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哺乳類動物著絲點 DNA 的分子結構及功能之研究(3/3)

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中文摘要：

關鍵詞：著絲點、衛星 DNA、著絲點蛋白、3D 立體結構

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。

著絲點在染色體上是以一種收縮的結構型態顯現，而其分子結構的基本元素包括鹼基序列及特殊的蛋白質結構名 Kinetochore(是以一種三層板狀的結構所構成，最外層是和紡錘絲連結，最內層是相連於著絲點 DNA，中間是一層狹窄的透明區)。就目前所知，在高等的真核生物內，存在著高度複雜性的著絲點 DNA 族群，其中已確定的著絲點 DNA 主要是由重複性的衛星 DNA 所組成，舉例來說，阿爾發衛星 DNA 是最主要著絲點 DNA 且存在每個人類染色體著絲點上，而且阿爾發衛星 DNA 的某些單元體內具有 17 個鹼基對的 CENP-B 鍵結序列。因此曾認為是支配人類染色體著絲點功能的重要且必須的衛星 DNA。可惜的是，後來在某些異常染色體 (marker chromosomes)上發現具有功能的新生著絲點上並沒有阿爾發衛星 DNA 的存在，而且近來研發的人造染色體構築中，若只是以重複的阿爾發衛星 DNA 作為著絲點 DNA 的基本架構元素，通常無法穩定存在人造染色體的轉殖細胞中。從低等到高等的生物體，著絲點的衛星 DNA 往往差異相當大，且在不同種族甚至同一種不同個體間也都不同；然而，著絲點的蛋白質的功能及構造卻保留相當大的一致性。正因如此，使得著絲點的構造及功能更加複雜且不易了解。

由於著絲點衛星 DNA 在各種族中差異相當大，因而推算著絲點衛星 DNA 在演化過程中變化極為快速，因此著絲點衛星 DNA 的差異度可作為種族間血緣遠近的依據。在尋找具有著絲點功能的著絲點 DNA 的同時，也可研究著絲點衛星 DNA 的演化過程。鹿科的染色體變化差異極大，而且在先前的研究中，發現衛星 DNA 在山羌的核型演化中扮演極重要的角色。在此次的計劃中，我們更進一步研究原先已選殖到的三種印度山羌衛星 DNA (分別為 I、II 和 IV)在著絲點上的分布結構:在原位螢光雜交及 3-D 影像建構的結果中,顯示衛星 II DNA 及衛星 IV DNA 是以相同的螺旋方式圍繞在著絲點上，圍繞二圈而在 X+3 複合著絲點上則圍繞四圈，每一圈約由六個原位螢光訊號所形成；而 Kinetochore 仍是以兩條平行對稱的方式排列在衛星 DNA 的外側，每條約有四個免疫螢光訊號在 X+3 的複合著絲點上。此一結果說明了衛星 DNA 的螺旋結構可能是為了將著絲點染色質絲表現在染色體的外側，使其能夠支配 kinetochore 的蛋白質的包裝，且和紡錘絲作用。然而這並不足以說明這兩種衛星 DNA 可和 kinetochore 上的蛋白質直接鍵結。因此我們利用染色質絲免疫沉澱法直接找到 kinetochore 蛋白質的鍵結 DNA 片段，再以此一 DNA 當作探針搜尋 BAC 基因庫以找到完整的 kinetochore 鍵結序列。首先我們已成功取得 kinetochore 上建構蛋白質的鍵結 DNA 片段，至於基因庫的部分，我們已得到涵蓋 2 個基因組的 BAC 菌株，目前仍繼續建構中。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。另外我們也利用一致性相當高的著絲點衛星 IV DNA 和衛星 I、II DNA 加以鑑定中國山羌及台灣山羌的血緣關係。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。在此次計

劃執行中，共有四篇已發表的論文，一篇論文已送出，一篇正在撰寫的論文，已有涵蓋 2 個印度山羌基因組的 BAC 菌株及許多由染色質絲免疫沉澱法得到的 DNA 菌株，目前正在分析這些 DNA 在染色體上的分布情形及和 CENP 鍵結的可能性。

英文摘要。

Keyword: centromere, satellite DNA element, centromeric proteins (CENPs), kinetochore binding DNA.

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.

Several detailed analyses have demonstrated that the centromere contains various centromeric DNA and an exquisite and dramatic proteinaceous structure, the kinetochore, which in turn interacts with the spindle microtubules. Satellite DNA is a predominant and ubiquitous centromeric DNA in mammals. Alphoid satellite DNA, the major centromeric satellite DNA of human chromosomes, was considered a sufficient component for a functional centromere in the construction of human artificial minichromosomes. It was not questioned until alphoid satellite DNA was not found in some mitotically stable markers with functional centromere. Moreover, taken the available data from yeast to man, the centromere sequence and sequence organization have diverged significantly, even amongst different chromosomes of a single organism; however, overall centromere and kinetochore components might be significantly more conserved than thought previously. 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 diversity of the centromeric satellite DNA among species resulted from the quick evolution. Therefore, satellite DNA can be traced during genome evolution and speciation. Several studies had used satellite DNA a marker to identify the karyotype evolution and phylogenetic relationship. In the mean time searching a functional centromeric DNA, there should be a lot of novel satellite DNAs identified. Those novel satellite DNAs can be further characterized to use in karyotype evolution and speciation. The chromosomes of the Indian muntjac (*Muntiacus muntjak vaginalis*) is unique among mammals due to their low diploid number (2N=6♀,7♂), giant size, and unusual large centromeres. Particularly, the centromere of X+3 chromosome appears having a compound kinetochore. Moreover, the karyotype of Indian muntjac resulted from the centric fusion. Therefore, we are interested in the functional and molecular structure study of centromeric DNA of Indian munjtac. In this project, we not only endeavor to find a functional centromeric DNA, but also characterize the role of the found novel satellite DNA in muntjac's karyotype evolution. We further characterized the 3D structure of three previously isolated cervid centromeric satellite DNA elements (satellite I, II and IV) of Indian muntjac. In the simultaneous 3D-FISH and immunofluorescence study, the CENP immunofluorescence signals parallels along with centromere as well as both satellite II and IV signals are organized into a spiral structure. The spiral structure may be to present centromeric chromatin to the exterior of the chromosome, where

