Research Paper

C-Band Variation in Indian Caecilians (Amphibia: Gymnophiona)

ZOOLOGY

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ABSTRACT Application of C-banding on the chromosomes of caecilians has enabled in discriminating family- level organization rather than throwing some clues on either interspecific or intraspecific speciation. C-bands were particularly conspicuous at their centromeric region of almost all members of those species belonging to Ichthyophis, wherein both biarmed and uniarmed chromosomes were shown to represent positive C-band discrepancies, but were not so apparent in the cases of species belonging to Uraeotyphlus and Gegeneophis. All the biarmed chromosomes were shown to profile a scarce representation in the case of Uraeotyphlus and whereas none in the case of Gegeneophis, whie acrocentrics did possess C-bands at their centromeric regions; in the case of Uraeotyphlus caecilians but not in the Gegeneophis.

INTRODUCTION

Caecilians (G ymnophiona) comprise one of the three extant orders of Amphibia. A distinctive, elongate and limbless body form distinguishes them from other amphibians and a gymnophion monophyly is supported by numerous morphological attributes (Taylor, 1968; Nussbaum and Wilkinson, 1989; Hass et al., 1993; Hedges et al., 1993; Hay et al., 1995; Wilkinson, 1997; Feller and Hedges, 1998; Zardoya and Meyer, 2000; Gower and Wilkinson, 2005). The majority of the approximately 180 currently recognized caecilian species (Frost, 2013; Nishikawa et al., 2013) are fossorial, inhabiting soft soil throughout much of the moist wet tropics. Due to their secretive life style, they are invisible members of tropical herpetofauna and it is widely recognized that many aspects of biology of these animals are poorly known (Wilkinson and Nussbaum, 2006; Frost et al., 2006).

Karyotype is an important biological attribute that represents total genomic content of a species in the form of chromosomes. In many instances it provides individuality and cytotaxonomical status to a species. Study of a karyotype may serve as to throw light on evolutionary relationships between species. Thus, the study of karyotypic differentiation among taxonomically closely related species provides insights into the still intriguing problems of chromosomal changes and thus offers cytogenetic evidence to infer evolutionary change.

In the light of karyological analyses thus far made in caecilians pertaining to species differentiation based on mitotic metaphase chromosomal morphology and of sporadic report on meiosis, a basic diploid number of gymnophions is in the range of 20 – 42. Of the ten known families in gymnophiona (Kamei et al., 2012) no cytological information is available for the family Rhinatrematidae (primitive) and Scolecomorphidae (advanced). On the other hand, in the other eight families, cytogenetic data derived were sparsely represented and fragmented for assessing rightful phylogenetic inferences either within or outside of caecilians (Nussbaum, 1991; Venu, 2008) at large.

Beta karyology (White, 1978) of caecilians (and of other amphibians) is well documented but literature pertaining to typical banding studies in caecilians 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 the present investigation, an attempt has been made to localize highly repetitive sequences onto caecilian chromosomes belonging to three genera and the role of heterochromatin in the context of evolution is highlighted.

Materials and Methods

C-bands highlight centromeric region staining components of chromosomes demonstrating constitutive heterochromatin components. C-banding technique involves extraction of non C-band DNA and the retention of C- band DNA on chromosomes.

For C- bands, the staining procedure of Sumner (1972) was adopted. The slides were treated for 1 hour with 0.2N HCl at room temperature and then briefly rinsed in distilled water. The slides were then treated for 4-5 minutes with 5% aqueous solution of Barium hydroxide at 50^0C which was followed by a thorough washing in running tap water for a few minutes. The slides were then incubated for 1- 4 hours in 2XSSC solution at 60^0C and then stained for 1-2 hours in dilute 2% Giemsa solution (buffer at pH 6.8).

Wild type:

In certain cases, the incubation temperature was extended to 20 or more minutes prior to routine staining, is called wild treatment.

Results

In order to avoid repetition of the similar-looking homologic C-bands in the constituent species within each family, only representative species-karyotype is highlighted in the present case.

