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RESEARCH ARTICLE

Studies on Silver staining of chromosomes of caecilians (Amphibia:Gymnophiona) of Western Ghats of India

***Venu G**

Centre for Applied Genetics, Department of Zoology, Bangalore University, Bangalore, Karnataka, India.

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****Corresponding Author***

Venu G

Abstract

Chromosomal localization for the ribosomal cistrons in the mitotic metaphase complements of caecilian chromosomes was examined by the application of silver nitrate staining, thereby highlighting of NOR regions. At least, two primary Ag-NORs were localized on the telomeric regions of two pairs of chromosomes in the complement, invariably; one, two or more secondary NORs were seen on different chromosomes that seemed to pose a common occurrence. The number and locations of Ag-NO_3 has generally been found characteristic although of variable nature throughout their genome. Interchromosomal, intercellular and interindividual variability of NORs, is a common sight, may reprimand of their genetic significance that could not be dismounted.

Introduction

Seshachar (1937a, b, 1939, 1944a, b) who had pioneered in the Indian caecilian cytogenetic analyses based on alpha karyological assessment involving three genera of Western Ghats of India, suggested a common Fundamental Number (FN) of 52, perhaps occluding to variable cytological manifestations implied to have occurred during the course of chromosomal derivation processes. The chromosomal reduction hypothesis put forward by Morescalchi (1973, 1975) for amphibian phylogeny, referring in particular to caecilians (Morescalchi, 1977, 1980) emphasizes on the gradual reduction of basic number and further upon consequent loss of microchromosomes as one road towards the advanced forms (however, with certain exceptions). This is obvious in the light of some additions made in the field (Nussbaum, 1991; Venkatachalaiah and Venu, 2002; Venu and Venkatachalaiah, 2005, 2006; Matsui *et al.* 2006; Venu, 2008). Support to this view derives from the basic karyotype of the more advanced caecilian family Typhlonectidae and Caeciliidae consists of low basic number involving meta and submetacentric chromosomes (Nussbaum, 1991). In contrast, the primitive families are distinguished by comparatively high chromosome number and the presence of medium sized acrocentric to telocentric chromosomes as well as very small microchromosomes. Experiments based on detailed analyses of conventional stained karyotypes have indicated that the evolution of primitive Ichthyophiidae and Uraeotyphlidae to the higher Caeciliidae has resulted in a reduction of the number of acrocentrics and microchromosomes in favour of larger metacentric and submetacentric chromosomes and with reduction in chromosome number. This process can be interpreted to have been occurred by centric fusions and tandem translocations which took place between telocentrics and acrocentrics and/or microchromosomes (Venkatachalaiah and Venu, 2002; Venu and Venkatachalaiah, 2005, 2006; Venkatachalaiah et al., 2006; Venu, 2008, 2013). Considering Indian caecilians as a group the conventional staining of chromosomes and the karyotypes prepared from such chromosomes would provide criteria counting upon the number and morphology of the chromosomes in the metaphase complement (relative length, centromere index and arm ratio). The data obtained from these investigations gave first hand information indicating the processes that had taken place during the course of chromosome evolution. From these, it is also possible to infer in general that only the degree of chromosomal relatedness between individual species could be determined. However, in order to obtain a more detailed knowledge of the characteristics of individual caecilian chromosomal lineages, the use of differentially stained preparations is

not only imminent but also obligatory, since these methods could help in procuring multiple cytogenetic datasets that could eventually lead to eliciting cytogenetic data based on molecular architecture of chromosomes.

