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衛星 DNA 的功能研究及人造染色體的建構

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中文摘要：

關鍵詞：著絲點、衛星 DNA、BAC 基因組資料庫

著絲點在有絲分裂及減數分裂的過程中扮演了相當重要的角色，它負責將姐妹染色分體平均分配至兩個子代細胞中有關。而功能不正常或失去功能的著絲點通常會有染色體不穩定的現象發生，而造成所謂的 Aneuploid。在細胞遺傳學上的研究報告指出 aneuploidy 和自發性的流產，先天性的染色體疾病，如唐氏症和透納氏症，甚至與某些癌症的發生有關。近年來，有許多實驗室為了改良基因治療載體，於是致力於研究人類人造染色體的建構上。但想要了解 Aneuploid 形成的機制及成功地建構人類人造染色體為基因治療的載體，就必須先透澈著絲點的功能及構造。

在人造染色體構築研究中以重複的阿爾發衛星 DNA 作為基本的架構元素時，發現人造染色體雖無法穩定存在轉殖細胞中，但是它卻是形成人造染色體的必要因子；因此更加認定衛星 DNA 是支配人類染色體著絲點功能的重要且必須的要素，但其真正的機制至今未明。又因為著絲點衛星 DNA 在各種族中差異相當大，但卻可和一致性相當高的著絲點蛋白結合形成有功能的著絲點。此一謎團，只有解開不同種族的著絲點上所有 DNA 的組成，才能透澈了解真正有功能的著絲點 DNA 為何？在各種種族中以印度山羌的著絲點是最獨特的 X+3 複合著絲點，而且它擁有最少的染色體數目（2N=6♀,7♂）、最大的染色體及奇特的核型演化。為了了解印度山羌的著絲點 DNA 組成及其核型的演化，建立印度山羌的 BAC 基因組資料庫是必須的。因此在此次的計劃中，我們已完成建立涵蓋 4 個基因組的印度山羌 BAC 基因組資料庫，共有 126,336 個印度山羌 BAC 菌株，平均每個 BAC DNA 約含有 80kb 印度山羌的基因組片段。目前有 591 個 BAC DNA 已用螢光原位雜交法定位在印度山羌的染色體上，有 31 個是位在著絲點上且有六種不同的著絲點分布情形；其中，有一個螢光原位雜交的訊號和著絲點蛋白的免疫螢光訊號是一樣平行位於著絲點兩側，因此推測此一 BAC DNA 中含有和著絲點蛋白鍵結的特定序列，此一序列可能扮演著絲點功能重要的角色，之後我們將針對此一 BAC DNA 做進一步的結構及功能分析。另一方面，在找尋功能性著絲點 DNA 的過程中，我們從 BAC 菌珠中找到了 Y 特異性的衛星 DNA，由於它只存在亞洲系的鹿類中，因此更加確認了印度山羌核型是由 2n=70 類似中國水鹿的核型演化而來。為了更加確認印度山羌的核型演化和中國水鹿的核型的關係，我們更進一步分析了三種衛星 DNA（I、II、III）在中國水鹿染色體中的分布情形，此部份的結果已發表在 2006 Cytogenetics and Genome Research 中。在這一年的計劃執行中，共有一篇已發表的論文，一篇正在撰寫的論文，已有涵蓋 4 個印度山羌基因組的 BAC 菌株，目前仍繼續分析這些 BAC DNA 在不同鹿類染色體上的分布情形，以推測其核型的演化；另外也會進一步分析位於著絲點的 BAC DNA 和 CENP 鍵結的可能性及在著絲點功能中的角色，以利將來人造染色體的建構作為基因治療的載體。

英文摘要。

Keyword: centromere, satellite DNA element, BAC library

Centromere plays a pivotal role during mitosis and meiosis. Malfunctional centromere would result in prematured centromere division (PCD) that causes aneuploidy, an aberrant chromosome number in the complement. Epidemically reported that aneuploidy is associated with disorders such as spontaneous abortion, birth defect (e.g. Down syndrome, Klinfelter syndrome, Tuner syndrome, etc.) and some neoplasia. Recently, several laboratories have endowed in the construction of human artificial chromosomes with centromeric DNA for gene therapy intervention. In order to unveil the enigma of aneuploidy and to construct successfully artificial chromosomes for gene therapy intervention, it is an important and necessary to understanding the molecular architecture and composition of centromere.