it can mediate kinetochore assembly and interactions with the spindle. However, it is no enough to prove that the satellite II or IV can directly bind with kinetochore protein. Therefore, we would uncover the whole DNAs that exactly associate with kinetochore protein. First step, we have successfully use chromatin immunoprecipitation to isolate the DNA fragments that associate with kineotchore protein. Subsequently, we would use the isolated DNA fragments as a probe to screen the Indian muntjac's BAC library. We have constructed 2 coverages of library. The BAC library is still under construction to achieve 6 coverages. During finding a functional satellite DNA, there is a novel Y-specific satellite DNA found. This satellite DNA existed 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. We also confirmed the phylogenetic relationship between Formosan muntjac and Chinese muntjac using satellite I, II, III as a tracing marker. Totally, we have four published papers, one submitted paper, and one preparing paper in this project.

Contents:

Introduction:

Centromere is a specialized and primarily constricted structure of eukaryotic chromosome. This constricted structure is presented as 30nm parallel fibers in several electron microscopic studies of mammalian centromere (Rattner and Lin 1985; Wanner and Formanek 2000). Early cytogenetic staining showed that the centromeric chromatin is packed into the constitutive heterochromatin (Heitz. 1928). Later, ultrastructure analyses had shown that a trilaminar disc plate-like structure, designated as the kinetochore, parallels the lateral surface of the centromeric heterochromatin block (Brinkley and Stubblefield 1966; Rieder 1982; Rattner 1986 and 1987; McEwen et al 1993). The trilaminar structure is composed of an electron-dense inner plate that is connected with the centromeric heterochromatin DNAs, an electron-translucent interzone and an electron-dense outer plate which surface is a fibrous corona where the microtubules of the mitotic spindle attach (Pepper and Brinkley 1977; Mitchison and Kirschner 1985; Rattner 1987; Cooke et al. 1993). Electron microscopic analyses of serial sections showed that the caffeine-induced detached kinetochores appear as fragments derived from whole kinetochores. A repeat subunit model is suggested to interpret the organization of a centromere-kinetochore complex (Zinkowski et al.1991). Thanks to the available anticentromere antibody from the patient with CREST disorders (**c**alcinosis, **R**aynaud's phenomenon, **e**sophageal dysmotility, **s**clerodactyly, **t**elangiectasia) variant of scleroderma (Moroi et al. 1980; Brenner et al. 1981; Earnshaw and Rothfield 1985) or with watermelon-stomach disease (He et al. 1998), proteinaceous kinetochore could be stained and identified by immunofluorescence microscopic analysis with this antibody. Subsequently, simultaneous immunofluorescence and fluorescence in situ hybridization study further supported the model that the subunits of stretched kinetchores arrange in a linear array and a repetitive pattern along a centromeric DNA fiber consisting of tandemly repeated subunits interspersed by DNA linkers (Zinkowski et al.1991).

In view of molecular building blocks, centromere is known to compose of centromeric DNA associated with a unique proteinaceous structure, the kinetochore (Rieder 1982). In human centromere, more than 20 different proteins found are divided into two groups: one is the constitutive centromere protein and the other is the transient centromere protein. The constitutive centromeric proteins, such as CENP-A, B, C, G, H and I, are responsible for the structure of centromere and for the assembly of kinetochore. The transient centromere/kinetochore proteins, such as CENP-E, F and INCENP, are essential for promoting spindle microtubules capture, proper chromosome congregation, sister chromatid cohesion, and appropriate movement of chromosomes to opposite poles. The constitutive centromeric proteins appear to be needed to fulfill the intricate and precise dynamic function of the centromere-kinetochore complex when various transient kinetochore proteins are recruited during different stages of the mitosis (Choo 2000; Pidoux and Allshire 2000; Tyler-Smith and Floridia 2000). Specifically, CREST antiserum was known to interact with the inner kinetochore region by recognizing three human "constitutive" centromere proteins CENPs-A, -B, and –C that are located in this region (Brenner et al. 1981; Earnshaw and Rothfield 1985). Additionally, human anti-CREST serum is also found to label centromeric regions of other species as well (Saffery et al. 1999; Hoopen et al. 2000) thus suggesting that the constitutive centromeric proteins are functional and evolutionary conservation.

From low to high eukaryotes, there are many centromeric DNAs found and characterized. In the budding yeast (*Saccharomyces cerevisiae*), the centormere occupies a 125-bp DNA region, comprising three elements (CDEI, CDEII, and CDEIII) with well characterized function and conferring mitotic stability after it is inserted into plasmid vector (Clarke and Carbon 1980; Clarke 1990; Schulman and Bloom 1991; Hegemann and Fleig 1993; Pluta et al. 1995). The *Drosophila*