1. C-banded karyotype of *I. beddomei*

The C-stained karyotype of *I. beddomei* exhibits a large number of constitutive heterochromatic regions of metaphase chromosome regions in complement. All the 21 regular chromosome pairs can be identified by the characteristic C-band patterns located at the centromeric regions of all the constituent chromosome pairs. Furthermore, prominent interstitial C-bands can be recognized in the distal end of long arm of chromosome number 1 which is quite evident and some non-specific C-bands are also apparent in some members in the complement. In the meiotic preparations, more specifically of pachytenes, diplo-diakinetic and metaphase I plates, demonstration of conspicuous centromeric region stained bands are very distinct. In some animals, faintly stained centromeric region of some acrocentrics (number 3, 14 and 17 bivalents) are of noteworthy features. Concomitantly, bivalent 1 is conspicuous by its distal end of the long-arm by its distinct interstitial C-band.

In certain, C- staining preparations, C-band staining property obviously points towards demonstrating kinetochore region specificities at the centromeric region, thereby eliciting kinetochore region rather than paracentromeric region, eventhough most of acrocentric C-bands represents in usual terms involving inclusion of centromere plus short arm region of the chromo-

some in the complement.

2. C-banded karyotype of *I. kodaguensis*
C-banded karyotype of *I. kodaguensis* has more similarity to *I. beddomei* karyotype except for chromosome number 3 in the complement in which extreme submetacentric 3 replaces acrocentrics. Chromosome number 1 carries a very prominent interstitial C-band at the telomeric region of the long arm. Besides, in certain populations chromosome number 4 is distinguished by deeply stained prominent band at the proximal to distal end of the long arm consummarating with the occurrence of tandem fusion that could have taken its origin from one of short-arm components of ancestral C-group metacentric (no. 10) chromosomes.

3. C-banded karyotype of *Uraeotyphlus gansi* and *U. narayani*
In all animals examined, a very streaky C-band are present in most of submeta and metacentric (biarmed) chromosomes in metaphase complement, whereas all the acrocentric pairs are represented by very deeply stained C-bands, at the centromeric regions.

4. C-banded karyotype of *Gegeneophis ramaswamii*
In all species belonging to the genus *Gegeneophis*, C-bands were not conspicuous, thereby eliciting very faintly stained constitutive heterochromatic regions especially of the meta and submetacentric chromosomes which form the major component of the karyotype. Distinct C-bands were obvious to notice on the centromeric region of acrocentric pairs, which of course varies according to species-specific patterns; hence resulting in displaying 1-3 pairs which could be accounted for their presence in the complement. In the karyotypes of *G. ramaswamii, G.* cf. *ramaswamii* and *G. nadkarnii,* the countable number of C-bands pertaining centromeric regions of all the acrocentric chromosomes were prominent, whereas in other longer chromosomes it is represented by very indistinct profiles at centromeric region.

Discussion
It is well-known that constitutive heterochromatin confrontment is composed of site–specific, highly repetitive DNA sequences and its chromosomal distribution is generally revealed by means of conventional C-banding techniques. The chromosomal distribution and nucleotide sequences of the repetitive DNA and their organization in the genome provide information on the structure of the genome, the process of chromosomal evolution and the phylogenetic relationships could be established as evidently shown among many familiar animal and plant examples (Hsu et al., 1975; Babu, 1988; John, 1988).