In attempt to constructing a human artificial chromosome, alphoid satellite DNA was considered a necessary but not a sufficient component for a functional and stable centromere. However, taken the available data from yeast to man, the centromeric satellite sequence has diverged significantly, even amongst different chromosomes of a single organism. It is unclear mechanism how a diverse satellite DNA does associate with conserved kinetochore proteins to form a conserved functional centromere. The centromeric DNAs found so far are quite complex so that it becomes even less clear about the structure and function of the mammalian centromeres. In order to understand completely the centromere structure and function in mammals, it will require detailed sequence analysis of centromeric DNA.

The X+3 compound centromere of the Indian muntjac (*Muntiacus muntjak vaginalis*) is unique among mammals, in addition to their low diploid number (2N=6♀,7♂), giant size, and a quick karyotypic evolution. Therefore, we are interested in the functional and molecular structure study of centromeric DNA of Indian muntjac and in karyotypic evolution of muntjac. To question these two issues, first of all is to establish Indian muntjac BAC library. In this project, we have finished constructing 4 coverages of library with 126,336 BAC DNA clones having average 80 kb insert size. The chromosomal location of 591 BAC clones had been each mapped onto the Indian muntjac metaphase chromosomes by fluorescence in situ hybridization (FISH), thus providing a high density FISH BAC clone map never been achieved of the species. Among these clones, 31 BAC clones were mapped onto the centromeric region. On the other hand, during finding a functional satellite DNA, there is a novel Y-specific satellite DNA found only in Asian deer species. This found Y-specific satellite DNA further interpreted that the karyotype of Indian muntjac derived from the Chinese water deer-like karyotype. For understanding the karyotype relationship between Indian muntjac and Chinese water deer more clearly, we identified the chromosomal distribution of satellite I, II, and III DNA in Chinese water deer. This part of results had been published in 2006 Cytogenetics and Genome Research. Totally, we have one published paper and one preparing paper in this one-year project. In future, we will continue to map the BAC clones onto the different deer species for predicting the karyotypic evolution of deer species. Moreover, the mapped centromeric BAC clones will provide an excellent resource for the isolation of new centromeric satellite DNA sequences and evaluating the role in centromere function by an artificial-chromosome assay. This could lead to the construction of function artificial mammalian chromosomes with gene therapy potential.

Introduction:

Satellite DNA is composed of tandemly repetitive sequences and ubiquitous present in the eukaryotic genome up to 10% -20% (Beridze 1986). It is located mainly in centromeric region, and up to tens of megabases can reside in a single human centromere (Choo 1997). Therefore, it is considered to play a role in centromere structure and function. Additionally, satellite DNA is a very dynamic component of the genome (Slamovits and Rossi, 2002). The evolutionary dynamics of satellite DNAs could possibly occur in the course of the speciation process, thus forming a species-specific profile of satellite DNAs (Ugarković and Plohl 2002). Taken together, satellite DNA has a particular mode of evolution, species-specific profile, and specific distribution in the genome by itself; therefore, it is recognized as a valuable marker for phylogenetic relationship and karyotypic evolution studies (Laursen et al 1992; Garrido-Ramos et al. 1999, Li et al. 2000).