centromere appears to be composed entirely of repeated DNA. *Neurospora crassa* centromeric DNA contains repeated sequences in the manner similar to that of *Drosophila*. The repeated sequences of flies appear as the remnants of transposable elements and are interspersed between different types of simple repetitive sequence (Cambareri et al. 1998). Mammalian centromeres typically encompass several million bases of DNA (Willard 1990; Tyler-Smith and Willard 1993; Farr et al. 1995; Sun et al. 1997; Harushima et al. 1998) and are consisted predominantly of tandem repetitive DNAs (e.g. satellite DNAs). Major and minor satellite DNA families are ubiquitous in the centromere of mouse (*Mus musculus*) (Horz and Altenburger 1981; Manuelidis 1981; Wong et al. 1990; Garagna et al.1993). There are eight different satellite DNAs well characterized in calf centromeric hetereochromatin region (Singer, 1982). In cervid species, three satellite DNA families I, II and III are found to localize in centromeric heterochromatin region (Bongerberger et al. 1985; Lin et al. 1991; Lee et al. 1997b; Qureshi et al. 1995; Vafa et al. 1999; Li et al. 2000a, b) and most recently, a novel satellite IV DNA family has been identified in several deer species (Li et al. 2002). In human, satellite DNA families (e.g. classical satellite DNAs, alpha satellite DNAs, beta satellite DNAs, gamma satellite DNAs etc.) are the major components of the centromeric DNAs along with some interspersed repetitive DNAs (e.g. SINEs and LINEs). Specifically, unlike other repeated DNA such as telomeric or rDNA, these identified and characterized repetitive centromeric DNAs appear quite complexly organized and display a high degree of variation among species (Lee et al. 1997c). Interestingly, a functional conserved centromeric domain is made up of the diverse repetitive centromeric DNAs and highly conserved kinetochore proteins. Some of repetitive centromeric DNAs from various species are identified to be associated with a specific kinetochore protein. Alphoid satellite DNA, the most predominant human centromeric DNA family, is present in every centromeric heterochromatin region of the human chromosome complement and is capable of binding with CENP-B (Pluta et al. 1992; Haff et al. 1994); therefore, it is thought of as the preferred substrates for kinetochore assembly (Ikeno et al. 1994). The cervid satellite II DNA could be immunoprecipitated with anti-CENP-A serum and also presents in every centromere of deer species studied; thus suggesting this given satellite DNA may be a candidate functional centromeric DNAs in cervid species (Vafa et al. 1999). *Mus musculus* minor satellite DNAs contains the 17 bp CENP-B binding box (ATTCGTTGGAAACGGGA) similar to that of alphoid satellite DNAs and is associated with CENP-B proteins in centromere region by simultaneously immunofluorescence and FISH analysis (Broccoli et al. 1990; Mitchell 1996). More recent studies on the construction of human artificial chromosomes (HACs) each containing an array of the human α- satellite DNA together with the genomic and telomeric DNA elements indicated that the HACs could acquire mitotic stability in cell lines (Harrington et al. 1997; Ikeno et al. 1998; Schueler et al. 2001). Together with these findings, the ubiquitous presence of repetitive sequences at the centromeres of higher eukaryotes led to the suggestion that sequence repetitiveness are needed for centromere function. Moreover, it has recently been proposed that the conservation of centromere functions with diverse repetitive centromeric DNAs could be the result of being able to form higher order structures, such as a secondary or tertiary structure. Such a higher order structure could bind to key centromeric proteins, or serve as targets for critical DNA modification, rather than on particular DNA sequence itself (Copenhaver and Preuss 1999). The requirement of at least some repetitive DNAs for centromere function has not been called into question until the recent observation of stable human marker chromosomes with functional "neocentromeres" that appeared to lack of any repetitive DNA (Voullaire et al. 1993; Depinet et al. 1997; Vance et al. 1997; Barry et al. 1999). Therefore, whether centromeric satellite DNA has a definitive functional role may be still debatable (Choo 2000; Henikoff et al. 2001). On the other hand, it also could not completely rule out the possibility that a conserved sequence nucleates centromere formation in higher eukaryotes**,** unless a complete DNA sequence analysis in any higher eukaryotic centromere had been

accomplished. One should reconsider the idea whether the centromere function is dependent on a specific DNA sequence itself or a high-order structure of sequence. Even though, to certain extend, -satellite sequences could be sufficiently responsible for the construction of human artificial chromosomes (Harrington et al. 1997; Ikeno et al. 1998; Warburton and Cooke, 1997; Henning et al. 1999; Schueler et al. 2001), one should pay attention to the fact that stable human marker chromosomes with "neocentromere" lack any repetitive DNA (Voullaire et al. 1993; Depinet et al. 1997; Vance et al. 1997; Barry et al. 1999). Therefore, it is still controversial whether the -satellite DNA sequence itself is essential for a functional centromere. Additionally, the diverse centromeric DNAs and conserved centromeric proteins have been found to be in the centromere (Sunkel and Coelho1995). To unveil the puzzle, the first step is to sequence the complete centromeric DNA.

The diversity of the centromeric satellite DNA among species resulted from the quick evolution. Therefore, satellite DNA can be traced during genome evolution and speciation. Several studies had used satellite DNA a marker to identify the karyotype evolution and phylogenetic relationship. The chromosomes of the Indian muntjac (*Muntiacus muntjak vaginalis*) is unique among mammals due to their low diploid number (2N=6♀,7♂), giant size, and unusual large centromeres. The centromere of X+3 chromosome appears having a compound kinetochore. Moreover, the karyotype of Indian muntjac resulted from the centric fusion (Lee et al. 1993; Yang et al. 1997; Li et al. 2000). Therefore, centromeric DNA of Indian muntjac is a good resource for studying the functional and molecular structure of centromere and the role in karyotype evolution.

In this project, we further characterized the 3D structure of three previously isolated cervid centromeric satellite DNA elements (satellite I, II and IV) of Indian muntjac. The simultaneous 3D-FISH and immunofluorescence result showed that the CENP immunofluorescence signals parallel along with centromere as well as both satellite II and IV signals are organized into a spiral structure. The spiral structure may be to present centromeric chromatin to the exterior of the chromosome, where it can mediate kinetochore assembly and interactions with the spindle. However, it is no enough to prove that the satellite II or IV can directly bind with kinetochore protein. Therefore, we would like to sequence the whole DNAs that exactly associate with kinetochore protein. First step, we have successfully use chromatin immunoprecipitation to isolate the DNA fragments that associate with kineotchore protein. Subsequently, we would use the isolated DNA fragments as a probe to screen the Indian muntjac's BAC library. We have constructed 2 coverages of library. The BAC library is still under construction to achieve 6 coverages. During finding a functional satellite DNA, there is a novel Y-specific satellite DNA found. This satellite DNA existed 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. We also confirmed the phylogenetic relationship between Formosan muntjac and Chinese muntjac using satellite I, II, III as a tracing marker.