The C-banded karyotype (although it cannot be that precise and definitive by the description of the C-bands it possess) based on conventional C-banding technique, provides a fundamental basis for further cytogenetic evaluation and gene mapping and in interspecies comparative genome studies. Position of C-bands identifies regions of constitutive heterochromatin which contains transcriptional curtailment and consequently of highly repetitive DNA sequences. As exemplified by many vertebrate and insect genomes, C-banding heterochromatin is represented not only in most centromeric and telomeric regions but also in some interstitial regions. In certain cases, the telomeric sequences have been detected outside of true telomeres. Most of the telomeric sites of telomeric repeats are located at pericentromeric areas within or borders of the regions of the constitutive heterochromatin are at interstitial chromosome locations (Meyne et al., 1990). It has been suggested that these interstitial sequences are the vestiges of true telomeres which remain after chromosome rearrangements, such as Robertsonian processes, tandem fusions and pericentric inversions. These types of chromosome rearrangements often distinguish closely related species and populations and seem to have played an important role in the karyotype evolution in several mammalian examples (Holmquist and Dancis, 1979; King, 1993; Searle, 1998). In congruence with the perception that interstitial telomere sites often represent and serves as remnants of ancient fusion points in mammals (Meyne et al., 1990; Vermeesch et al., 1996; Hart-

mann and Sherthan 2004). Li et al. (2002) suggested that most ancestral chromosome fusion sites result from breaks in satellite II, telomere or other sequences. It has been observed earlier (Brinkley et al., 1984; Elder and Hsu, 1988; Nanda et al., 2002; Santani et al., 2002) that the rearrangements of supernumerary centromeres and particular makeup of repetitive sequences and centromeres and telomeres has contributed to the chromosome rearrangements in the evolution of several mammalian examples (King, 1993; Castaglia et al., 2002; Garagna et al., 2002; Foster and Bridger, 2005).

In essence, the studies pertaining to C-banding patterns of three caecilian genera (*Ichthyophis, Uraeotyphlus* and *Gegeneophis*) studied differ enormously in their content with regard to amounts and limits of constitutive heterochromatin. Examples of Ichthyophiidae that possess the greatest amount among the caecilians examined thus far, while the short arms along with centromeric region of several smaller chromosomes (acrocentrics) and of all the major chromosomes at centromere and interstitial regions being entirely heterochromatic (i.e., C-band positive). In addition, the large blocks of heterochromatin that occurs near the centromeres of every member in the complement (i.e. pericentromeric heterochromatin). In the genera *Uraeotyphlus*, the major chromosomes (both submeta and metacentrics) seemed possess less conspicuous amounts of heterochromatin in at least 11-12 pairs, but not as much as occurs in the corresponding chromosomes of ichthyophiids, whereas, the smaller acrocentrics demonstrate deeply stained constitutive heterochromatin component. Among those species of Indotyphlidae, the metacentric chromosomes are characterized by their possession of very small amount of constitutive heterochromatin. Only 8 or 9 pairs of chromosomes seem to possess easily observable bands and the very best preparations reveal small speck of staining including pericentromeric heterochromatin localized in the majority of the chromosomes of these species. The C-banded chromosomes of *Gegeneophis* possess even greater amounts of heterochromatin in case of many small chromosomes, rather than the longer, biarmed chromosomes, than that of euchromatic portions. In all the caecilian taxa studied, high quality C-bands could be demonstrated both from somatic metaphase and some meiotic stages thereby allowing precise identification of the constitutive heterochromatin component in the genomes predominantly at their centromeric region in the complement.

The classical C-banding patterns depicted by means of conventional C-banding technique revealed rather faintly stained centromeric heterochromatin component, as is evident in some animals but not in others. Whereas the metacentric and submetacentric chromosomes that form major part of the genomes of caecilians especially of derivative forms (i.e. higher caecilians), while deeply–stained acrocentric chromosomes at the centromeric regions form a distinct entity and contrive to be an important component. Discrete and specific C-staining is evident in acrocentric chromosomes in the complement; but the same study has failed in disclosing interspecies differentiation. The conventional C-staining technique expedites only those components of the chromosome that were enriched with highly repetitive or rapidly reassociating category of DNA sequences. However, at present it is not clear whether the cytologically definable discrete staining profiles include intermediate and middle repetitive sequences but could also be induced from the same chromosome. It is more appropriate to convene studies that highlight application of reassociation kinetic studies delineating various sequence-specific classes including interspersed DNA sequences organization that may prevail in metaphase chromosomes that being considered during the implication for further characterization in the classification of heterochromatin compartment.

