; (**C**) C-banding. The arrow indicates the NORs. The arrowheads indicate the interstitial and telomerical heterochromatins. Note the complete metaphase plates beside the karyogram. Bar=10 μm.

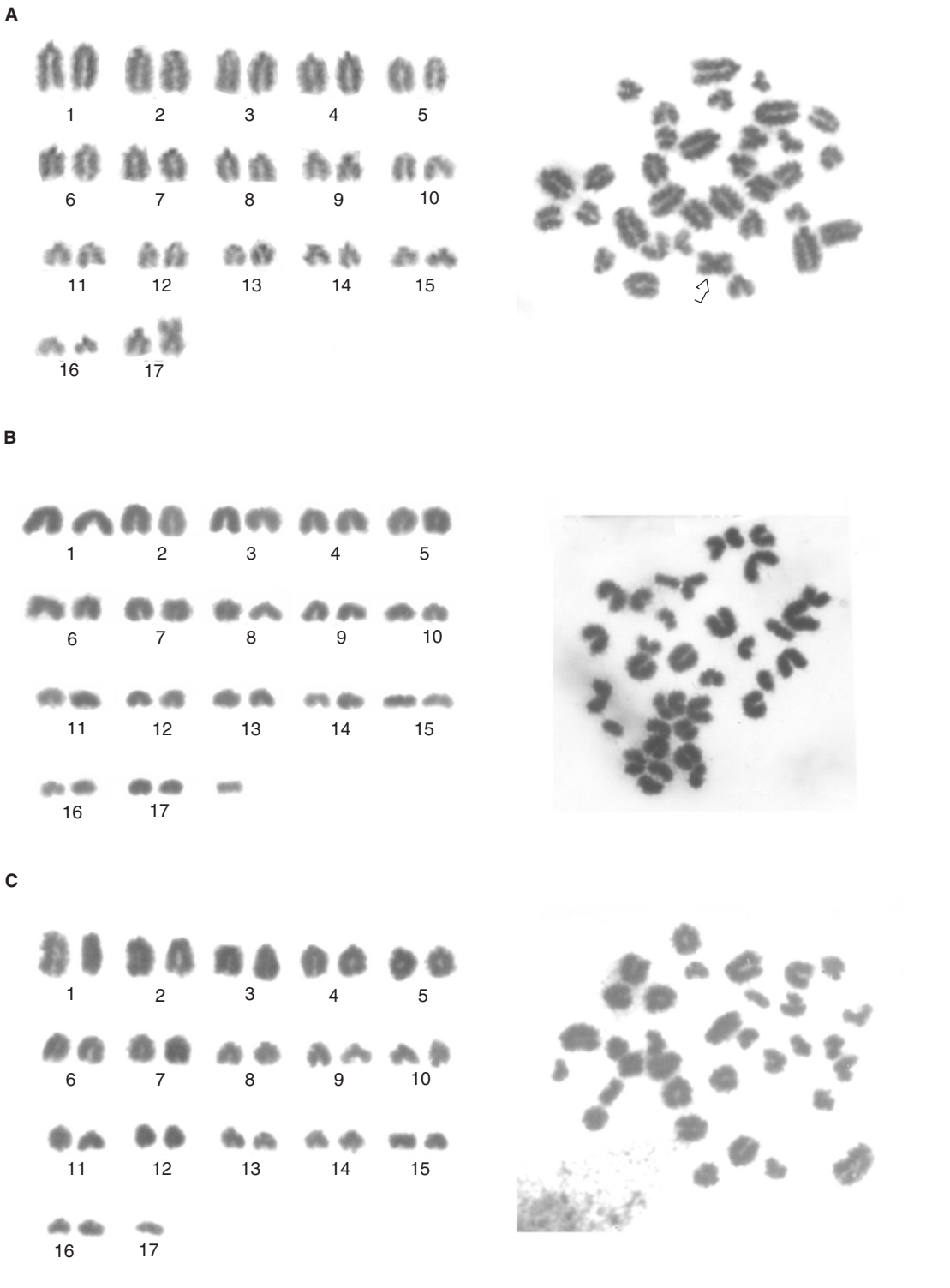

Fig. 4A–C. Giemsa stained karyotypes of *Pristimantis fenestratus* from Rio Branco: (**A**) 34 chromosomes, FN=35, with a metacentric chromosome; note that the chromosomes number 17 could not be paired with any other chromosome of the complement; (**B**) 35 chromosomes, FN=35, with an extra telocentric chromosome; (**C**) 33 chromosomes, FN=33 with absence of a chromosome. The arrow indicates the metacentric chromosome in the metaphase plate. Bar=10 μm.

Table 1. *Number of metaphases in specimens of P. fenestratus from Rio Branco population.*

	Specimens (acc. no.)	34T FN=34	33T FN=33	35T FN=35	33T+1M FN=35	Total of analyzed metaphases	% of altered metapahses in each specimen
Female	13300	37	–	–	–	37	–
	13301	58	5	2	–	65	10,8%
	13302	67	5	–	1	73	8,2%
	13307	6	–	–	–	6	–
	13316	18	1	–	2	22	13,6%
	13320	18	2	–	–	20	10,0%
	13322	23	1	–	–	24	4,2%
	13325	25	–	–	–	25	–
	13326	16	2	–	1	19	15,8%
	14103	43	2	1	–	46	6,5%
	14104	56	–	–	–	56	–
	14106	34	–	–	–	34	–
	14107	27	–	–	–	27	–
	14109	19	2	–	1	22	13,6%
Male	13304	5	–	–	–	5	–
	13308	30	–	–	–	30	–
	13309	32	–	–	–	32	–
	13311	60	2	–	–	62	3,2%
	13312	36	3	–	–	39	7,7%
	13314	114	7	4	1	126	9,5%
	13315	56	–	–	–	56	–
	13317	88	–	–	–	88	–
	13321	54	5	4	–	61	11,5%
	13323	6	–	–	–	6	–
	13324	26	2	–	–	28	7,1%
	14105	16	–	1	–	17	5,9%
Total no.		971	39	12	6	1028	5,4%
Total %		94,4%	3,8%	1,2%	0,6%	100,0%	

