Genetica **118:** 11–16, 2003.

Conservation of chromosomal location of nucleolus organizer in American marsupials (Didelphidae)

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Received 12 February 2002 Accepted 26 June 2002

Key words: Ag-NORs, Didelphidae, FISH, marsupials, NORs, rDNA

Abstract

The distribution and expression of nucleolus organizer regions (NORs) were analyzed in seven species of marsupials representative of the three karyotypes ($2n = 14$, 18 and 22) found in the American family Didelphidae. Analyses comprised silver-staining of NORs and fluorescence *in situ* hybridization with an rDNA probe. In addition to confirming the variability in number and distribution of NORs in Didelphidae, we demonstrated the conserved location of NORs on one autosome pair in the three karyotypes. In *Monodelphis domestica* ($2n = 18$), the NOR on the X chromosome was not inactivated in females.

Introduction

Marsupials represent an extreme example of karyotypical conservation in which a 'basic karyotype' with $2n = 14$ occurs in most of the roughly 270 living species that inhabit Australasia and the American continent. Rofe and Hayman (1985) stressed the interspecific variability in the amount of constitutive heterochromatin, size of sex chromosomes, and the number and location of Ag-NORs, in contrast with the conservation of G-banding patterns in 15 marsupial species with $2n = 14$. NORs may be located at secondary constrictions, as in Australian Macropodidae, or may be less conspicuous and only detectable after specific staining. There are species with NORs on one or more autosome pairs, on the sex chromosomes and autosomes, restricted to the X and Y chromosomes or to the X chromosome (Hayman, 1990).

To date, the study of NORs in American marsupials has been limited to silver-staining analysis, a technique that reflects NOR expression. The distribution of Ag-NORs was described in 11 species, comprising the three known diploid numbers ($2n = 14$, 18 and 22) (Fernandez-Donoso, Berrios & Pincheira, 1979; Yonenaga-Yassuda et al., 1982; Merry, Pathak & Vandeberg, 1983; Seluja et al., 1984; Casartelli, Rogatto & Ferrari, 1986; Souza, Maia & Santos, 1990). Ag-NORs varied from two to nine and were located on autosomes, except for the NOR on the X chromosome of *Monodelphis domestica*. NORs were never observed in association with conspicuous secondary constrictions. *In situ* hybridization, that allows the detection of ribosomal cistrons independently of their activity, has not been performed.

In the present study, we analyzed the distribution and activity of NORs in seven species of Didelphidae, belonging to different genera and representative of the three diploid numbers found in this family, through fluorescence *in situ* hybridization with an rDNA probe, and silver-staining.

Materials and methods

NOR distribution was analyzed after *in situ* hybridization of rDNA and silver-staining in 13 specimens belonging to seven genera of Didelphidae collected in different localities in Brazil (Table 1).

Chromosome preparations were obtained directly from bone marrow or from kidney or tail cells, cultured in DMEM/L-15 1:1 (Gibco-BRL/Interlab), supplemented with 20% of fetal bovine serum (Interlab).

Table 1. Number and distribution of NORs in Didelphidae

Species	Specimens	$2n$/FN*	No. of NORs		NORs location	No. of cells analyzed	Origin of cells
			In situ hybridization	$AgNO_3$			
Marmosops incanus	1M	14/24	2	2	6p	39	CC
Metachirus nudicaudatus	1M	14/20	2	2	5p or 6p	43	CC
Caluromys philander	2M	14/20	2	2	6p	85	BM
Micoureus demerarae	1M	14/20	6	4–6	5pq; 6p	60	CC
Monodelphis domestica	1M/2F	18/20	4	2 and 4	5p; Xp	84	CC, BM
Philander opossum	2F	22/20	4	4	5p; 7q**	122	CC, BM
Didelphis marsupialis	1M/2F	22/20	8	4–8	Autosomal	125	BM

F = Female; M = Male; p = Short arm; q = Long arm; CC = Cell culture; BM = Bone marrow; FN = Fundamental number.
* Short arms of subtelocentric chromosomes not included.
** According to Yonenaga-Yassuda et al. (1982).

NOR expression was analyzed after silver-nitrate staining (Howell & Black, 1980). Probe HM456, which contains part of the 18S and 28S rDNA of *Xenopus laevis* (Meunier-Rotival et al., 1979), was used for *in situ* hybridization. Probe hybridization and detection were performed according to Viegas-Péquignot (1992).

Results and discussion

NOR distributions in species representing the three diploid numbers found in Didelphidae ($2n = 14$, 18 and 22) are shown in Table 1.