Beta karyology (White, 1978) of caecilians (and of other amphibians) is well documented but literature pertaining to typical banding studies in this group is not recorded. This fact is dogged by difficulties in obtaining good quality G-bandings as well as other differentially stained sequences in this group of animals. It is well–known that Giemsa and fluorochrome based differentially stained chromosomal preparations helps in providing significant information about chromosomal substructures (Sumner, 1990, 2003). Such an extension of staining protocols helps tremendously in the identification of individual chromosomes in the complement as well as in assessing their role in chromosomal rearrangements, and thereby helping in establishing chromosome homeology (as compared to the normally prevailing homology) and in highlighting of status of sex chromosomal differentiation. Thus, a great deal about the cytogenetic changes have been incurred during speciation and karyotypic evolutionary processes (White, 1973, 1978; Schmid, 1980; King, 1990; Sumner, 2003; Kasahara et al., 2003). Until such time, any attempts on karyotypic assessments of caecilians will have to rely only upon beta karyological information.

In addition to classical chromosomal study, differentially banded sequences (such as C-, Q-, G-, R-, NOR bands) and other traditional bands are likely to contribute substantially in amphibian phylogenetic research with an emphasis on the conserved linkage groups and the mechanism of sex determination. Some linkage groups are conserved throughout vertebrates and others show levels of variability within and among families of amphibians. Recent work on chromosome based sex determination in amphibians has revealed a wealth of information that takes derivation from studies of amphibian phylogeny (Hillis and Green, 1990). In fact, the boundary between molecular genetics and cytogenetics has closed to the point that it is difficult to distinguish the two fields. As molecular technological approaches invade into cytogenetics, it will have ample opportunity to contribute to furthering our knowledge of higher levels of amphibian phylogeny (Schmid, 1980; King, 1990; Schmid et al., 1990; Schmid and Steinlein, 1991; Mancino, 1994).

In the present paper, an attempt has been made to localize secondary constriction regions onto interphase nuclei and mitotic metaphase chromosomes of caecilian species procured from different parts of Western Ghats of India and their role in evolution is discussed.

Materials and Methods

For the staining of active NOR regions of metaphase chromosomes of three genera of caecilians namely *Ichthyophis*, *Uraeotyphlus* and *Gegeneophis*, a silver impregnation technique of Elder and Pathak (1980) was followed. 50% $AgNO_3$ solution was prepared in deionized water and a 3% formalin solution was added to it. Four to five drops of this fresh mixture were placed on each horizontally placed slide and incubated in a water bath at 50^0C till the coverslip turned golden yellow. The slides were washed thoroughly in distilled water and a counter staining was done with a 2% solution of dilute Giemsa stain for about a minute. The slides were rinsed in deionized water and air dried. Well-spread mitotic metaphase spreads were photographed using Leitz Orthoplan photomicroscope. Karyotypes were prepared in accordance with Levan et al. (1964), Venkatachalaiah and Venu (2002) and Venu et al. 2011.

Results

One of the interesting aspects of the $AgNO_3$ staining of the Indian caecilian chromosomes has been the conserved characteristics and of the invariable and consistent presence of $AgNO_3$ spots on the short arm of chromosome number 9 in the complement and inadvertently and occasionally of their presence on the longer arm terminal region bear an additional NOR region.

1. NOR studies in Ichthyophiidae

As demonstrated by the conventional silver nitrate staining technique, NORs were detectable in two additional chromosome pairs predominantly and in lesser conspicuousness in 1 or 2 other chromosomal pairs. All the species of *Ichthyophis* seem to bear a general similarity in stainings.

Two primary AgNORs were located on the telocentric regions on the long arm of chromosome pairs 17 and 19. On rare occasions, one to two secondary NORs were also observed. The secondary AgNORs were seen on different chromosomes. There was variability in expression patterns as regards to one or other chromosomes in the pair as well as the sizes of AgNORs which are highly variable and found at inter-individual and inter-populational level.
The number and locations of Ag-NORs has generally been found to be characteristic, although variable among caecilians. Both *I. beddomei* and *I.* cf. *beddomei* are characteristics for possessing a prominent secondary constriction on the distal end of long arm of chromosome pair 1, but was not represented by the expected classical NOR site, which is intriguing to a certain extent (Fig. A).