Methods:

Chromosome preparation:

Male Indian muntjac fibroblast cell line (obtained from ATCC) is grown in Ham's F-10 medium supplemented with 10% FBS, 1% penicillin and streptomycin, and 1% L-glutamine at 37oC, 5% CO2 incubator. For chromosome preparation, the detailed procedure to be used was described in Li et al. 2000a.

Dual color FISH:

The fixed cells are drop-splashed onto glass slide and stand at room temperature for two days aging. Biotin-labeled satellite II probe (Mmv-0.7; AF170123) and digoxigenin-labeled satellite 1kb probe (MMV-1.0; AY 064466) are cohybridized to metaphase chromosomes of Indian muntjac. The biotin signals are observed by means of incubating with Cy3-conjugated avidin, biotinylated anti-avidin and Cy3-conjugated avidin, sequentially. The digoxigenin signals are detected using mouse anti-DIG antibody, FITC-conjugated rabbit anti-mouse antibody and FITC-conjugated goat anti-rabbit antibody, sequentially. Subsequently, samples are mounted in antifade mounting medium with DAPI (vector).

Simultaneously immunofluorescence and FISH:
The Indian muntjac cells are treated with colcemid (0.1ug/ml) for 3 hours prior to harvesting. The harvested cells are incubated in 0.075 M KCl for 15 minutes at room temperature and subsequently cytospin onto slides. CREST sera, specific for anti-CENP-A,B and C antibodies (a gift from Dr. J.B. Rattner, University of Calgary, Canada) will be used to identify kinetochore domains. The primary CENP antibodied are detected with FITC-conjugated rabbit anti-human IgG. After immunofluorescent experiment, cells on the slide are immediately fixed in 10% formalin/KCM buffer (120mM KCl, 20 mM NaCl, 10 mM Tris-HCl pH 7.6, 0.1 % Triton X-100) for 10 min and then in 3:1 methanol/acetic acid for another 15 min. The following FISH is carried out as the single color FISH experiment with biotin labeled either MMV-1.0 or Mmv-0.7 probes mentioned earlier (The detailed procedure was described in Li et al. 2002).

Isolating the complete sequence of a functional centromeric DNA:
To studying the functional centromeric DNA, we would isolate the BAC clone using CENP-binding DNA fragment as a probe that is extracted from chromatin immunoprecipitation. First, we would establish the BAC library of Indian muntjac. Second, we would isolate the CENP-binding DNA fragment using chromatin immunoprecipitation. The BAC library has been under construction. Briefly, the 10^6 cells were harvested and embedded in low melting agarose. The embedded cells were digested with proteinase K and *Not*I to get the digested DNA without nick. After pulse field gel electrophoresis, elute DNAs from the agarose with 100kb range. Clone the eluted DNAs into pCC1BAC vector and transform into competent cells. The chromatin immunoprecipitation were referred to Lo et al. (2001) with briefly modified. The nuclei were isolated by dissolving cell membrane with 0.4% NP-40 in buffer A (10mM Tris-HCl, 10mM KCl, 0.1mM EDTA) and were sonicated. The sonicated nuclei were incubated with specific anti-ACA serum for overnight at 4^oC. The immunocomplex were captured by agarose protein A for overnight at 4^oC. Finally, the DNA fragments associated with kinetochore protein were purified by phenol/chloroform.

Results and Discussion:

I. Isolation and identification of a novel satellite DNA family highly conserved in several Cervidae species (appendix 1)

In the course of amplifying Cervid satellite II DNA from the genome of two muntjac species (Indian muntjac and Chinese muntjac) with a pair of primer sequences derived from the white tailed deer satellite II clone (OvDII), a prominent 1kb product was achieved in addition to the expected 0.7kb satellite II DNA elements in each species. Later, the ~1kb fragments had also been obtained in the black tailed deer and Canadian woodland caribou. Southern blot analysis, DNA sequencing, PCR-Southern hybridization and FISH studies indicated the ~1kb elements are belong to a new cervid centromeric satellite DNA family which is 60% AT-rich. It is different from all previously identified cervid satellite DNAs, I, II and III that were GC-rich. The novel ~1kb satellite DNA sequences of Indian muntjac, Chinese muntjac, caribou and black tailed deer were deposited to Gene

Bank data base with accession numbers AY064466, AY064467, AY064468 and AY064469 and designated as cervid satellite IV. Sequence comparison showed extremely high sequence conservation of satellite VI among deer species studied. Such sequence conservation had never been reported in other found satellite DNA families. Zoo-blot result showed that 1kb satellite DNA may also be present in various other mammalian species (e.g. *Homo sapiens*, rabbit and mouse). FISH studies revealed that the Indian muntjac ~1kb satellite IV DNA (MMV-1.0) is colocalized with satellite II DNA in the centromeric heterochromatin region and in some interstitial centromeric heterochromatin sites. Simultaneous FISH and immunofluorescence experiments demonstrated that the ~1kb satellite DNA signals are co-localized with specific immunofluorescence signals produced by anti-CENPs sera. Due to the facts that the ~1kb satellite VI DNA clones from all deer species share extremely high sequence homology and are located closely to the kinetochore region, this novel centromeric DNA family is suspected to have a functional role. These findings had been published in *Chromosoma* 111:176-183, 2002 (Li et al. 2002).