NORs in the karyotypes with 2n = 14

The karyotypes of the four species with $2n = 14$ include three pairs of large submetacentric autosomes (pairs 1–3), one pair of medium metacentrics (pair 4) and two small pairs (pairs 5 and 6). The two smallest autosome pairs are acrocentric in *Caluromys philander*, *Metachirus nudicaudatus* and *Micoureus demerarae* (= *Marmosa cinerea*), and submetacentric in *Marmosops incanus*. With the exception of the metacentric X of the latter species, sex chromosomes are acrocentric, with interspecific variation in size (Svartman & Vianna-Morgante, 1999).

In situ hybridization with the rDNA probe revealed ribosomal cistrons just on the short arms of a small autosome pair in *C. philander*, *M. incanus* and *M. nudicaudatus* (Figure 1(a and b)). This was identified as pair 6 in the two former species based on the analysis of the same cells after DAPI staining. In *M. incanus*, interstitial telomere sequences are present at the pericentromeric region of pair 5, but not of pair 6, which is of similar size (Svartman & Vianna-Morgante, 1998). This allowed further confirmation of pair 6 as the NOR-bearing chromosome. Pairs 5 and 6 of *M. nudicaudatus* are of similar size and morphology, which prevented precise identification of the NOR-bearing autosomes. In *M. demerarae*, hybridization signals were present on the short arms and telomeric region of the long arms of pair 5 and on the short arms of pair 6, totaling six NORs (Figure 1(c)).

In the three species with NORs on one pair of autosomes, both of them were active in all cells, as demonstrated by silver-staining (Figures 2(a and b)). In *M. demerarae*, the only species with $2n = 14$ presenting more than two NORs, the number of active NORs per cell varied: out of 60 cells analyzed, 47 showed activity of the six NORs, 11 had four Ag-NORs and two presented five Ag-NORs (Figure 2(c)).

This is the first description of NORs for *M. incanus* and *M. nudicaudatus*. In *C. philander*, Souza, Maia and Santos (1990) reported the presence of two Ag-NORs as herein described, although the authors had classified the NOR-bearing autosomes as pair 5.

In *M. cinerea* (= *M. demerarae*), Casartelli, Rogatto and Ferrari (1986) and Souza, Maia and Santos (1990) reported the presence of four Ag-NORs. In the specimen that we analyzed, a total of six NORs was observed with both techniques and the difference is probably due to variations in NOR activity.

NORs in the karyotype with 2n = 18

The karyotype of *M. domestica* ($2n = 18$) includes four large submetacentric/metacentric auto-