Analyzed mitotic metaphases with regular diploid number (2n=34) and with chromosome number variation (33 or 35 telocentrics). T=Telocentic; M=Metacentric; FN=Fundamental Number.

the NOR number was detected by silver staining in 23 out of 26 specimens and confirmed by the in situ hybridization with the rDNA probe. The number of NOR-bearing chromosomes ranged from three to five (Fig. 6A–C, G–K). In four individuals, metaphases with NOR size heteromorphism was detected between homologues and between sister chromatids (Fig. 6B, 6G–M).

DISCUSSION

Karyotypes

The diploid number of 34 telocentric chromosomes, as observed in *P. fenestratus*, was previously reported in the *Pristimantis* species *P. altamazonicus* (Bogart 1970a, 1970b, DeWeese 1975), *P. bogotensis* (DeWeese 1975), *P. conspicillatus* (Bogart 1970a, 1973a, DeWeese 1975), *P. gaigeae* (Bogart 1973b), *P. lacrimosus* (DeWeese 1975), *P. variabilis* (Bogart 1970a), *P. ridens* (DeWeese 1975; Miyamoto 1984), as well as in *Eleutherodactylus* (*Eleutherodactylus*) *varians* (Bogart 1970a). All the *Pristimantis* 2n=34 karyotypes are highly similar to *P. fenestratus* regarding their chromosome morphology; however, these species differ in the fundamental number, which is 34 in *P. fenestratus* and 36 in all the other mentioned species, which have one metacentric or one submetacentric pair. Only one species of this genus, *P. crepitans,* showed a lower number of 2n=22 chromosomes and this is also the only species *Pristimantis* living in xeric habitats, which led Siqueira et al. (in press) to suggest that this species is not closely related to its congeneric species.