II. Karyotyping and satellite DNA analysis of Formosan muntjac (Muntiacus reevesi micrurus) (appendix 2 and 3)

Formosan muntjac (*Muntiacus reevesi formosan*) is indigenous to Taiwan; however, relatively little known about its karyotype and satellite DNAs. In present study, G-banding, C-banding and NOR-staining chromosome analysis were performed. The detailed G-banded karyotypes of the species, 46, XY in male and 46, XX in female, are constructed and found to resemble to the DAPI banded Chinese muntjac karyotype (Yang et al. 1996). The male chromosome complete has 22 pairs of acrocentric autosomes and one pair of acrocentric sex chromosomes with X and Y. Constitutive heterochromatins contribute predominantly in the every centromeric region of chromosome. One rRNA gene cluster is located in the medial of chromosome 1 and the other one is located in the terminal of chromosome 5 that is also similar as Chinese muntjac. The satellite I, II and 1kb-satellite IV homologous clones were also isolated by PCR-cloning, PCR amplification of which was carried out with primer pairs, specific for satellite I, II and IV respectively. Sequence comparison show that 82%, 85% and 98% similarity to satellite I, II and IV of Chinese muntjac, respectively. FISH study demonstrated that satellite I is present in every centromeric regions with the exception of that of chromosome 3 and Y. The hybridization signals of satellite II are observed in every centromeric regions except the Y and those of satellite 1kb are shown in a similar manner as satellite II. Taken together, it should be reasonable to support the classification that Formosan muntjac is a subspecies of Chinese muntjac. Results of this study had been published in two papers. *Cytogenet Genome Res* 105:100-106, 2004 (Lin et al. 2004) and *Zoological Studies* 43:749-758, 2004 (Chiang et al. 2004).

III. Karyotypic evolution of a novel cervid satellite DNA family isolated by microdissection from the Indian muntjac Y-chromosome (appendix 4)

A mini-library was constructed from DOP-PCR products using microdissected Y chromosomes of Indian muntjac as DNA templates. Two microclones designated as IM-Y4-52 and IM-Y5-7 were obtained from negative screening of all three cervid satellite DNAs (satellite I, II, and IV). These two microclones were 295- and 382-bp in size respectively, and shared approximately 70 % sequence-homology. Southern blot analysis showed that the IM-Y4-52 clone was repetitive in nature with a ~ 0.32-kb register in *Hae*III digest. Sequence comparison revealed no similarities to DNA sequences deposited in the GeneBank database, suggesting that the microclone sequences were from a novel satellite DNA family designated as cervid satellite V. A subclone of an Indian muntjac BAC clone which screened positive for IM-Y4-52 had a 3325-bp insert containing 6 intact monomers, 4 deleted monomers and 2 partial monomers. The consensus sequence of the monomer was 328-bp in

length and shared more than 80% sequence homology with every intact monomer. A zoo-blot study using IM-Y4-52 as a probe showed the strong hybridization with *Eco*RI digested male genomic DNA of Indian muntjac, Formosan muntjac, Chinese muntjac, sambar deer and Chinese water deer. Female genomic DNA of Indian muntjac, Chinese water deer and Formosan muntjac also showed positive hybridization patterns. The satellite V was found to specifically localize to the Y heterochromatin region of the muntjacs, sambar deer and Chinese water deer, and to chromosome 3 of Indian muntjac and the X-chromosome of Chinese water deer. These findings had been published in *Chromosoma* 114:28-38, 2005 (Li et al. 2005).

IV. 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 has been submitted to *Chromosoma*.

V. Reconstructing the 3D-spatial structure of mammalia centromere

Centromere plays a pivotal role during mitosis and meiosis. Malfunctioned centromere would result in aneuploidy that is associated with disorders such as spontaneous abortion, birth defect and some neoplasia. In order to unveil the enigma of aneuploidy, it is an important and necessary to understanding the molecular architecture and composition of centromere. Interestingly, a functional conserved centromeric domain is made up of the diverse repetitive centromeric DNAs and highly conserved kinetochore proteins. Does a various centromeric satellite DNA be organized into a specific structure to associate with conserved kinetochore proteins in mammals?

The simultaneous immunofluorescence and FISH study showed that the satellite II and IV were found to co-localize in muntjac centromere and to associate with kinetochore. Furthermore, the 3D- FISH and immunofluorescence were carried out on the polyacrylamide embedded, formaldehyde fixed cells using either biotinylated satellite II probe and digoxigenin-labeled satellite IV probe or one of them and CREST serum. The images acquired from the different Z-axial sections were deconvolved and reconstructed. The reconstructed 3D image showed that the CENP immunofluorescence signals parallels along with centromere whereas both satellite II and IV signals of X+3 centromere are organized into a spiral structure with four turns, each of which showed six fluorescence signals. The spiral structure may be to present centromeric chromatin to the exterior of the chromosome, where it can mediate kinetochore assembly and interactions with the spindle.

Parts of the results from this study had been presented in the 53rd Annual Meeting of the American Society of Human Genetics, Los Angel, Nov. 2003, abstract program no. A838 and supported by grants from NSC91-2320-B-040-037 and NHRI-EX92-9207 SI. A manuscript about this study is in preparation for submitting to *Chromosoma*.

VI. Isolation of kinetochore binding DNA from Indian muntjac by chromatin immunoprecipitation.

In order to shed more lights on the interaction between the centromeric DNA and CENP, we use the strategy of chromatin immunoprecipitation to isolate the CENP-binding DNA. The ACA (anti-centromere antibody), anti-CENP-H and anti-CENP-I were used to probing CENPaca-, CENPh-, and CENPi-associated DNA, respectively. There are three different CENPassociated DNAs obtained. The CENPaca-associated DNAs were labeled with biotin and were hybridized onto metaphase chromosomal spreads of Indian muntjac. The FISH result showed that most of the isolated CENPaca-binding DNA colocalized with kinetochore. It is similar to kinetochore signals, which were observed by ACA immunofluorescence parallel along the centromere. It is different to the satellite II and IV signals where located on the centromere region. It suggested that the chromatin-immunoprecipitation method could successfully isolate the specific centromeric DNA from genomic DNA. Furthermore, we used the extracted CENPaca-associated DNAs to construct a mini-library by introducing into pSK vector. We ruled out the clones containing cervid satellite I, II, IV, and V by performing colony hybridization using satellite I, II, IV, and V as probes. The remaining negative clones would be used as probes to screen the BAC library for obtaining the complete organization of a novel functional satellite DNA. In the meantime, we also randomly pick up 30 clones for sequence analysis. The DNA sequence results showed that most clones were retroelements. One of the clones contained two parts of elements: one is satellite I DNA and the other is a novel DNA sequence, not a retroelements that is no similar to any DNA sequences of NCBI gene bank. We are carrying out subcloning this clone and performing the FISH experiment to identify the chromosomal localization of this novel DNA sequence. We will further carry out the south-western analysis to identify whether these retroelements and this novel sequence could associate with CENP. We anticipate that we can get a novel putative functional centromeric DNA. Furthermore, we will use this candidate DNA fragment to probing the BAC clones containing the complete centromeirc DNA element, which is essential for a functional centromere. The novel centromeric DNAs will be evaluated for their role of in centromere function using an artificial-chromosome assay. In artificial-chromosomal assay, the BACs are transfected into cultured cells and analyzed for their ability to form de novo centromeres (Harrington 1997; Ikeno 1998). The resulting mitotically stable microchromsomes have been used to evaluate the necessary elements of a functional human chromsome, with the hope that one day such vectors might prove useful for gene therapy (Calos 1996; Warburton 1999).