As the human genome project enters a phase focusing on comparative and functional genomics, further emphasis will be placed on understanding our genes, their regulation and expression and their complex interactions, primarily as they pertain to human health and medicine. It will be important to resolve the evolutionary history of the human genome, as this has affected both genome organization and chromosomal architecture. Comparative G-banding studies (Shi et al. 1980; Yunis et al. 1980), somatic cell hybrid mapping (Lalley et al. 1978; O'Brien et al. 1997), and Zoo-FISH mapping (Wienberg and Stanyon 1995; Yang et al. 1995; Fronicke and Scherthan 1997; Muller et al. 2000) had been used to infer homologies of whole chromosomes or subregions between species and even across mammalian orders. The genome organizations of eight phylogenetically distinct species from five mammalian orders had been compared. These comparative gene-mapping data collected to date indicated that most of chromosomes present as a highly conserved synteny, whereas other chromosomes show more extensive rearrangement in each mammalian lineage. In multispecies studies, the sequence alignment analysis of an evolutionary breakpoint (an interval region between two homologous synteny blocks) showed that nearly 20% chromosome breakpoint regions were reused during karyotypic evolution; these reuse sites were also enriched for centromeres that were associated with the formation of acrocentric centromeres in other species (Murphy et al. 2005). Additionally, sequence analyses at the junction of human conserved segments showed a preponderance of highly repetitive elements existing (Puttagunta et al. 2000). The highly repetitive elements at the evolutionary breakpoints might be a remaining of ancient acrocentric centromere DNA during forming a new chromosome by tandem fusion in karyotypic evolution. As previous evidences, the interstitial telomere and centromeric satellite DNA signals were observed in Indian muntjac using the telomeric and centromeric satellite DNA as probes for FISH studies suggesting that the chromosome fusion events occurred in the karyotypic evolution of Indian muntjac (Lin et al. 1991; Lee at al. 1993; Scherthan 1995; Li et al. 2000). In human, several de novo "neocentromeres" found in abnormal chromosomes (Amor and Choo 2002) might derive from a cryptic interstitial centromere at the evolutionary breakpoint. This observation addressed a possible mechanism for chromosome evolution and the appearance of reuse breakpoints, whereby these evolutionary breakages preferentially occur at sites of ancestral centromeres or cryptic interstitial centromeres in independent lineages. Alternatively, reuse breakpoints may represent unstable chromosomal sites that, after breakage, will tend to form a new centromere or telomere. Taken all data together, it affirms that the centromeres are dynamic, rapidly evolving structures that can be repositioned among closely related species and have evolutionary importance in karyotype evolution and speciation.

As we known, the repetitive satellite DNAs is mainly composition of centromere and is diversity among species. Generally, most similar satellite DNAs are restricted to closely related species. In other words, the related (or congeneric) species share a "library" of related and similar satellite sequences, some of which could be amplified onto a major satellite DNA in some particular species (Salser et al. 1976; Meštrović et al. 1998). The evolutionary dynamics of satellite DNAs could possibly occur in the course of the speciation process, thus forming a species-specific profile of satellite DNAs (Ugarković and Plohl 2002). The species-specific profile of centromeric satellite DNA is possible able to be explained now by means of comparative genome finding that a

large fraction of centromeres analyzed were repositioned either by independent chromosomal rearrangements or by de novo centromere emergence in the independent lineage to become unique to a species (Murphy et al. 2005). The DNA composition at the repositioned centromere might continuous origination and amplification to form a new highly repetitive DNA after a sufficient evolutionary time. As Nijman and Lenstra (2001) proposed, in the course of satellite DNA evolution, newer or younger satellite sequences could have derived from preexisting DNA sequences to replace or coexist with the old satellites via the three-phases of evolution processes. In summary, satellite DNA has a specific mode of evolution, species profile, and distribution in the genome by itself; therefore, it was recognized as a valuable marker for phylogenetic relationship and karyotypic evolution studies (Laursen et al 1992; Garrido-Ramos et al. 1999, Li et al. 2000 and 2005). To further appreciate the "library" hypothesis, collecting and identifying more novel satellite DNAs in the related species will be essential. The mapping and sequencing of satellite DNAs from phylogenetically closely or divergent species will provide a better understanding on the relationship of extant species, and thus frame evolutionary hypotheses. Additionally, it can elucidate an important structural and functional role of breakpoint regions in a broader range of mammalian taxa. With the use of comparing the sequence among breakpoint regions and centromeric satellite DNAs, it will shed more lights on the mechanism for centromeric satellite DNA evolution and on the mechanism for formation of a de novo "neocentromere". Furthermore, it could also unravel the puzzle of a functional conserved centromere in the face of rapidly evolving DNA sequence.