2. NOR Studies in Uraeotyphlidae

In the four species of *Uraeotyphlus*, two pairs of chromosomes (no. 9 and 13) bear AgNORs on the distal region of the short arm. Occasionally, other chromosomes (for example, chromosome pair 1) and invariably on the smaller acrocentrics, minor NORs could be traced (Fig. C).
Most populations of the species of *U. gansi* have two NORs on the long arm proximal regions of chromosomes 17 and 19; otherwise chromosome 10 bears NORs which appear to be polymorphic in nature (Fig. B).

3. NOR Studies in the genus *Gegeneophis*

AgNORs in the genus *Gegeneophis* present an intra-and inter-specific variation in number and a remarkable size heteromorphism. The number of AgNORs ranges from a single (*G. krishni*) (Fig. F) to 2-4 in other species (*G. ramaswamii* (Fig.D), *G. seshachari* (Fig. E), *G. madhavaorum* (Fig.G), and *G. nadkarnii* (Fig. H)) and even 5-6 sites invariably show commonly a secondary constriction on the short arms of 13th pair. The size variation of such constrictions resulted in heteromorphism between homologues. Interindividual variability in number and position of AgNOR was detected in *G. nadkarnii* and *G. danieli*. There were occasions where Ag-NORs ranged from 2-8 on telomeric regions of some metaphase chromosomes in each of the four species examined as a frequent occurrence. Whereas *G. ramaswamii* karyotype is characterized by a distinct telomeric NOR regions on the long arm of chromosome number 2.

4. NORs in diploid nuclei

In the diploid interphase nucleus from the somatic tissues (for example bone marrow, liver) of the present study, revealed that there are distinctly stained AgNOR blocks. Since in majority of diploid karyotypes a maximum of 3-4 Ag-NOR blocks could also be expected especially in interphase nuclei. These blocks could be representing apparently fusion of one-two or perhaps more homologous NORs (Fig. I).