VII. Establishing BAC library of Indian munjtac genome DNA

Comparative studies of chromosome content and organization are providing insight into fundamental questions of genome evolution and speciation. Existing BAC library resources sample major branches of the phylogenetic tree. This resource has tremendous potential to inform specific questions pertaining to cnetromeric and pericentromeric evolution, primate genome evolution, and the mechanisms and consequences of karyotypic change between closely related species. The six large chromosomes of Indian muntjac mark the smallest diploid chromsome number of any mammalian species studies to date (Levy 1993). Indian muntjac could hybrid with the morphology-similar Chinese muntjac with 46 chromosome numbers (Shi et al. 1980). It was thought that the karyotype of the Indian muntjac evolved from Chinese munjtac karyotype. Four

years ago, we proposed that the Indian muntjac karyotype may have evolved directly from a 2n=70 ancestral karyotype rather than from an intermediate 2n=46 Chinese muntjac-like karyotpe. For understanding the evolution of muntjac species and genome comparative analysis, we started to construct an Indian muntjac BAC library since the second year of this project. This was a new task for us and in the beginning we spend a great deal time to overcome a number of technical problems in constructing the BAC library. Lately, we have successfully established the complete protocol for constructing such a library. At present, we have obtained 2.7 coverages of an Indian muntjac BAC library (see the following table). We will continue constructing to achieve 4 coverages at least in the coming years.

Insert size	Total whit colony NO.	Insert/white colony NO.	False positive%	Coverage
＜ 20kb	5959	86.4%	13.6%	0.04
～20kb	3632	77.3%	22.7%	0.032
～50kb	23222	90%	10%	0.46
～100kb	55268	63.2%	36.8%	2.2

In addition to informing the muntjac's genome evolution and to understanding the mechanisms and consequences of karyotypic change between closely related species, the studies of centromeric evolution will involve the isolation of BACs containing sequences likely to be responsible for centromere function in non-human primates and other mammals. These studies will be expanded to include candidate non-primate mammalian centromere sequences from animals currently serving as model organisms in biomedical and agricultural research.

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Self-evaluation:

In this project, our aims are to study the molecular structure and function of centromeric DNA. First of all, it needs to isolate and characterize novel satellite DNA elements. We had isolated a highly conserved satellite DNA element in 2002. The result of characterization had been published in Chromosoma (2002) and briefly described in the part I of results of this report. This novel satellite DNA along with other two satellite DNAs in Formosan muntjac had been analyzed. Analysis of these three satellite DNA elements in Formosan munjtac had been finished and published in Cytogenet Genome Res (2004). Furthermore, phylogentic comparisons were carried out between closely related Formosan munjtac and Chinese munjtac by satellite DNA and karyotype analysis. This part result had been published in Zoological studies (2004) and briefly described in the part II of results of this report. Additionally, a roe deer-specific satellite III DNA was also found in Chinese water deer. This part of result was briefly described in the part IV of results of this report and had been submitted to Chromosoma lately. The studies in molecular structure of centromeric DNA had been finished. The result has been briefly described in the part V of results of this report and its manuscript is in preparation for submitting to journal “Chromosoma”. In functional study of centromere, first of all, it needs to establish BAC library and to prepare CENP-associated DNA as probes. We had finished the preparation of probes and constructing 2 coverages of Indian muntjac BAC library. During construction of BAC library, we isolated a Y-specific satellite DNA from BAC library. The result has been briefly described in the part III of results of this report and published in journal “Chromosoma” (2005). Therefore, our studies meet the aims of the original project and achieve the progress of the project. Totally, we have four published papers, one submitted paper, and one preparing paper during carrying out this project.

Appendix 1:
Isolation and identification of a novel satellite DNA family highly conserved in several Cervidae species

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***Chromosoma* 111**:176-183 (2002)

Abstract In an attempt to amplify cervid satellite II DNA from the genomes of Indian muntjac and Chinese muntjac, a pair of primers derived from the white tailed deer satellite II DNA clone (OvDII) yielded a prominent ~1 kb PCR product (in addition to the expected 0.7 kb satellite II DNA fragments) in both species. The ~1 kb products were cloned, sequenced, Southern blot analyzed and FISH-studied to reveal that the ~1 kb cloned sequences indeed represent a previously unknown cervid satellite DNA family which now designated as cervid satellite IV DNA. ~1 kb PCR clones were also obtained from the genomes of the black tailed deer and Canadian woodland caribou with similar primer pairs. Extremely high sequence conservation (over 90% homology) was observed among the clones generated from all four deer species and PCR-Southern hybridization experiments further verified the co-amplification of two kinds of satellite DNA sequences with a same pair of primers. This satellite DNA was found to co-localize with CENPs at the kinetochore by simultaneously FISH and immunofluorescence study. Due to its highly sequence conservation and close association with kinetochores, the newly identified satellite DNA may have a functional centromeric role.