Comparative studies of chromosome content and organization are providing insight into fundamental questions of genome evolution and speciation. BAC library has tremendous potential to facilitate such studies to shed light on the temporal relationships of extant species by comparative sequence analysis and cytogenetic mapping of genome rearrangement, expansion, and loss (Gomyo et al. 1999; Srinivasan et al. 2002). In addition to decipher genome evolution, BAC resources could be used to address specific questions pertaining to centromeric and pericentromeric evolution, and the mechanisms of karyotypic change between closely related species. The Indian muntjac (*Muntiacus muntjac vaginalis*) has the lowest chromosome number in mammalian species with 6 chromosomes in female and 7 in male. Recent molecular cytogenetic studies landed support to the hypothesis that the karyotype of the Indian muntjac could evolve from an ancient deer species with a karyotype 2n=70 through extensive tandem fusions and several centric fusions (Li et al. 2000). Moreover, the centromere of X+3 chromosome of the species is compound and unique. Therefore, it is an ideal species for studying chromosome rearrangement, comparative genomic and karyotype evolution. In an attempt to explore the centromeric evolution and function, and to elucidate the fusion orientations of conserved chromosome segments and the underlying mechanism of tandem fusion of Indian muntjac chromosomes, we therefore constructed a BAC library of Indian muntjac.

Methods:

Construction of a Indian muntjac genomic BAC Library. Male Indian muntjac fibroblast cells (CCL-157, American Type Culture Collection) were used to prepare the high molecular weight (HMW) genomic DNA. Briefly, 8X106 cells (corresponding 40ug of DNA) were embedded in low-melting agarose (0.5%) in PBS for each plug. The cells in the plug would be digested by proteinase K to extract the genomic DNA. After extraction, the genomic DNA in the plug would be run a 1% agarose gel using a PFGE apparatus in 0.4X TBE buffer at 12°C and 120V/cm for 10hrs with a 5-sec pulse time to remove the mitochondria DNA. Subsequently, the genomic DNAs in plugs were partial digested with *Eco*RI/*Eco*RI methylase (New England Biolabs) at 37℃ water bath for 16hrs. The partially digested DNAs were double-size fractionized by pulsed-field gel electrophoresis (PFGE) in a low-melting agarose to obtain HMW genomic DNA. Gel slices containing the DNA fragments in the length range of about 60-100kb and 100kb-150kb were excised. The HMW genomic DNAs were eluted and were ligated with pCC1BACTM *Eco*RI cloning-ready vector (Epicentre, U.S.A.) by Fast-LinkTM DNA Ligase (Epicentre, U.S.A.). Ligation were drop dialyzed against 5% PEG or TE buffer with Millipore VS 0.025 uM membranes

for 1hr. The dialyzed ligation DNAs were electroporated into 33ul of *E.* coli DH10B competent cells (Epicentre, U.S.A.). After electroporation, cells were incubated in 600ul 2XLB medium containing 1mM MgCl2 and 20mM glucose at 37℃ with gentle shaking for 1hr and spread on 2XLB plates containing chloramphenicol (12.5ug/ml), X-gal (40ug/ml) and IPTG (100ug/ml). The plates were incubated at 37℃ overnight. Blue and white color selection was used to identify the recombinants. Approximately 20 transformations were carried out to obtain >6,000 BAC clones. White positive BAC clones were picked manually to 96-well microtiter plates containing 100ul freezing media (0.5% w/v NaCl, 1% w/v Bacto-Tryptone, 0.5% w/v Bacto-extract, 13mM KH2PO4, 36mM K2HPO4, 1.7mM sodium citrate, 6.8mM (NH4) SO4, 4.4% v/v glycerol, 0.4mM MgSO4・7H2O and 12.5ug/ml chloramphenicol). The microtiter plates were incubated at 37℃ with 350rpm shaking for 18-20hrs, two copies of each 96-well microtitre plate were prepared and stored at -80℃ at different locations. The detailed protocol was described in Peterson et al. (2000).