The specimens of the three studied *Pristimantis fenestratus* populations are similar in their phenotype and in chromosome morphology. However, they diverge in

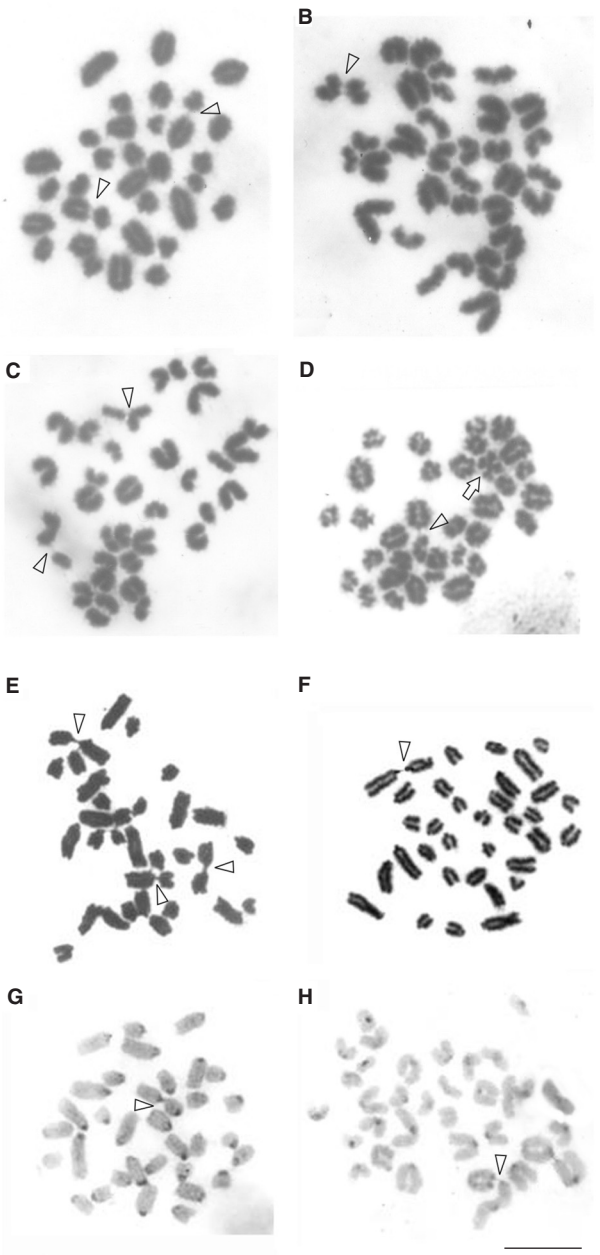

Fig. 5A–H. Metaphases of *Pristimantis fenestratus* from Rio Branco: Giemsa-stained (**A**–**F**) and C-banded metaphases (**G**–**H**) showing chromosomes associated by thin filaments (arrowheads). The arrow indicates a metacentric chromosome. Bar=10 μm.

number and position of NORs, and in the C-band pattern. Because Borba is the *P. fenestratus* type-locality, we suggest that the individuals from Rio Branco and from Manaus might be two undescribed species. The cytogenetic data allied to their differences in the acoustical characteristics (Lima unpubl.) strongly support this hypothesis and indicate that a reevaluation of the taxon *P. fenestratus* is necessary.

The analyses of chromosome morphology, NOR localization and heterochromatin pattern have been successfully used to distinguish anuran cryptic species. For instance, Medeiros et al. (2003) discriminated *Dendropsophus nanus* and *D. sanborni* based on their differences in the number of telocentric and submetacentric chromosomes and primary NOR-bearing chromosomes. These cytogenetic characteristics allowed unambiguous identification of syntopic individuals of those two species that are similar morphologically. In a previous study, two distinct karyotypes, including sexual chromosomes distinguished specimens within a *Physalaemus petersi* population in Acre, Brazil (Lourenço et al. 1999). *Scythrophrys* populations were discriminated by karyotypes as well, and the presence of two distinct taxonomic units in those populations was proposed by Lourenço et al. (2003a). In *Paratelmatobius*, a new species was described in the *P. cardosoi* group based on differences in the NOR and heterochromatin location (Lourenço et al. 2003b).