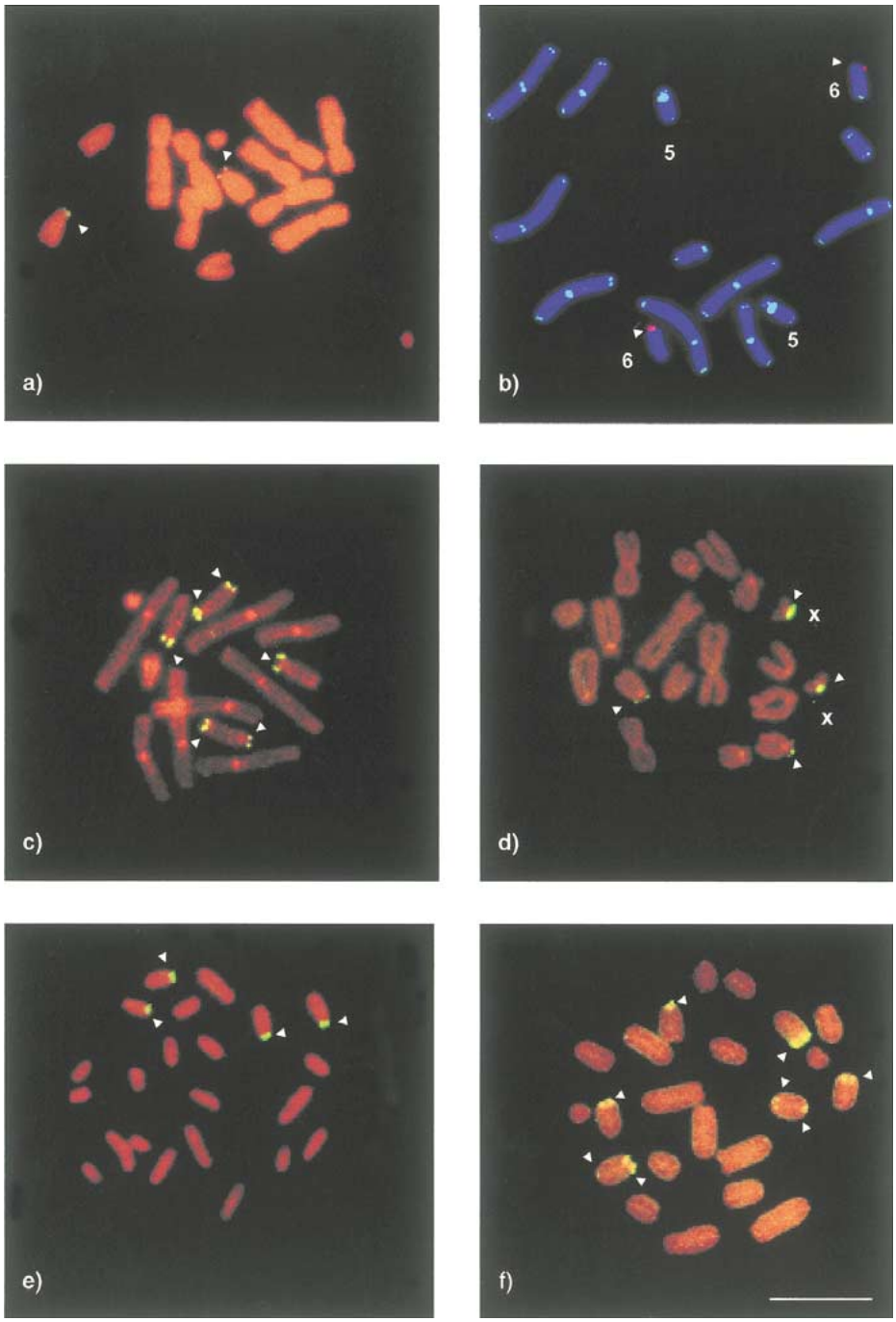

Figure 1. After *in situ* hybridization with rDNA: (a) two signals are observed in a male *Metachirus nudicaudatus* ($2n = 14$); (b) simultaneous hybridization with rDNA, in red, and with a telomere sequence, in green, in a male *Marmosops incanus* ($2n = 14$) confirms NOR location at 6p (see text); (c) six NORs are observed in a male *Micoureus demerarae* ($2n = 14$); (d) four NORs, at 5p and Xp, are evidenced in a female *Monodelphis domestica* ($2n = 18$); in $2n = 22$ karyotypes, (e) a female *Philander opossum* shows four signals and (f) a female *Didelphis marsupialis* presents eight signals. NORs are indicated by arrowheads. Bar = 10 μm.