Characterization of the BAC Library. **Insert size estimation.** Randomly picked recombinant clones were pre-grown in 2ml 2XLB medium containing 12.5ug/ml chloramphenicol for 5hrs and then 2ul of 1000X CopyControl Induction Solution (Epicentre, U.S.A.) was added. Inoculation continued at 37℃ with 250 rpm shaking for another 18-20hrs. The bacteria broth was collected for isolating the BAC DNA. The BAC DNA was isolated by the standard alkaline lysis protocol. The purified BAC DNA was digested by *Not*I to excise the insert. The insert was fractionated by running pulsed-field gel electrophoresis in 0.4X TBE buffer at 12℃ and 6.0V/cm for 15.5hrs with 1- to 15-sec pulse time using a PFGE apparatus. **Fluorescence in situ hybridization (FISH)** was used to evaluate the degree of chimerism in this library and physical mapping of BAC clones. Metaphase chromosomes were prepared from the male Indian muntjac cell line and the BAC DNA probes were labeled with either digoxigenin-11-dUTP or biotin-16-dUTP (Roche, Basel, Switzerland) by nick-translation. The procedures for denaturation, hybridization, post hybridization washing and signals were described in detail elsewhere (Lee et al. 1999). Fluorescent signals were captured on a Leica ALM fluorescence microscope equipped with appropriate filter sets and a cooled charge-coupled device (CCD) camera. The images were normalized and enhanced using the FISH software (Applied Spectral Image, Isrel).

Results and Discussion:

I. Chromosomal distribution and organization of three cervid satellite DNAs in the centromeric heterochromatin of Chinese water deer (*Hydropotes inermis*)

The species-specific profile and centromeric heterochromatin localization of satellite DNA in mammalian genomes imply that satellite DNA may play an important role in mammalian karyotypic evolution and speciation. A satellite III DNA family, CCsatIII was thought to be specific to roe deer (*Capreolus capreolus*). In this study, however, this satellite DNA family was found also to exist in Chinese water deer (*Hydropotes inermis*) by PCR-Southern screening. A satellite III DNA element of this species was then generated from PCR-cloning by amplifying this satellite element using primer sequences from the roe deer satellite III clone (CCsatIII). The newly generated satellite III DNA along with previously obtained satellite I- and II- DNA clones were used as probes for FISH studies to investigate the genomic distribution and organization of these three satellite DNA families in centromeric heterochromatin regions of Chinese water deer chromosomes and resting nuclei. Satellite I and II DNA were observed in the pericentric/centric regions of all chromosomes, whereas satellite III was distributed on 38 out of 70 chromosomes. The distribution and orientation of satellite DNAs I, II and III in the centromeric heterochromatin regions of the genome were further classified into five different types. The existence of a *Capreolus*-like satellite III in Chinese water deer implies that satellite III is not specific to the genus *Capreolus* (Buntjer et al. 1998) and supports the molecular phylogeny classification of Randi et al. (1998) which suggests that Chinese water deer and roe deer are closely related. The result had been published in *Cytogenetics and Genome Research (2006) 114:147-157.*