C-banding pattern was found to be identical and homologous within several species of *Ichthyophis* and *Gegeneophis,* but it varies specifically at generic and family level studies. These kinds of approach to elucidate variation disclosed by this group of caecilians, is in contrast to the most commonest situation encountered in anura, in which there is an enormous amount of

interspecific variation found both in distribution and in the content of constitutive heterochromatin; which might be the reason for the application, induction and implication, a inevitability factor by means of high resolution C-banding techniques (Green, 1986; King, 1991; Aguiar et al., 2002) very much warranted although attempts made to disclose intraspecific level variations by means of C-banding patterns have described for reasons for some species (Odierna et al., 2000; Silva et al., 2000).

Significant findings of the present study have been that karyotypic study of the taxa involving incidental cases from *Ichthyophis* to *Gegeneophis*, it was found evident to learn that there is a gradual reduction of acrocentrics and consequent increase in biarmed chromosomes, as is evident that the amount and nature of constitutive heterochromatin seemed to play direct role along with lineages involved that they vary along with chromosome structural variations. It becomes imminent to point out that the chromosomal structural rearrangements initiated by Robertsonian and non-Robertsonian processes thereby gradual debilitating in the consequent reduction of heterochromatic content in the genome. It is evident from some mammalian examples including primates that C-banding staining of heterochromatin may have disappeared completely in some metacentric chromosomes (Dutrillaux *et al.* 1986; Volbouev *et al.* 1988, 1995; Wichman *et al.* 1991).

In spite of these assertions, it appears tantamount in the present findings, that it becomes necessary to delineate more about chromosome constitutional secrets that they contend with major component in the complement (for example, biarmed chromosomes in the case of *Uraeotyphlus* and *Gegeneophis*) that are not amenable for C-band expression, although to a certain extent they are accessible in the case of ichthyophids. This situation may point towards implying to a secretive box that needs to crack open.

In the present case, it was observed that the genomes with high amounts of constitutive heterochromatin (C-positive bands) and no or less gross chromosomal rearrangements to one such as those described above, with tiny amounts of highly repetitive DNA but with massive chromosomal reorganization.

In the end it becomes imperative enough to appraise that in caecilians implicated in C-staining of their genome led to the possibility that compositional heterogeneity in constitutive heterochromatin content might have affected in driving at divergence of karyotypes and hence such a speciation event.

