Heredity **75** (1995) 10–16 *Received 24 August 1994*

Cytogenetic peculiarities in the Algerian hedgehog: silver stains not only NORs but also heterochromatic blocks

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Hedgehogs belong to one of the several mammalian taxa in which karyotype differences are based on variations in heterochromatin content. Furthermore, the number and location of nucleolar organizer regions (NORs) can also vary widely. In the present study these cytogenetic features were investigated in the Algerian hedgehog, *Erinaceus (Aethechinus) algirus*. The heterochromatin and NOR distribution patterns in the karyotype of this species are new among hedgehogs, whereas the euchromatic regions, including their G-band pattern, are similar to those reported by others. In addition, silver staining revealed a cytogenetic feature exclusive to the heterochromatic blocks of *E. algirus*: their silver staining with standard cytogenetic procedures. Because no similar phenomenon has been described previously in a mammalian species, several hypotheses about the significance and specificity of silver staining to NOR sites are discussed. Finally, the existence of different types of heterochromatin in the species analysed here, lead us to propose that what hedgehogs have inherited from their common ancestor is a mechanism which permits the accumulation of heterochromatin on specific chromosomes, rather than the heterochromatin itself.

Keywords: *Erinaceus algirus*, heterochromatin variation, karyotype evolution, NOR variation, silver staining.

Introduction

Hedgehogs have been the subject of several interesting cytogenetic studies, mainly devoted to geographical variations in the content of autosomal constitutive heterochromatin and their connection with the number and localization of nucleolar organizer regions (NOR) (for a review, see Zima & Kral, 1984). Several karyotypic variants have been described in European species of the genus *Erinaceus* (*E. europaeus* and *E. romanicus*), which differ in the presence and location of large autosomal blocks of constitutive heterochromatin (Gropp, 1969; Mandahl, 1978; Sánchez *et al.*, 1994). The distribution of NORs in these species was studied by Mandahl (1979), who showed the existence of intraindividual, interindividual and interpopulational variations for the distribution of these chromosome regions in karyotypes.

The karyotype of the Algerian hedgehog, *Erinaceus* (*Aethechinus*) *algirus*, has been analysed by Gropp & Natarajan (1972), who presented evidence for the presence in this species of large heterochromatic blocks. In this species, however, their relationship with NORs could not be established, as Ag-staining techniques were not available when this work was done.

In this paper, we report karyotypic analyses in this species with newer, more powerful cytogenetic techniques such as Ag-staining of NORs, C-banding, fluorescent staining and *in situ* hybridization, which make it possible to characterize more precisely both constitutive heterochromatin and NORs, and to establish possible relationships between them. We discuss several interesting cytogenetic peculiarities of the Algerian hedgehogs such as silver stainability of the heterochromatic blocks and the existence of inter- and intraindividual variability for the number and size of NORs.

Materials and methods

A total of five individuals (two males and three females) of the species *Erinaceus algirus* were trapped live in northern Morocco. Chromosome preparations were made following our standard air-drying procedure (Burgos *et al.*, 1986), either directly from bone marrow cells or after culture of spleen lymphocytes.

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Chromosomes were G-banded according to our combined method (Burgos *et al.*, 1986), and C-bands were revealed by the method of Sumner (1972). For Ag-staining, standard chromosome preparations were placed in a moist chamber at 60°C and exposed for 3–5 min to a 100 per cent (w/v) solution of silver nitrate in deionized water adjusted to pH 3.1 with formic acid (Sánchez *et al.*, 1989a).

For *in situ* hybridization to fixed metaphase chromosomes we used a Syrian hamster rDNA probe consisting of sequences corresponding to the 3′ end of the 18S gene, the internal spacer, and almost the entire 28S gene (see Wahl *et al.*, 1983). Nick translation labelling with digoxigenin and hybridization were done according to the protocol recommended by the supplier (Boehringer Mannheim).

Results

Karyotype

Because *E. algirus* has the same number of chromosomes ($2n = 48$) as the rest of the species in this genus (e.g. *E. europaeus*), karyotypes were arranged according to Mandahl (1978) to facilitate comparisons (Fig. 1). Autosomal chromosome pairs 1–11 represented a series of metacentric elements of decreasing size; pairs 12–14 were large subtelocentrics; pairs 15–20 were medium-sized submetacentrics; pair 21 was a very small telocentric, and pairs 22 and 23 were small metacentric chromosomes. Sex chromosomes were represented by a meta-submetacentric X and a dot-like metacentric Y. On the basis of this karyotype arrangement, morphological differences with respect to other karyotypic forms described in hedgehogs were found in pair 14 (a large subtelocentric) and pair 21 (a small telocentric) in *E. algirus*. In addition, secondary constrictions were located centrally on the long arm of pair 13, and near the centromere in pairs 21 and 22. These secondary constrictions were variable in size, and consequently, they were not clearly distinguishable in all metaphase plates.