Fig. A. Silver stained metaphase karyotype of *Ichthyophis beddomei*

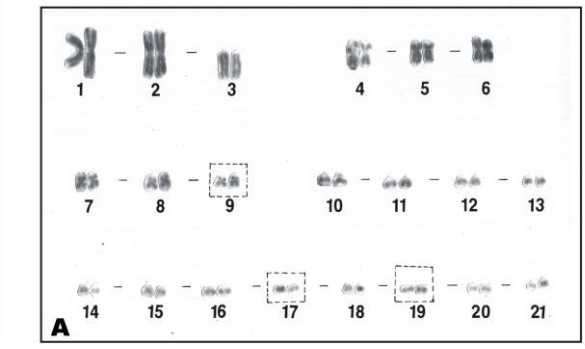


Fig. B. Silver stained metaphase karyotype of *Uraeotyphlus gansi*

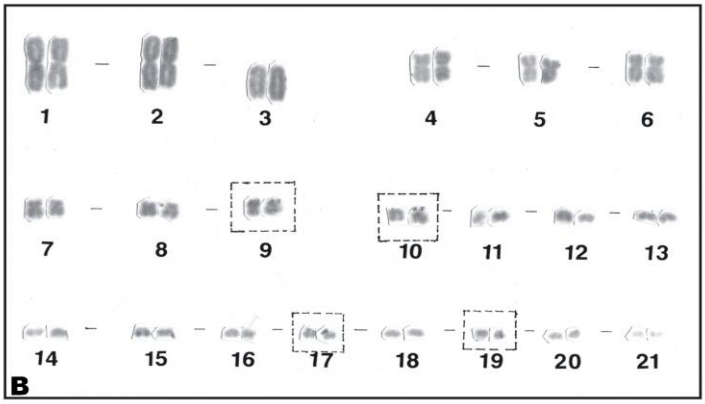


Fig. C. Silver stained metaphase karyotype of *Uraeotyphlus interruptus*

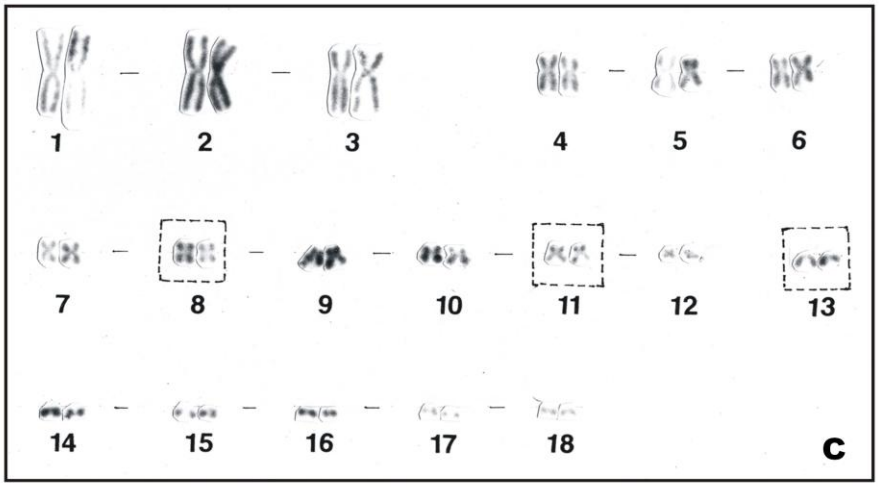


Fig. D. Silver stained metaphase karyotype of *Gegeneophis ramaswamii*

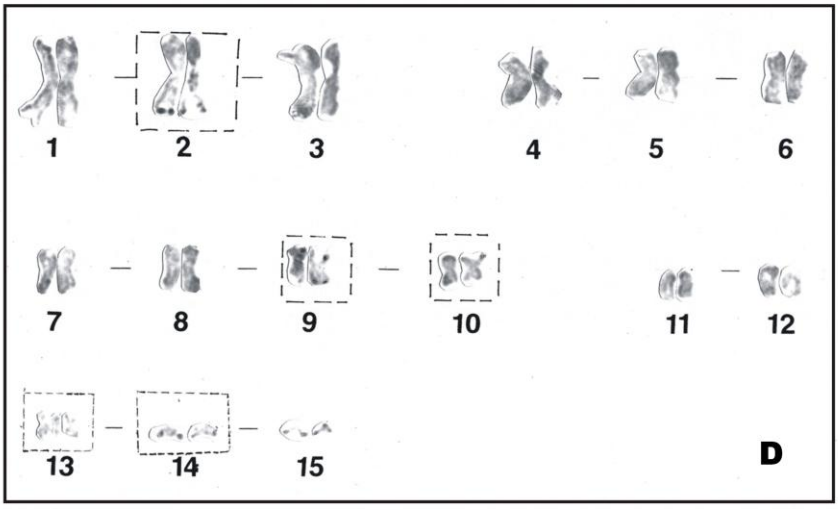


Fig. E. Silver stained metaphase karyotype of *Gegeneophis seshachari*

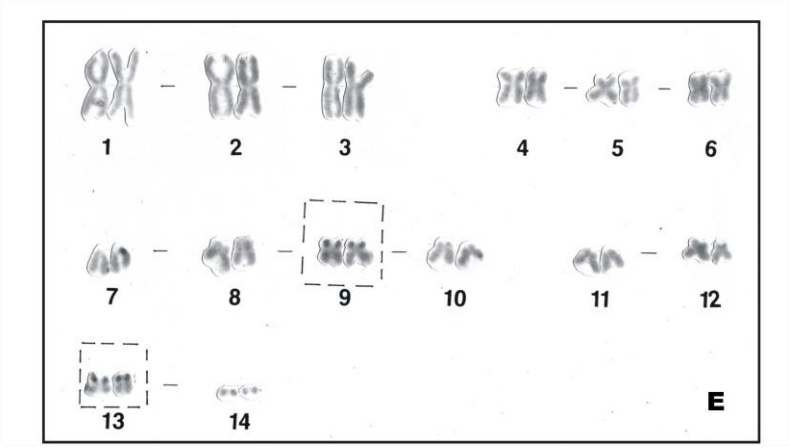


Fig. F. Silver stained metaphase karyotype of *Gegeneophis krishni*

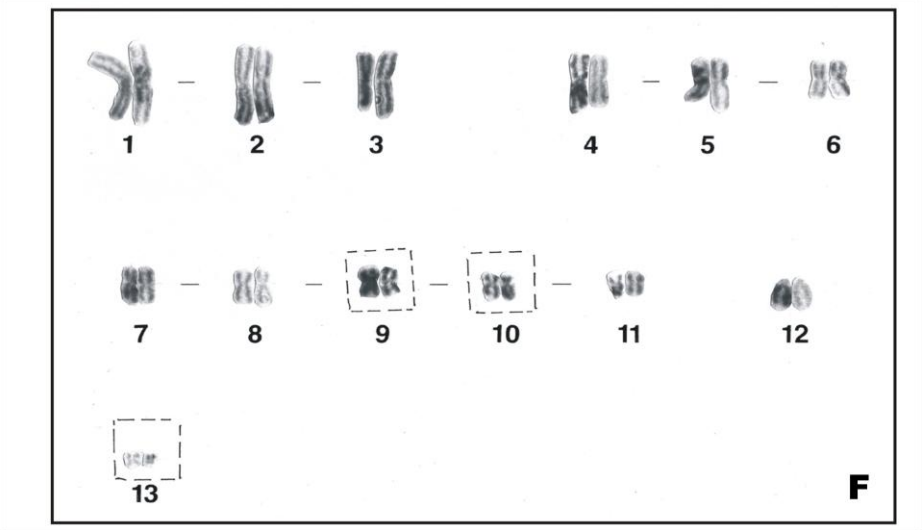