II. High density BAC clone mapping and defining the karyotype evolution of Formosan sambar deer (appendix 1)

In this study, the insert DNA fragments of BAC were generated from a male Indian muntjac fibroblast cell line (ATCC) and were treated with *Eco*RI/*Eco*RI methylase. The high-molecular-weight DNA fragments were cloned into the *Eco*RI site of pCC1BAC (Copy ControlTM cloning system). In total 126,336 individual BAC clones have been obtained. The frequency of clones with inserts is 88% and the average size of the inserts was estimated at 80 kilobases by analyzing ~545 randomly chosen clones using *Not*I digestion followed by Pulsed Field Gel Electrophoresis (PFGE). Assuming that the Indian muntjac genome contains $2.2X10^9$bp, the total library constructed corresponds to 4X genome coverage. The chromosomal location of 591 BAC clones was each mapped to the Indian muntjac metaphase chromosomes by fluorescence in situ hybridization (FISH), thus providing a high density FISH BAC clone map never been achieved of the species. Among these clones, 31 BAC clones were mapped onto the centromeric region. The genomic organization of satellite DNA in these clones should shed light on the structural aspect of centromeric satellite DNA. Furthermore, the centromeric BAC clones will also provide an excellent resource for the isolation of new centromeric satellite DNA sequences and their role in centromere function that can be evaluated by an artificial-chromosome assay. This could lead to the construction of function artificial mammalian chromosomes with gene therapy potential. 21 clones were selected and mapped onto the Formosan Sambar deer by comparative FISH mapping, the results showed that large synteny segments were conserved between Indian muntjac and Formosan sambar deer. Comparison of the orders of mapped BAC clones on Formosan Sambar deer and Indian muntjac enabled us to determine the orientation of the more ancestral chromosomal segments on the Indian muntjac chromosomes that could subsequently define the mode of tandem fusions in the events of karyotypic evolution of the Indian muntjac. The partial results had been presented in the 56^{th} annual meeting of American Society of Human Genetics, New Orleans, Oct. 2006, abstract program no. A840 and supported by grants from NSC94-2320-B-040-042 and NHRI-EX94-9207 SI. A manuscript about this study is in preparation for submitting to *Animal Genetics*.

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Self-evaluation:

In this project, our aims are to establish an Indian muntjac BAC library for studying the molecular structure and function of centromeric DNA. We had constructed 4X coverage of Indian muntjac BAC library. Additionally, a roe deer-specific satellite III DNA was also found in Chinese water deer. The results regarding the analysis of three satellite DNA elements in Chinese water deer had been finished and published in Cytogenet Genome Res (2006). Therefore, our studies meet the aims of the original project and achieve the progress of the project. Totally, we have one published papers and one preparing paper during carrying out this project.

Appendix 1:

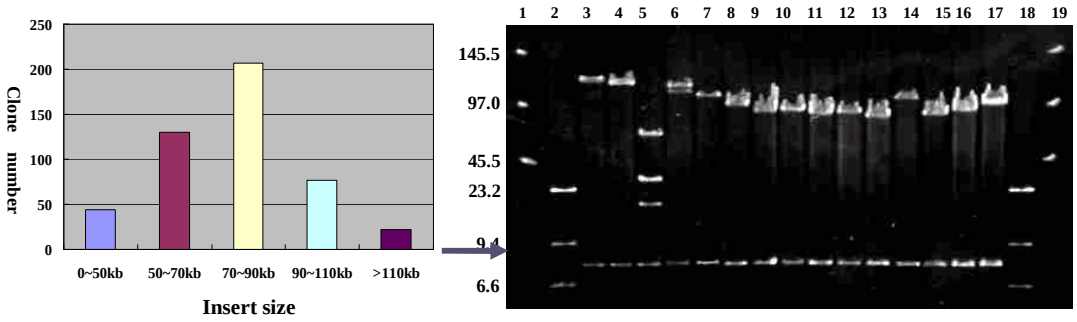

Figure 1.
(A) The insert size distribution of the Indian muntjac BAC library. Insert sizes were determined for 545 BAC clones by performing PFGE after *Not* I digestion. The horizontal axis refers to the size ranges in kb, while the vertical axis indicates the number of clones. (B) Typical examples of *Not* I restricted BAC clones after PFGE analysis (Lane 3 to 17). Lane 1 and 19 denoted the PFGE standard DNA marker; Lane 2 and 18 were λ-*Hin*dIII marker; The row of vector bands was at 7.5 kb (denoted by arrow).