This description coincides basically with that reported by Gropp & Natarajan (1972) for this species, although these authors presented a different karyotype arrangement.

G-banding

G-banding patterns were nearly identical in Algerian and European hedgehogs, except, of course, in those chromosome pairs in which morphological differences were found (Fig. 2). Interestingly, two large G-negative segments were found on chromosome pairs 13 and 14, denoting sensitivity to trypsin in these regions.

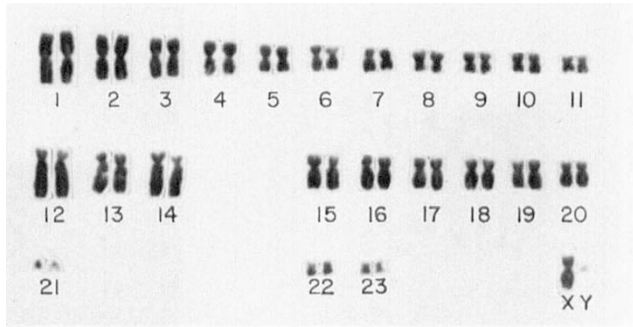

Fig. 1 Conventional Giemsa-stained karyotype in *Erinaceus algirus* ($2n = 48$). In this cell, only secondary constrictions on chromosome pair 13 are clearly visible.

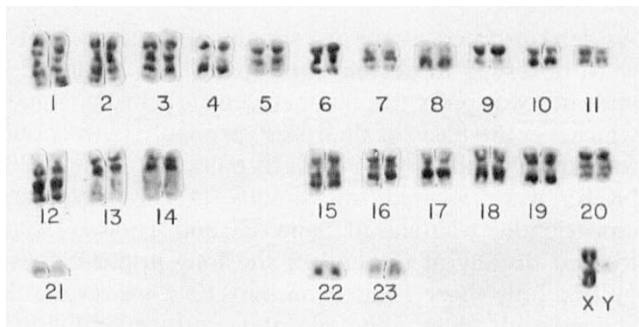

Fig. 2 G-banding pattern in the Algerian hedgehog. Note the G-negative condition of two large segments on pairs 13 and 14, the entire chromosome of pairs 21 and 23, and the Y chromosome.

Similarly, pairs 21 and 23 as well as the Y chromosome were also entirely G-negative.

C-banding

In this species, heterochromatin is clustered in two large C-positive blocks, located distally on pairs 13 and 14 (Fig. 3a), and coinciding with the large G-negative regions described above for these chromosomes. The rest of the chromosomes are C-negative.

Fluorochrome staining

Chromomycin A_3 showed that these heterochromatic segments are chromomycin positive, which implies that they are C–G rich (Fig. 3b). The G-negative small chromosome pairs 21 and 23 were also chromomycin positive, despite the fact that they are not heterochromatic. The secondary constrictions on pair 13 showed the strongest fluorescence, indicating that they are particularly rich in C–G base pairs.

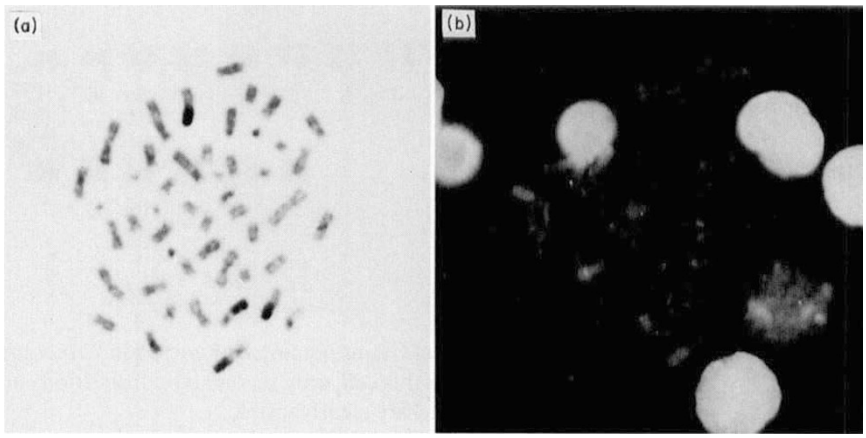

Fig. 3 Constitutive heterochromatin of *Erinaceus algirus*. (a) C-banding showing two large C-positive blocks on pairs 13 and 14. (b) Staining with chromomycin A_3 showing that the heterochromatic blocks on pairs 13 and 14, and the small chromosome pairs 21 and 23, are rich in C–G base pairs; the secondary constrictions on pair 13 showed the brightest fluorescence, suggesting that they are especially rich in C–G base pairs.