Fig. G. Silver stained metaphase karyotype of *Gegeneophis madhavaorum*

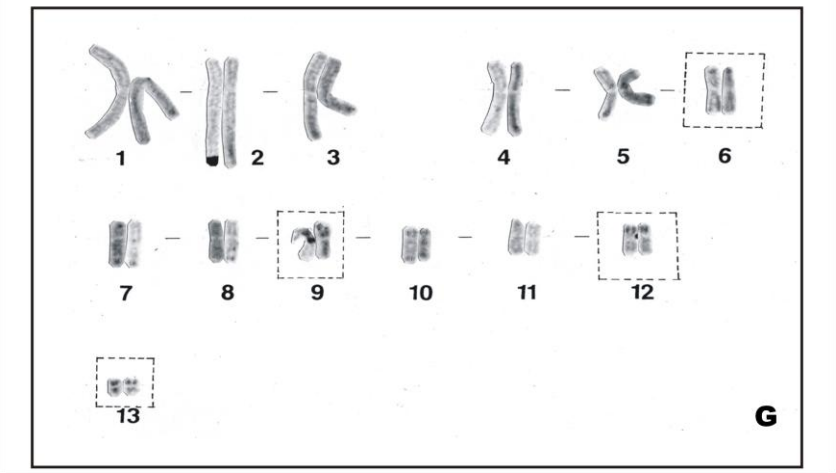


Fig. H. Silver stained metaphase karyotype of *Gegeneophis nadkarnii*

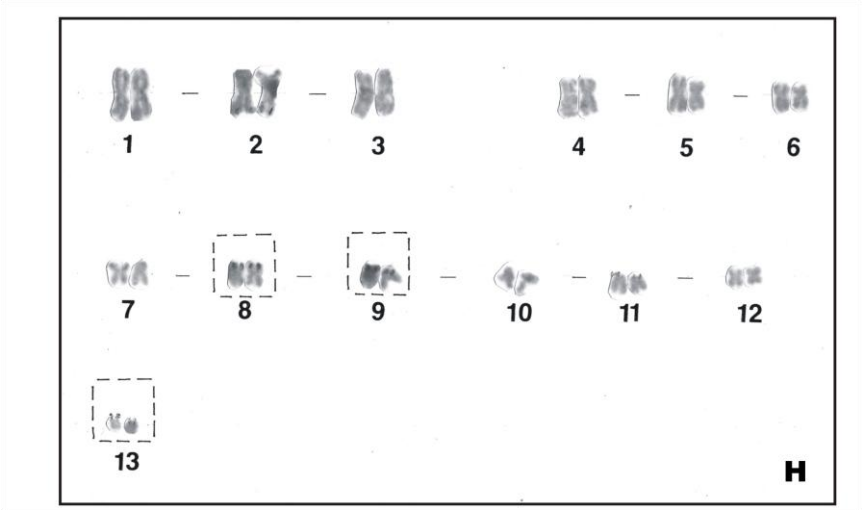
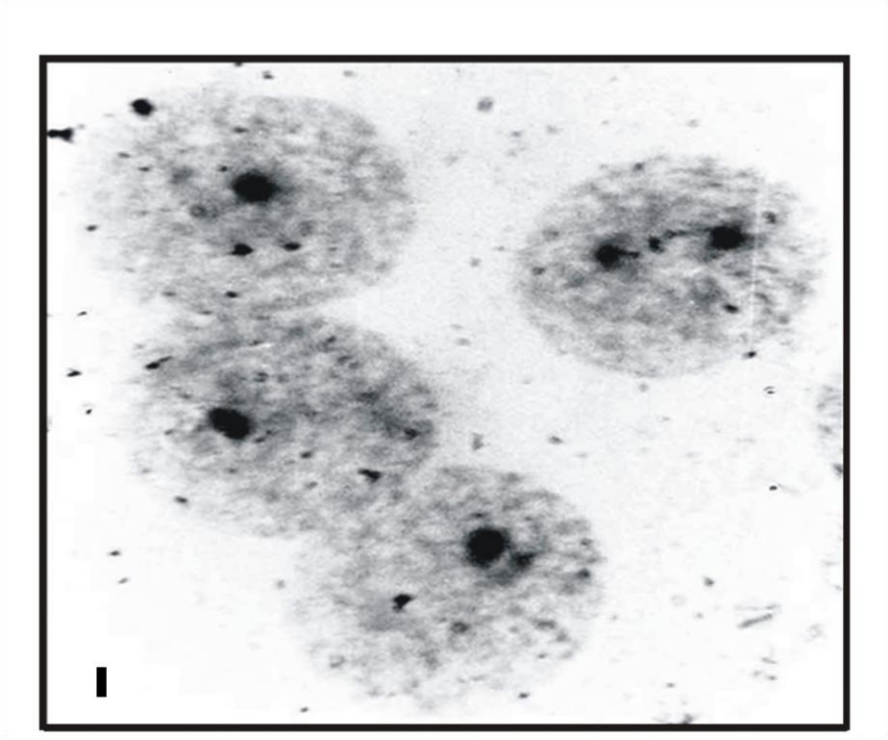


Fig. I. Silver stained interphase nuclei of *Ichthyophis beddomei*



Discussion

The silver nitrate (AgNOR) staining of metaphase chromosomes only stains those Nucleolor Organizer Regions (NOR) which have expressed themselves during the last interphase, because silver binds to a complex of acidic proteins associated with the nucleolus and nascent pre-RNA (Jordan, 1987). These proteins are largely non-histone in nature and their strong reaction with silver is thought to be due to their sulfhydril and disulfide groups (Buys and Osinga, 1980). The black blocks of Ag-stained NORs presumably consist of precipitated metallic Ag which is derived from the reduction of the Ag+ ions by the sulfydryl and disulfide groups of the NOR-associated protein. In somatic mitoses the Ag-stained proteins tend to remain associated with the NORs for a variable length of time after

NOR-inactivation. Those residual rRNA-associated proteins entrapped around the inactivated and condensed rDNA make it possible to define the NORs even in the somatic metaphase chromosomes.