Interchromosome connections and intra-individual variation

The data described herein represent a first report in anurans of thread connections between chromatids and between chromosomes in mitotic cells, and of intra-individual variation in chromosome number and morphology.

The unusual intra-individual variation in NORs and chromosome number of the *P. fenestratus* population from Rio Branco could be possibly related to the presence of heterochromatin associated with interchromosome thread connections. The presence of an extra free telocentric, a metacentric or a monosomy in mitotic cells of the *P. fenestratus* population from Rio Branco might be a consequence of a mitotic missegregation of sister chromatids. This hypothesis could explain the intra-individual occurrence of metaphases with FN=33 and FN=35, because the metaphases with FN=35 had a free extra telocentric chromosome. Consequently, metaphases with FN=33 may have arisen from a cell that lost a telocentric through the anomalous segregation during the mitosis. Alternatively, the metacentric (FN=35) could be an isochromosome.

Intra-individual chromosome variation was previoulsy reported in one specimen of the fish *Trichomycterus davisi* (2n=54) that exhibited metaphases ranging from 2n=52 to 2n=56 chromosomes. This variation was attributed to a post-zygotic non-disjunction of a metacentric chromosome, followed by a spontaneous centric fission or to chemical or physical agents, possibly favored by the

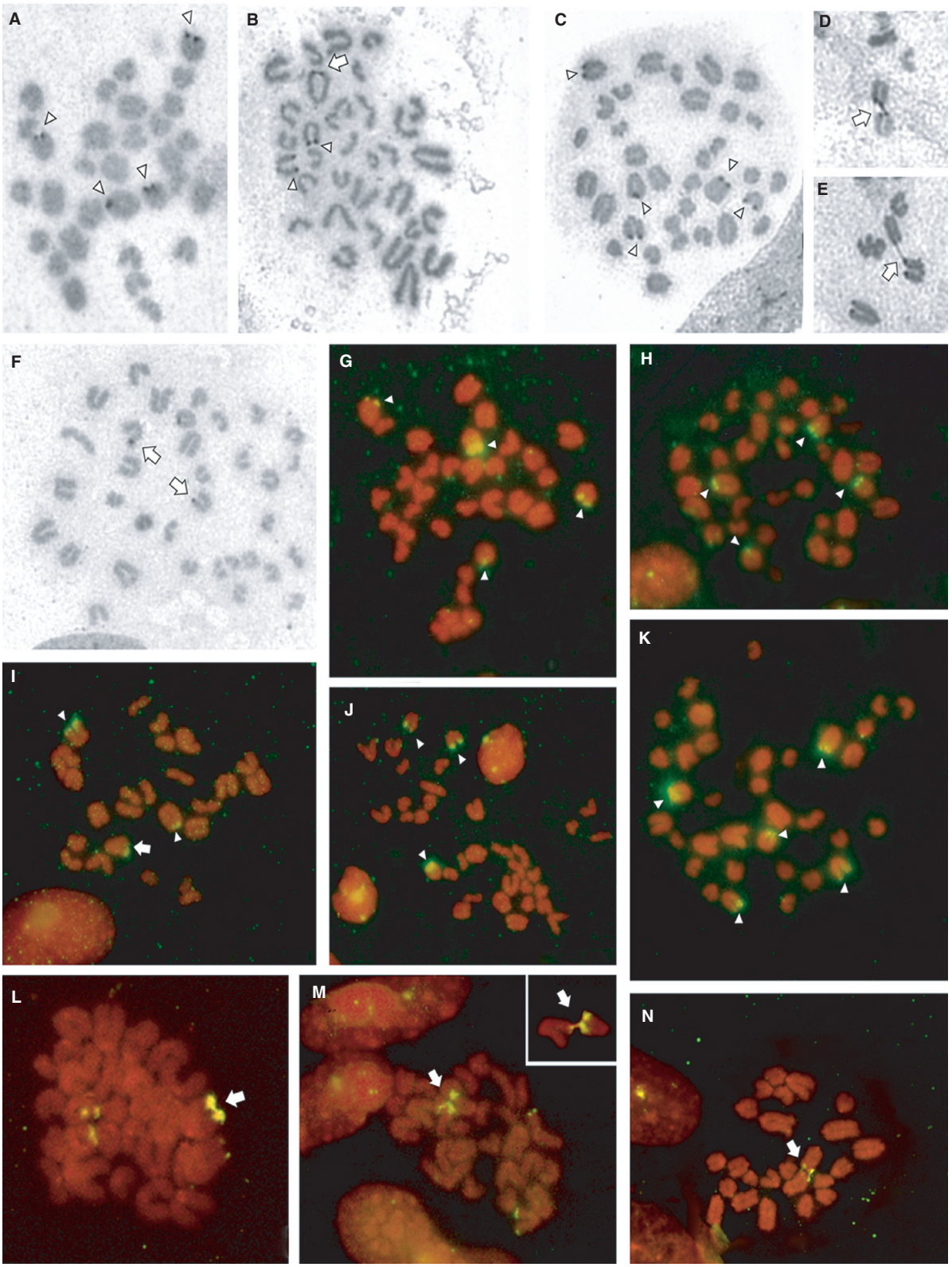

Fig. 6A–M. Metaphases of *Pristimantis fenestratus* from Rio Branco: Ag-NOR staining (**A–F**) and in situ hybridization (**G–N**). Note the four NORs (**A**, **G** and **H**), five NORs (**C** and **K**), and three NORs (**B**, **I** and **J**) indicated by arrowheads; NOR thread connections (**D**, **E**, **M** and **N**), NOR heteromorphism between chromosomes (**G**, **J**, **K** and **L**) and between sister chromatids (**F**, **I** and **M**) indicated by arrows.

intense aggression to the urban effluent Iguaçú river in the Paraná State of Brazil (Borin and Martins-Santos 2000). In the present study, the intra-individual chromosome variation of the *Pristimantis fenestratus* population from Rio Branco was observed in 14 (52%) out of the 27 specimens, and there was no evidence an environmental or any other exogenous contributing factor.