Silver staining

As demonstrated by the Ag-staining technique, NORs were observed in chromosome pairs 13, 21 and 23, and in two pairs of submetacentric chromosomes which, on the basis of their size, probably correspond to pairs 15 and 16 (Fig. 4a). In pairs 13, 21 and 23, NORs were located interstitially at the secondary constriction, whereas in pairs 15 and 16 they were located distally at the end of the long arm. Of these NORs, only those located on pair 13 were invariably stained with silver, although inter- and intraindividual size variability was observed. However, staining of the rest of the NORs was variable depending on both the individual and the cell. This variability arose mainly from the NOR-bearing submetacentric chromosomes as there were clear interindividual differences with respect to the most frequently found number of these chromosomes that were positive for Ag-staining.

In addition to showing the position of NORs (see below), Ag-staining provided another unexpected finding. In most cells observed in chromosome preparations treated for NOR detection, the heterochromatic blocks on pairs 13 and 14 appeared clearly stained by silver, a finding which has not been reported before in mammalian chromosomes (Fig. 4b).

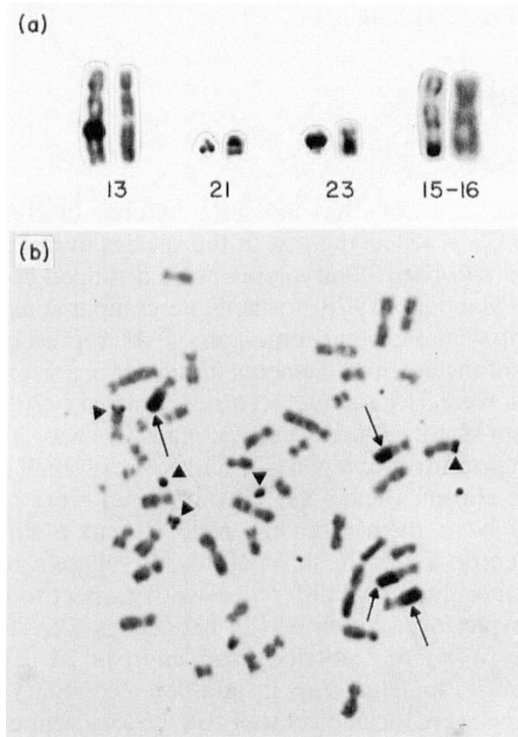

Fig. 4 Silver staining of *Erinaceus algirus* chromosomes. (a) Location of NORs on chromosome pairs 13, 21 and 23 (interstitially), and on pairs 15 and 16 (distally). To compare chromosomal morphology and the position of the secondary constrictions, conventional Giemsa-stained chromosomes are shown to the right of silver-stained chromosomes. (b) Metaphase plate showing Ag-staining of the entire heterochromatic blocks on chromosome pairs 13 and 14 (arrows) and several stained NORs (arrowheads).

In situ *hybridization*

This technique corroborated the position of NORs shown by silver staining and demonstrated that variability in Ag-staining may result from the existence of polymorphism in the number of ribosomal cistrons in different NOR sites. Thus, heteromorphism for the hybridization signals was frequent, mainly in chromosome pair 13. Furthermore, one of the individuals analysed showed only three, instead of the expected four submetacentric chromosomes with evident hybridization signals, suggesting that one of them had lost most, if not all, of its ribosomal cistrons (Fig. 5). In addition, *in situ* hybridization showed that the heterochromatic blocks lack ribosomal cistrons in their entire length, except in the secondary constriction on pair 13, where a NOR is located. Hence, silver stainability cannot be attributed to a putative NOR condition of these heterochromatic blocks.

Table 1 summarizes the results obtained with all these different techniques.