In both metaphase and interphase nuclei, application of silver nitrate staining allow, in addition to the identification of NORs, a distinct labeling of heterochromatic regions, such as, heterochromatin, experimentally decondensed heterochromatin, chromosome cores, synaptonemal complex in meiotic nuclei and kinetochore.

All species examined in the present investigation showed two Ag-positive NORs in their diploid karyotype. A maximum of two Ag-stained nuclei was accordingly observed in the nuclei of somatic tissues (bone marrow, liver). In some animals there were distinct differences in size of the homologous Ag-stained NORs on the metaphase chromosomes, which were also expressed in size differences between the two Ag-stained nucleoli in somatic cell nuclei. These heteromorphism between homologous NORs were demonstrated in all metaphases from different tissues, thus constituting an intra-individual characteristic.

The Ag-NOR staining has been a useful chromosome marker, polymorphism of which, including number, location and size are often species-specific. It has been suggested that Ag-NORs, indicate the metabolic activity of the cell, which in turn, can depict the physiological status of the cell affair, because NOR activity found to be higher in younger and developing stages than in adult. However, the silver staining method does not indicate whether the observed variations in number of Ag-NORs are caused by differences in transcriptional activity of rDNA or by structural changes on the bearing chromosomes such as deletions, duplication or translocations of the regions containing the rDNA genes. It has been suggested that chromosomal NORs can play a role in inferring phylogenetic relationships mainly based on a primitive pair of NOR bearing chromosomes in most vertebrate examples (Amemiya and Gold, 1990).

The NOR location is a conserved characteristic. This is in concordance with Schmid et al. (1990) study, who asserted that in closely related species, the NORs are almost always located in the same chromosomal regions, although many exceptions have been found (for example, Dendrobatid species of the genus *Epipedobates*, (Aguiar et al. 2002). No other species of *Hylodes* and *Crassydactylus* have been analysed using Ag-NOR labeling (FISH). It seems reasonable to suppose that the NORs are also located in the same chromosome region in the remaining species since the NOR carrying secondary constrictions has counterparts in all of the already karyotypically studied species of other genera. In the case of *Megaelesia* (Rosa et al. 2003), although the NOR is located on different chromosome pairs, the chromosome region is also obviously homeologous among the three karyotypes described and may have arisen via a series of structural rearrangements which changed its position among the karyotypes.

Generation of the observed multichromosomal location of NORs remain obscure. Transposition of NORs to new chromosome sites through translocation of rDNA loci between homologous or nonhomologous chromosomes, or after duplication of those loci by unequal crossing over between sister chromatids could represent the probable mechanisms as has been suggested by earlier authors (Schmid et al. 2002). Such chromosome rearrangements may have been facilitated by transposable elements that can often provide substrates for recombination (Schmid et al. 2003).

Size differences between homologous NORs, like those found in the majority of the amphibians tested with specific labeling (mithramycin, DAPI) (Miller and Brown, 1969; Macgregor et al., 1977; Schmid et al., 1986, 2003) showed differences at the chromatid level. Approximately 2/3rd of animals tested for NOR heteromorphism, revealed a tandem duplication or even triplication in one of their two NORs. Several comparative investigations although understudy have demonstrated that these NOR heteromorphism are caused by differences in their number of rRNA genes.

Number and frequencies of Ag-nucleoli between populations and between species were examined and were comparable to the results of Ag-NORs, in interphase cells. A maximum number of four nucleoli and frequencies of 1to 4 Ag-nucleoli similar to those of Ag-NORs were observed.