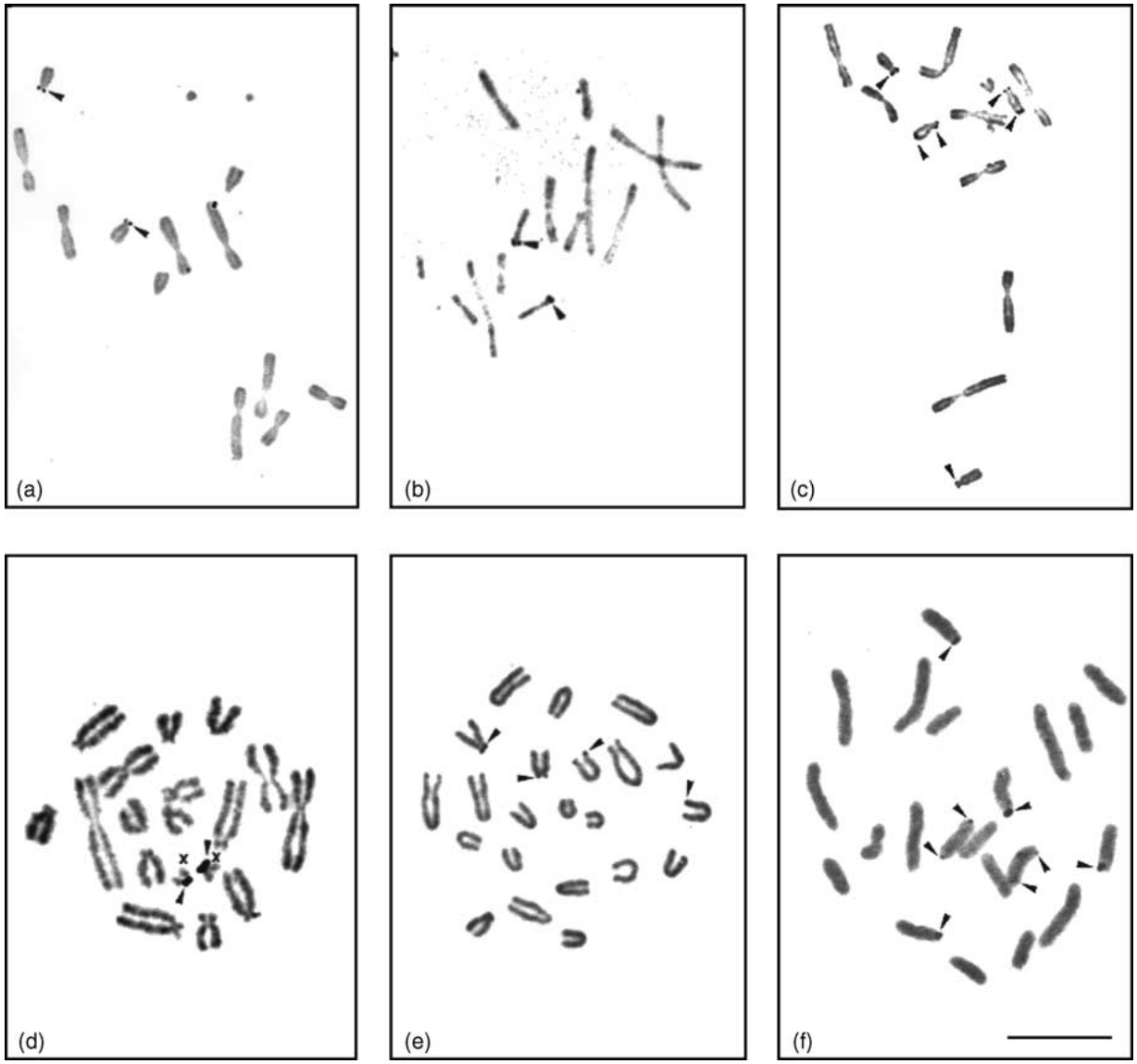

Figure 2. NORs after silver-staining in males: (a) *Metachirus nudicaudatus*, (b) *Marmosops incanus*, and (c) *Micoureus demerarae* (all with $2n = 14$) and in females: (d) *Monodelphis domestica* ($2n = 18$), (e) *Philander opossum* and (f) *Didelphis marsupialis* (both with $2n = 22$). NORs are indicated by arrowheads. Bar = 10 μm.

somes (pairs 1 and 2), four large subtelocentrics (pairs 3 and 4) and eight medium-sized subtelocentrics/acrocentrics (pairs 5–8). The X chromosome is a small acrocentric and the Y chromosome is dot-like (Merry, Pathak & Vandeberg, 1983).

After *in situ* hybridization, ribosomal cistrons were detected on the short arms of chromosomes 5 and X. Therefore, females have four NORs and males, only three (Figure 1(d)), as previously described by Merry, Pathak and Vandeberg (1983) and Pathak et al. (1993). In every cell analyzed in females, hybridization signals on the X chromosomes were clearly larger than those on the autosomes, pointing to a larger number of ribosomal cistrons on the sex chromosome. In addition, NORs varied in size between the two X chromosomes (Figure 1(d)). In the male, the signals observed on the autosomes and on the X chromosome had approximately the same size. Silver-staining revealed the activity of only two NORs on the short arms of the X chromosomes in 34 cells of the two females.

Differences in the size of Ag-NORs between the two X chromosomes were in accordance with those observed after *in situ* hybridization (Figure 2(d)). In only one cell, there were four NORs, two on the sex pair and two much smaller on the short arms of pair 5. In 49 cells from the male, three patterns were found: three NORs, located on the X chromosome and on pair 5 (24 cells); two NORs, one on the X chromosome and the other on one chromosome 5 (21 cells); and one NOR, located on the X chromosome (four cells).

Concerning Ag-NORs, our observations confirmed those of Merry, Pathak and Vandeberg (1983). Together, these results demonstrate that: (1) in male and female *M. domestica*, the NORs on the X chromosome are always active, while the autosomal NORs have variable expression; (2) the ribosomal cistrons on the X chromosome are not subject to inactivation, as female *M. domestica* always presented NOR activity on both X chromosomes.

NORs in the karyotypes with 2n = 22

All chromosomes are acrocentric in the karyotypes of *Philander opossum* and *Didelphis marsupialis* ($2n = 22$) (Yonenaga-Yassuda et al., 1982).