Discussion

Our results consistently support the view (Mandahl, 1978) that variations in the content and location of blocks of constitutive heterochromatin are a fundamental feature of the karyotypic evolution in the genus *Erinaceus*. This feature is shared by several other mammalian taxa (Fredga & Mandahl, 1973; Dev *et al.*, 1975; Ando *et al.*, 1980; Mandahl & Fredga, 1980; Baverstock *et al.*, 1982; Yosida & Kodama, 1983; Freitas *et al.*, 1984; Barros & Patton, 1985). A variety of karyotypic forms has been described for different species and geographical races of this genus, in which clear conservation of the euchromatic regions, as shown by the constancy in both chromosomal number and morphology and in the G-band patterns, contrasts strongly with marked variability in the number and location of their C-positive, heterochromatic blocks. On the basis of this variability, five karyotypic forms were established in hedgehogs from Northern Europe (Mandahl, 1978), an additional form was established in Southern Europe (Sánchez *et al.*, 1994), and a new variation was found in the Chinese hedgehog (Yongshan & Ficin, 1987). The karyotype described here, which basically coincides with that previously reported by Gropp & Natarajan (1972) for *E. algirus*, represents a further form.

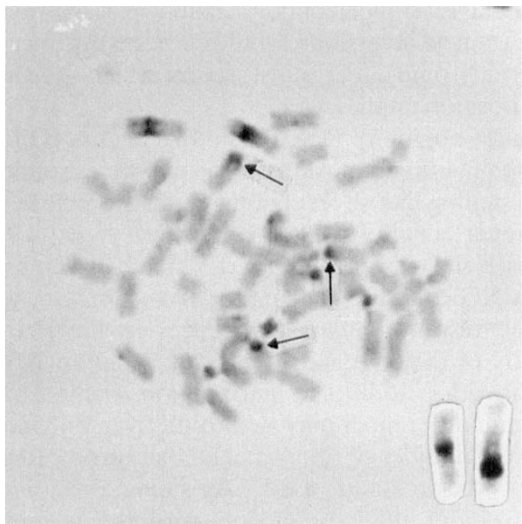

Fig. 5 *In situ* hybridization with an rDNA probe on chromosomes of *Erinaceus algirus*. Intraindividual heteromorphism between homologous chromosomes (inset, pair 13) and interindividual polymorphism for the number of NOR sites were frequent; in this cell only three (instead of the expected four) submetacentric chromosomes showed evident hybridization signals (arrows).

Table 1 Results of the application of different banding techniques to significant chromosomes or chromosome regions of *Erinaceus algirus*

Chr	G	C	CMA	AG	NOR	Location	ISH
13h	−	+	+*	+	+	i	+
14h	−	+	+	+	−		−
15	±	−	±		+	t	+
16	±	−	±		+	t	+
21	−	−	+		+	i	+
23	−	−	+		+	i	+

13h: Heterochromatic block of the chromosome 13.
14h: Heterochromatic block of the chromosome 14.
AG: Ag-staining of constitutive heterochromatin.
NOR: NOR carrier as detected by Ag-staining.
Location: Location of NORs; i, interstitial; t, terminal.
ISH: *in situ* hybridization with rDNA probe.
*: Secondary constriction showed the strongest fluorescence.

In addition to the variability in size and location of the heterochromatic blocks in hedgehogs, there seem to be some qualitative differences between them, at least in *E. algirus* heterochromatin. This idea is supported by the positive silver staining we have shown and the particular requirements for C-banding (Gropp & Natarajan, 1972). This qualitative heterogeneity may imply heterogeneity in the origin of the heterochromatic blocks, so that the situation in hedgehogs may be similar to that described in several species of the genus *Microtus*: large heterochromatic blocks are present on their sex chromosomes, and different types of heterochromatin have been found with molecular (Modi, 1993a,b) and cytogenetic analyses (Sperling *et al.*, 1985; Burgos *et al.*, 1988, 1990). Hence, as suggested for *Microtus* (Burgos *et al.*, 1992) it is possible that some chromosomes in *Erinaceus* acquired from a common ancestor the ability to accumulate constitutive heterochromatin, rather than the heterochromatin itself.

The nature of the heterochromatin in the C-positive blocks of *E. algirus* deserves further discussion, as it shows a feature unique among mammals, i.e. positive silver staining. This raises two points that have been widely debated for the last several years: the significance of silver staining of mitotic chromosomes, and its specificity for NOR sites. The view that silver specifically stains transcriptionally active NORs, based on the clear correlations between the two events (transcription activity and silver staining) (see Hubbell, 1985), has repeatedly been contradicted by a number of authors, who have suggested that silver stainability of NORs is exclusively dependent on the decondensed state of NOR chromatin (see Medina *et al.*, 1983;

Sánchez-Pina *et al.*, 1984). More recently, Jiménez *et al.* (1988) demonstrated that decondensation of NOR chromatin is necessary but not sufficient for silver staining to occur, and suggested that previous transcriptional activity may also be needed. However, the fact that silver also stains chromosomal regions other than NORs, as demonstrated in amphibians (Varley & Morgan, 1978), complicates the problem even further. The fact that this phenomenon has been observed exclusively in lampbrush chromosomes of amphibian oocytes seems to preserve the traditionally accepted hypothesis that silver specifically stains NOR sites in mammalian mitotic chromosomes (see King, 1980), but our finding that not only NORs but also heterochromatic blocks in *E. algirus* are silver stainable refutes this assumption.