Legend for Figures
1. C-banded mitotic metaphase karyotype of Ichthyophis beddomei (Scale bar =10 µm).
2. C-banded diplotene karyotype of Ichthyophis kodaguensis (Scale bar =10 µm).
3. C-banded mitotic metaphase karyotype of Uraeotyphlus gansi (Scale bar =10 µm).
4. C-banded mitotic metaphase karyotype of Uraeotyphlus oxyurus (Scale bar =10 µm).
5. C-banded mitotic metaphase karyotype of Gegeneophis ramaswamii (Scale bar =10 µm).
6. C-banded (wild) mitotic metaphase karyotype of Gegeneophis ramaswamii (Scale bar =10 µm).
7. C-banded mitotic metaphase karyotype of Gegeneophis cf. ramaswamii (Scale bar =10 µm).
8. C-banded mitotic metaphase karyotype of Gegeneophis nadkarnii (Scale bar =10 µm).
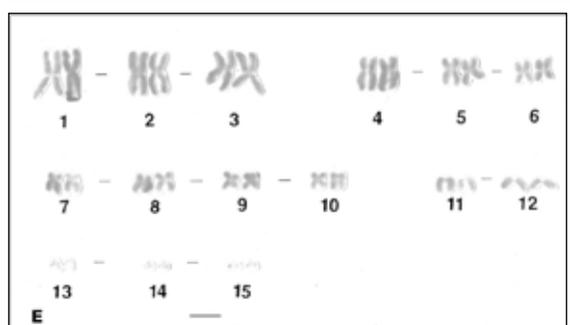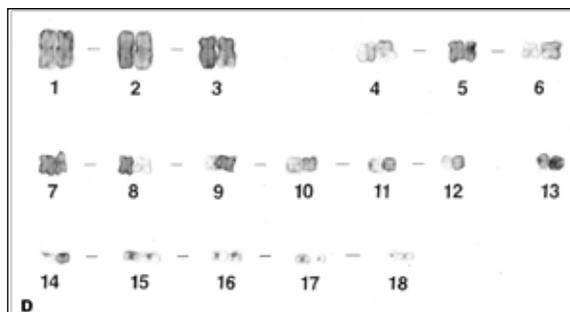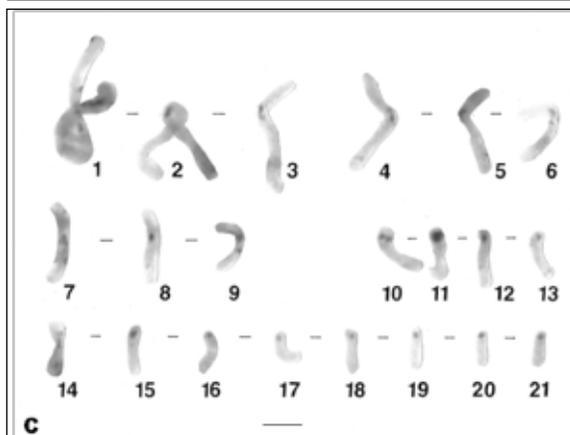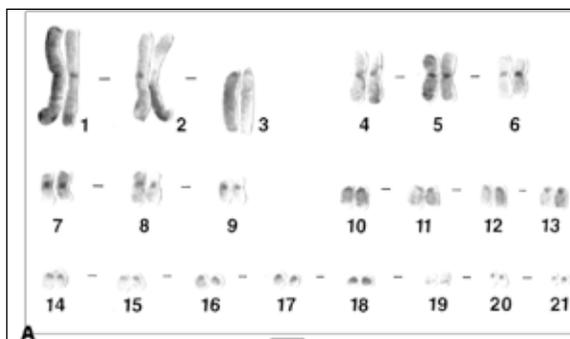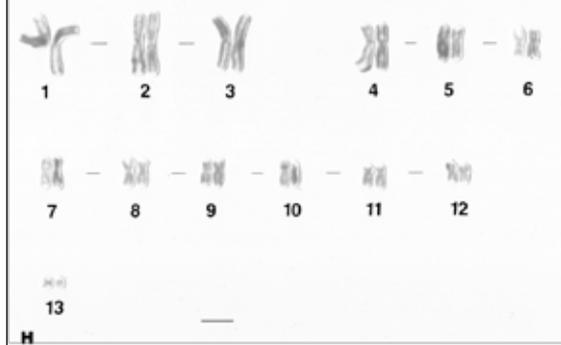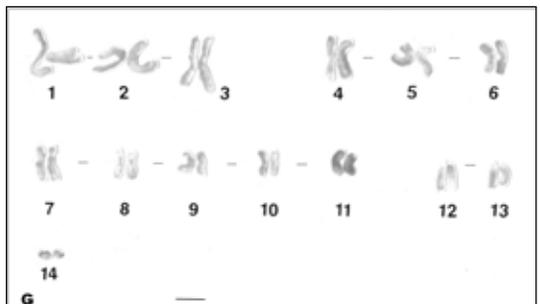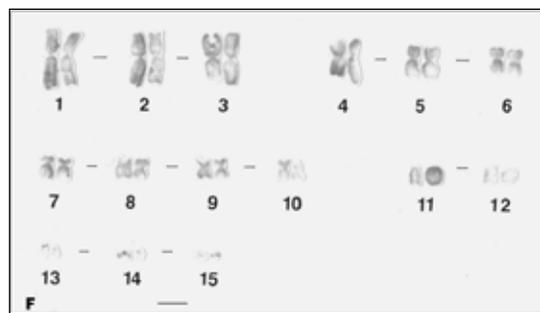
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