The intra-individual variation in number of NOR sites could also be explained by missegregation of the chromatids randomly associated by telomeres, since there were also thread connections between NOR sites at telomeres. Additionally, the chromosome number variation and NOR connections were clearly associated with heterochromatin, as demonstrated by C-banding, indicating that these connections involving telomeric and NOR heterochromatin could be repetitive DNA sequences such as satellite DNA, as observed by Kuznetsova et al. (2007) in mouse and human cell lineages.

In human cells, chromosome connections between NOR regions were reported by Tuck-Muller et al. (1984) as, most often, associated to chromosomal regions containing constitutive heterochromatin, especially that of centromeric regions. The authors suggested the occurrence of NOR transfer in humans, possibly by transposable genetic elements localized in heterochromatin sequences. Moreover, they considered that this phenomenon could explain the silver connection associations within heterochromatic regions in different cell lineages of mice and humans, and showed that these connections are composed of satellite DNA of the centromeric and telomeric heterochromatin. We have no conclusive evidence about the composition of the DNA involved in the associations in *P. fenestratus*, but the FISH and C-banding results suggested that the connections might have repetitive sequences related to rDNA and C-banding positive heterochromatin.

Conclusions

The karyotypical differences in the NOR position and banding pattern found in the *Pristimantis fenestratus* populations from Manaus and Rio Branco compared to *P. fenestratus* from the Borba type-locality indicate the existence of previously undescribed species in this taxon. The population from Rio Branco differed from the others by presenting an unusual intra-individual variation in chromosome number and in NOR number and position, not observed in the Borba and Manaus populations. The intra-individual variation in *P. fenestratus* specimens from Rio Branco suggests chromosomal instability and plasticity not previously reported for anurans. Such chromosomal rearrangements, when occurring in germinative cells, could contribute to karyotype differentiation among species.

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