Silver stainability of any biological material is strongly dependent on the conditions of fixation, Ag-NOR staining being specific for conventional cytogenetic alcohol–acetic acid fixed chromosomes (see Hubbell, 1985). However, silver staining of the heterochromatic blocks in *E. algirus* cannot be attributed to this effect, as the conventional methods we used for both fixation (methanol–acetic acid) and staining led to no significant modifications. This situation is clearly different from that reported by Mandahl & Fredga (1980) for mustelids, in which silver staining of heterochromatin was observed only after the chromosome preparations had been overexposed to the silver nitrate solutions.

Several investigations have led to the biochemical identification of the proteins involved in cytogenetic silver staining. Hubbell *et al.* (1979) isolated three different nucleolar proteins, designated C23, B23 and Ag-NOR, although it is not clear which of them, if any, is responsible for specific Ag-staining of mitotic NORs (Lischwe *et al.*, 1979; Williams *et al.*, 1982; Hubbell, 1985). In any case, because of their nucleolar origin, probably none of these proteins is responsible for the heterochromatin silver staining observed in *E. algirus* mitotic chromosomes. Since it is clear that silver stains some kinds of chromosomal proteins – probably those of an acidic nature (Schwarzacher *et al.*, 1978; Buys & Osinga, 1980) – a similar protein may be responsible for the silver staining of heterochromatin we observed, and may represent a unique characteristic with respect to the other types of heterochromatin studied to date. Further biochemical research will be necessary to test this hypothesis, and any other considerations on the composition and/or structure of this heterochromatin would be speculative for the moment.

With regard to the position of NOR sites in the karyotype, *E. algirus* also shows a feature unique among hedgehogs: this is the only species in which NORs are located interstitially, the rest of the species showing distally located NORs, generally associated with the heterochromatic blocks (Mandahl, 1979). To explain the marked variability in NOR location in the five karyotypic forms of hedgehogs from Northern Europe, Mandahl proposed a mechanism of transfer of NORs based on nonhomologous exchange between distal regions of the associated heterochromatin. Although a similar mechanism may have operated in *E. algirus* to permit interchromosomal transfer of NORs, additional rearrangements, probably pericentric and/or paracentric inversions, would be necessay to explain the intrachromosomal translocation which their interstitial position implies.

Finally, polymorphism for the size of Ag-NORs is a common feature in most mammalian species studied so far. Assuming that silver staining reflects previous transcriptional activity of NORs, Sánchez *et al.* (1989b) proposed the existence of interference between homologous NORs in the rodent *Eliomys quercinus*, which may be responsible for differences in the size and intensity of the Ag-bands. However, *in situ* hybridization provides additional information, demonstrating that these differences may also result from polymorphism in the number of ribosomal DNA cistrons. Regardless of the method used (Ag-staining or *in situ* hybridization), this type of polymorphism is common in animals, and has been described in a variety of taxa, including *Drosophila* (Ritosa & Spiegelman, 1965), amphibians (Schmid, 1982), turtles (Bickham & Rogers, 1985), fishes (Galetti *et al.*, 1985) and mammals such as marsupials and humans (Goodpasture & Bloom, 1975; Varley, 1977; Merry *et al.*, 1983). In general, the authors agree that the occurrence of deletions or duplications of the NOR chromatin is the simplest explanation for this polymorphism. Although these rearrangements probably take place by asymmetrical recombination during meiosis, thus giving rise to interindividual variability, recombination between homologous NORs and/or sister chromatid exchanges in somatic cells must also occur to give rise to the intercellular variability observed in most individuals analysed. Associations between homologous and nonhomologous NORs which persist until mitotic metaphase in European hedgehogs (Mandahl, 1978) and in other mammals such as the Spanish mole (*Talpa occidentalis*; our unpublished data) are consistent with this idea.

Acknowledgements

We would like to thank Dr Thelma Slezyger from the Universidad Simón Bolivar (Venezuela) for providing us with the rDNA probes used in this study, and Ms

Karen Shashok for revising the English style of the manuscript. This work was supported by the Spanish DGICYT through Project No. PB92-0951.

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