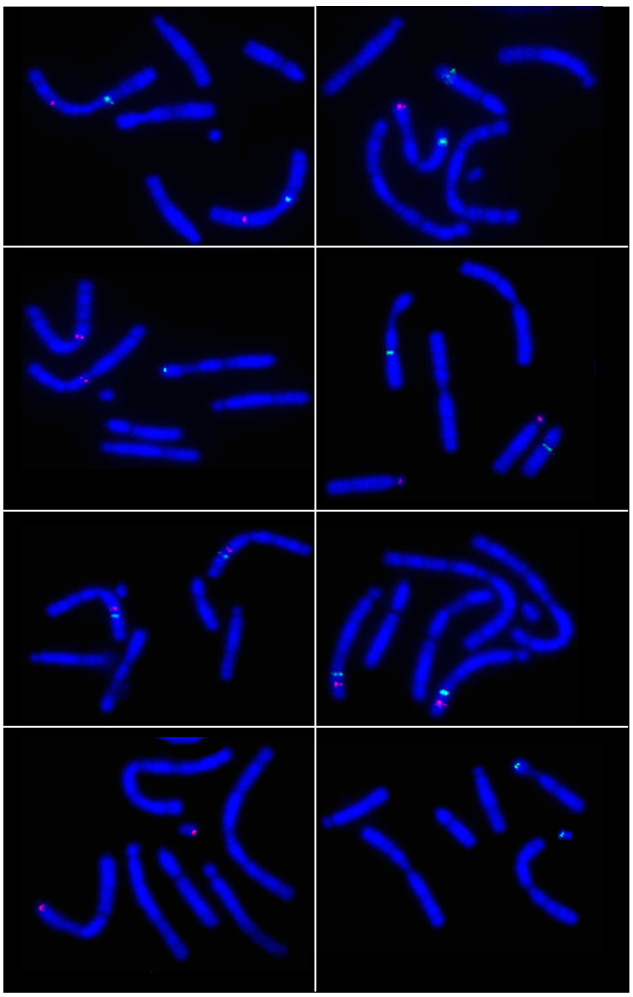

Figure 2: BAC clones mapped on the specific chromosomal sites of Indian muntjac by FISH.
(A) Clone 119A1 mapped onto the 1q23 (green) and clone 122A1 mapped onto the 1p14 (red). (B) Clone 121A1 mapped onto the Xp15 (red) and 124A1 mapped onto the 3q41(green). (C) Clone 41E6 mapped onto the 1p12 (red) and 50A1 mapped onto the Xp15(green). (D) Clone 3E6 mapped onto the 2p11 (red) and 4E6 mapped onto the 3q31(green). (E) Clone 127E61 mapped onto the 1q21 (red) and 130E6 mapped onto the 1q25(green). (F) Clone 15A1 mapped onto the 2q37(red) and 16A1 mapped onto the 2q35(green). (G) Clone 1121H12 mapped onto the pseudo-autosomal region of X and Y (red). (H) Clone 1154E6 mapped onto the pseudo-autosomal region of X and Y (green).

Figure 3: BAC clones mapped on the centromeric region of Indian muntjac by FISH.
(A) Clone 1128A1 (green) mapped onto the centromeric and interstitial regions of Indian muntjac chromosomes. (B) Clone 1158A1 (green) was observed to have similar patterns like the signals of Mmv-0.7 probe (cervid satellite II) also with centromeric and interstitial signals. (C and D) Clones 1189E6 and 1179H12 were only mapped onto the two sites of compound centromere of X+3. (E) Clone 1189A1(red) located to the Yq and centromeric region of chromosome 3 in Indian muntjac. (F) Clone 1296A1 (green) mapped onto all centromere regions, some of which appeared as kinetochore signals which parallel to two sides of centromere. (G) Clone 1241H12 (red) occupied all centromeric heterochromatin region. (H) Clone 747A1 (red) located in the middle region of centromere especially X+3.