Introduction

One unique feature of higher eukaryotic genomes is the universal presence of large quantities of tandemly repeated DNA (satellite DNA), which could account for up to 10 – 20% of some mammalian genomes (Beridze 1986). Satellite DNAs is located mainly in the centromere where up to tens of megabases can reside in a single human centromere (Choo 1997). Furthermore, the genomic organization of satellite DNAs in the centromeric region appeares quite complex (Lee et al. 1997b) and no sequence conservation can be found among a wide range of species. Whether centromeric satellite DNA has a definitive functional role is still debatable (Choo 2000; Henikoff et al. 2001) and due to its abundance and highly repetitive nature, few attempts have been made to completely sequence this region of the chromosome. The existence of a functional "core" centromeric DNA sequence, similar to that found in the budding yeast (*Saccharomyces cerevisiae*), has not been reported in higher eukaryotic species. However, it still cannot rule out the possibility that within the complex centromere, less prominent yet functional sequence motifs have gone undetected (Henikoff et al. 2001). Most recently, efforts have been made to map a ~450 kb region of the centromeric DNA on a human X chromosome and identify a candidate functional centromeric sequence that is comprised of ~3 Mb higher-order repeats of DXZ1 alpha satellite DNA (Schueler et al. 2001).

Three centromeric satellite DNA families have been identified in the Cervidae (Table 1). Satellite I is the predominant centromeric satellite DNA family found in all deer species examined (Bogenberger et al. 1987; Scherthan 1991; Lee et al. 1997a). This cervid satellite DNA has a repeat unit of 0.8 kb in plesiometacarpalia deer and 1 kb in telemetacarpalia deer (Lee et al. 1997a). These monomers are thought to represent the higher-order structures of internal 31 bp subrepeats (Bogenberger et al. 1985; Lee and Lin 1996). The cervid satellite II family was initially isolated from the white tailed deer, and is characterized by monomeric repeats of 0.7 kb (Qureshi and Blake

1995). It was later also found in the genomes of the Indian muntjac (Vafa et al. 1999; Li et al. 2000b) and other deer species (Li et al. 2000a). Cervid satellite II DNA appears to be complexed with CENP-A, as demonstrated by immunoprecipitation of Indian muntjac DNA with human anticentromere autoantibodies (Vafa et al. 1999). The cervid satellite III DNA family has a repeat unit of 2.2 kb and is so far only reported in the genome of the roe deer (*Capreolus capreolus*) (Buntjer et al. 1998).

In this study, we have obtained ~1 kb repetitive DNA fragments using a pair of primer sequences designed to amplify satellite II DNA from the Indian muntjac or Chinese muntjac genomes. Similar ~1 kb repeated DNA elements were also generated from the genomes of the black tailed deer and the Canadian woodland caribou using a modified pair of primers. The PCR products were cloned, Southern blot analyzed, sequenced and FISH-studied to suggest the identification of a novel cervid centromeric satellite DNA with a monomeric unit of 1 kb. The ~1 kb satellite DNA clones from these four cervid species share extremely high sequence homology and are located close to the kinetochore regions. These findings imply that this new cervid satellite DNA may be functionally relevant.

Materials and methods

Cell culture and DNA isolation

Four cervid cell lines were used in this study. Cell lines from a male Indian muntjac *(Muntiacus muntjak vaginalis)*, a male Chinese muntjac *(M. reevesi)*, a male Canadian woodland caribou *(Rangifer tarandus caribou)*, were established in our laboratories previously. The female black tailed deer *(Odocoileus hemionus hemionus)* cell line was acquired from American Type Culture Collection. Cells were grown in Dulbecco's modified Eagle medium (Gibco/BRL), supplemented with 10% fetal calf serum, 1% glutamine and 1% penicillin-streptomycin. Procedures for cell harvesting and genomic DNA isolation were described elsewhere (Li et al. 2000a).

PCR-amplification and cloning

PCR amplification of the ~1 kb fragment from genomic DNA samples of the two muntjac species was achieved by PCR using a set of primers (forward: GAGCTGCCTGACAGA CTCG, reverse: CAGAGCCGACCTAGGATCAC) (Li et al. 2000a) derived from the published white tailed deer *(O.virginianus)* satellite II sequence (OvDII) (Qureshi and Blake 1995). PCR was performed in 25 µl reaction volume with 10 mM Tris-HCl, pH 9.0, 50 mM KCl, and 0.1% Triton X-100, 1.5 mM of $MgCl_2$, 200 µM each of dNTP, 200 nM of each primer, 100 ng of the genomic template DNA, and 2.5 U of Taq polymerase (Promega). Predenaturation at 94^{o}C for 5 min was followed by 30 cycles of 30 seconds at 94^{o}C for denaturation, 30 seconds at 50^{o}C for annealing and 1 min at 72^{o}C for extension. The final extension was carried out at 72^{o}C for 10 min. The PCR products were electrophoretically fractionated on a 1.2% agarose gel, and prominent ~1kb-sized DNA fragments were excised, purified, and ligated into pGEMT-easy vector (Promega). The recombinant plasmids were used to transform XL1-blue competent cells. Subsequently, the transformants were screened and randomly chosen for further characterization following the standard procedure (Maniatis et al. 1982).

Amplification and cloning of the ~1 kb DNA fragment from the black tailed deer and the caribou genomes were similar to that mentioned above except the primer sequences were modified based on the sequence of the 1 kb clone obtained from the Indian muntjac (forward:

GACTGATTTCCTGGGTTAAGAG, and reverse: CACACAGAATGCTAGGAAATCC) and the annealing temperature was set at 48^{o}C.

DNA sequencing and analysis

~1 kb PCR clones from the genome of Indian muntjac, Chinese muntjac, caribou and black tailed deer respectively were designated as MMV-1.0, MR-1.0, RTC-1.0 and OHH-1.0 and sequenced from both ends using a dideoxy chain termination kit (United States Biochemical) and read on a Perkin-Elmer ABI DNA sequencer (model 377). The complete DNA sequences of these four clones were deposited into the GenBank database (accession numbers AY 064466, AY 064467, AY 064468 and AY 064469). Single base shift self-comparisons (Pluciennicza et al.1982) were also conducted to evaluate the presence of internal subrepeats.