In *P. opossum*, *in situ* hybridization with probe HM456 showed four NORs, two on the short arms of a pair of medium-sized autosomes and two at the end of the long arms of a slightly smaller autosome pair (Figure 1(e)). These regions showed activity in two females (80 cultured cells and 42 bone marrow cells), as documented after silver-staining (Figure 2(e)). These NOR-bearing chromosomes had already been identified as pairs 5 and 7 by Yonenaga-Yassuda et al. (1982) after simultaneous analysis of G-bands and silver-staining patterns.

In *D. marsupialis* (one male and two females), *in situ* hybridization revealed at least four NORs per cell, located at the ends of the long arms of two medium-sized autosome pairs. In several cells there were signals also at both ends of a medium-sized autosome pair, totaling eight NORs per cell (Figure 1(f)). These additional NORs were smaller than the other four consistently present in all cells. In some cells from females, we observed additional very small signals on the X chromosomes and at the ends of some autosomes. It is possible that they indicate small amounts of ribosomal cistrons, but we cannot exclude nonspecific hybridization.

The distribution of Ag-NORs was analyzed in 30 cells from one male and in 95 cells from one female of *D. marsupialis*. In both instances, at least four NORs were observed, at the ends of the long arms of two pairs of medium-sized chromosomes. The maximum number of Ag-NORs was seven in the male and eight in the female due to the presence of NORs on the long and short arms of another medium-sized pair (Figure 2(f)). Silver-staining of this pair was always fainter than that of the chromosomes with a single NOR, and corresponded to smaller signals of the rDNA probe. Yonenaga-Yassuda et al. (1982) reported one to nine Ag-NORs in cells from two specimens of *D. marsupialis*. NOR distribution was the same as in our specimens, but the presence of nine NORs in two cells led these authors to suggest that there was at least another pair with NORs. On the other hand, Casartelli, Rogatto and Ferrari (1986) observed NORs only at the ends of the long arms of three autosome pairs in this species. These results suggest that in *D. marsupialis*, besides larger and frequently expressed NORs located at the ends of the long arms of two autosomal pairs, there are NORs with smaller numbers of ribosomal cistrons showing variable expression that are generally located on the short arms of autosomes.

Two other species of American marsupials with $2n = 22$ had their NORs previously described: *D. albiventris*, with eight Ag-NORs, as observed in *D. marsupialis*, and *Lutreolina crassicaudata*, with four NORs, as in *P. opossum* (Seluja et al., 1984; Casartelli, Rogatto & Ferrari, 1986).

The evolution of NORs in Didelphidae

Analysis of NOR distribution in American marsupials with $2n = 14$ revealed that pair 6, the smallest autosome pair, has NORs on its short arms in all species in which this chromosome could be precisely identified. In species with more than two NORs, pair 5 frequently has NORs on the short and/or long arms. More rarely, additional NORs are present on larger chromosomes, as described in *Marmosa murina* (Souza, Maia & Santos, 1990). The NOR-bearing pair 5 in *M. domestica*, $2n = 18$, is homeologous to pair 6 in species with $2n = 14$ (Svartman & Vianna-Morgante, 1999), reinforcing the idea that the presence of NORs on this chromosome is a conserved condition.

Species with 22 chromosomes present very similar karyotypes and possibly have NORs at the same location. Thus, the NOR-bearing pairs 5 and 7 in *P. opossum* (Yonenaga-Yassuda et al., 1982), the species with the smallest number of NORs, may also be the pairs that always bear NORs in the two

species of *Didelphis*. Pair 7 in species with $2n = 22$ corresponds to pair 6 in species with $2n = 14$ and to pair 5 in the $2n = 18$ karyotype (Svartman & Vianna-Morgante, 1999). These homeologous pairs are NOR-bearing chromosomes in these three karyotypes, although NORs are located on the long arms in *P. opossum* and on the short arms in the species with $2n = 14$ and $2n = 18$ chromosomes. Therefore, in Didelphidae, in spite of the interspecific variation regarding the number and location of NORs, at least one NOR-bearing autosome pair is conserved in all karyotypes.

Monodelphis domestica is the only species of Didelphidae with a NOR on the X chromosome. Among Australian marsupials, several examples of NORs on X chromosomes were reported, as in the genus *Potorous* (Johnston, Davey & Seedbeck, 1984) and in *Trichosurus vulpecula* (Murray, 1977). The family Macropodidae is specially interesting, including about 30 species with NORs only on the X chromosome and, in some cases, also on the Y chromosome. When located on the X chromosome, NORs are active on both chromosomes in females.

Acknowledgements

The authors are indebted to Ligia S. Vieira and to Amauri C. Marcato for technical assistance, and to Dr Meika A. Mustrangi, Dr Helder José and Prof. Jenny Graves, who provided us with marsupial material. This work was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

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