It has been suggested that the presence of multiple NORs in the genome each containing a highly repeated ribosomal DNA gene sequences, which must be due to the strong positive selection, since the number of NORs generally appears to exceed the number of nucleoli (Flavell and Martini, 1982). The co-operation of several RNAs in forming a common nucleolus has been observed at prophase for a variety of different organisms including the present study,

wherein when several nucleolor chromosomes are attached to the same nucleolus by their NORs (Stahl, 1982). NOR numbers could be integrated into cell metabolism via the nucleolus due to their different physiological states between cells or between individuals, so that some enzymes like RNA polymerase is co-ordinated with combination of aminoacids, intracellular energy levels and rates of protein synthesis (Dixon *et al*. 1986). In order to localize rDNA genes and to examine the variability in both number and size in relation to copy number of rDNA genes it becomes imperative on our part to probe further in this issue through the use of CMA_3-staining and FISH analysis is very much warranted. It is possible to classify and verify GC/AT- rich content of DNA sequences that have been associated with NOR activities, as it was done in many vertebrate examples (Schmid, 1982; Pardo et al., 2001).

Zurita et al. (1998) showed that the level of transcriptional activity of NORs is directly related to the copy number of ribosomal genes. The variations in the copy number of rDNA genes is based on both directions i.e., addition or deletion of loci (Garrido-Ramos et al., 1995). Some studies have indicated that the variation in rDNA copy number is manipulated by selection.

Present data demonstrated that the immense presence of variable environmental (external) factors could have impacted upon implying to the extent that their higher level expression of NOR heteromorphism as well as variable presence of multiple loci of nucleoli, as when compared to the other anuran and urodelan examples, is in compromise. It is also interesting to note that this impact based on the genetical (internal) factors could also be solicited, wherein geniality is appended towards variations. To probe further on this problem one has to seek additional and innovative approach in order to test this proposition.

The NOR bearing chromosome pairs were different among the species analyzed suggesting the occurrence of several chromosomal rearrangements involving particular chromosomal segments. The presence of a single chromosome pair with NORs is a common factor as is evident in many vertebrates including amphibians (Schmid et al., 2002) and of course, the results of the present study. The data obtained in the present study indicate that the three families included exhibit conspicuous occurrence of many chromosomal rearrangements, hence, the discrepancy between family level comparisons probably relate to an evolutionary history between and among the divergent families of gymnophiona.

Based on the earlier extensive work carried out on amphibian examples (Schmid et al., 1978a. b, 1993) a clear indication is given that in the karyotypes of anurans actually only two large NOR pairs occur. One can speculate that karyotypes with a single large NOR pair are more ancestral than those in which the NORs are distributed over several chromosomes (Hsu et al., 1975). Accordingly, the number of NORs would have remained constant during the evolution of anurans, despite abundant speciation. By means of chromosomal rearrangements, the NORs would have been translocated to other chromosomes as complete unit, rather than as multiple smaller units distributed throughout the genome. Amphibian examples have also revealed to the content that most NORs identified preferentially take shelter at constitutive heterochromatin (Bogart, 1972). Since the NORs of most amphibians are always associated with constitutive heterochromatin, the probability of the breaks within the nucleolor constriction is slight. This conservative distribution pattern of two large NORs was not always maintained during the evolution of chromosomes in all groups of amphibia. The earlier results based on higher amphibians (Schmid et al., 1993, 2002) have revealed that in the karyotypes of many species there are upto 16 smaller NORs. In the karyotypes of species that belong to the same or closely related species- groups, the Ag-NORs always lie in comparable chromosomal sites. Many of the earlier work reveal to the extent that the pericentric region on the largest chromosome in the complement are the preferred site as the position of the designated NOR. A single pericentric inversion brought out these sites onto a terminal position, as is evident in many amphibian examples studied so far (Bogart, 1972; Blair, 1972; Schmid et al., 1987), by the support derived from interspecies hybrid experiments. The results of the present study endorse to the same view that many species revealed to the extent that the NOR sites are present on the terminal sites of many chromosomes in the complement.

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