Southern and slot blot analysis

For Southern blot experiments, 10-μg aliquots of genomic DNA of muntjac species were each digested with one of six different restriction endonucleases. Digested DNA samples were electrophoretically fractionated, transferred to a nylon membrane (Biodyne), and hybridized with [^{32}P]dCTP-labeled DNA probes. The conditions for hybridization, membrane washing and autoradiography were described previously (Li et al. 2000b). PCR-Southern hybridizations were similarly performed with the exception that DNA samples used were PCR amplified products from the four deer species. Hybridizations were carried out with either ^{32}P-labeled MMV-1.0 DNA probe or satellite II DNA probes. Slot blot hybridization procedures for copy number estimations of the 1 kb repeated DNA elements in the genomes of four deer species were also described in detail earlier (Li et al. 2000b).

Fluorescence in situ hybridization (FISH) and immunofluorescence microscopy

Chromosome preparations of the Indian muntjac, Chinese muntjac and black tailed deer were obtained from the established fibroblast cell lines following routine cytogenetic protocols. For single color FISH experiments, the ~1 kb cloned DNA probes (MMV-1.0, MR-1.0 and OHH-1.0) were labeled with biotin and hybridized to metaphase chromosomes of each of the respective species. The hybridization signals were detected with Cy3-avidin. In dual color FISH, the newly identified ~1 kb (MMV-1.0) DNA and a cervid satellite II DNA (Mmv-0.7) (Li et al. 2000b) were labeled with biotin and digoxigenin respectively. The biotin labeled probe was detected with Cy3-avidin whereas the digoxigenin-labeled probe was detected with FITC-conjugated antibodies. The protocol used for simultaneous immunofluorescence and FISH studies was described by Sullivan and Schwartz (1995) with brief modification. The Indian muntjac cells were treated with colcemid (0.1μg/ml) for 3 hours prior to harvesting. The harvested cells were incubated in 0.075 M KCl for 15 min at room temperature and subsequently cytospun onto slides. CREST sera, specific for anti-CENP-A, B and C antibodies (a gift from Dr. J.B. Rattner, University of Calgary, Canada) were used to identify kinetochore domains. The primary CENP antibodied were detected with FITC-conjugated rabbit anti-human IgG. After immunofluorescent experiment, cells on the slide were immediately fixed in 10% formalin/KCM buffer (120mM KCl, 20 mM NaCl, 10 mM Tris-HCl pH 7.6, 0.1 % Triton X-100) for 10 min and then in 3:1 methanol/acetic acid for another 15 min. FISH experiments were carried out in a manner similar to that described for single color FISH experiments with biotin labeled MMV-1.0 probes.

Results

Isolation and characterization of ~1kb repetitive DNA elements in the muntjacs

Primer pair sequences, designed to amplify cervid satellite II DNA, produced three expected bands of 0.7 kb register with the Indian muntjac DNA template, and a 1.4 kb band with the Chinese muntjac DNA template. In addition, two unexpected PCR products (a prominent 1.1 kb band and a 1.9 kb band) were observed using both the Indian and Chinese muntjac DNA templates (Fig. 1). However, only bands with expected size of 0.7 kb register were observed with template DNAs of black tailed deer and Canadian woodland caribou using a same pair of primer.

The ~1 kb PCR products of two muntjac species were cloned, screened and designated as MMV-1.0 for ~1 kb clone of Indian muntjac and MR-1.0 for that of Chinese muntjac. Southern blot hybridization with MMV-1.0 probe to Indian muntjac genomic DNA produced multiple hybridization bands with irregular patterns (Fig. 2). Although, there were no typical type A like pattern found in any particular digestion, a 1 kb register can be detected in *Pst*I digested DNA. Copy number estimations indicated that about 0.08% of the Indian muntjac genome and 0.16% of the Chinese muntjac genome contain this repeated DNA sequence, respectively.

The MMV-1.0 clone of Indian muntjac and the MR-1.0 clone of the Chinese muntjac were sequenced (MMV-1.0 was 1102 bp in length and MR-1.0 was 1099 bp in length). Both clones are 60% AT-rich and share an extremely high (96%) sequence similarity to each other (Fig. 3). No significant internal subrepeat was detected by the single pair shifts, self-comparison method of Pluciennicza k et al. (1982). These two clones also do not share significant sequence similarity with any DNA sequences currently deposited in the GenBank including known cervid or bovine satellite DNA families. These findings suggest that 1 kb repeated DNA sequence could represent a new cervid satellite DNA family which we now refer to as cervid satellite IV.

PCR-Southern hyridization and isolation of cervid satellite IV DNA in black tailed deer and Canadian woodland caribou

PCR-Southern blot hybridization was performed to further verify the co-amplification of both the ~1 kb repetitive DNA and the satellite II DNA from genomic DNA templates of the four deer species using a single primer pair derived from a satellite II clone (OvDII). The Southern blot of the PCR products was obtained after hybridization with ^{32}P-labeled MMV-1.0 clone (Fig. 4a, left panel). This probe hybridized very strongly with the corresponding 1.1 kb PCR product (Fig. 4a, right panel) in the two tested muntjac species. Two additional faint bands were also observed in the Chinese muntjac. However, these bands were not shown in the corresponding PCR products (Fig. 4a, right panel). The MMV-1.0 probe also detected a faint 1.1 kb band in both black tailed deer and Canadian woodland caribou that was not found in the corresponding gel (Fig.4a, left panel). Another Southern blot was achieved after hybridization with the satellite II DNAs (a mixture of Mmv-0.7-, Mr-0.7-, Ohh-0.7- and Rt-0.7- clone DNA) probe. The hybridization profiles showing bands of 0.7 kb register were observed in all four tested deer species (Fig. 4b, left panel). Together, these observations showed that both the ~1 kb repeated DNA and cervid satellite II DNA could be co-amplified using the same satellite II primer pairs from genomic DNA templates of four deer species studied.

Since only limited amounts (Fig. 4a right panel) of the 1 kb PCR products were obtained from genomic DNA templates of black tailed deer and woodland caribou with the initial OvDII-based primers, another primer pairs was designed based on the DNA sequence of the newly identified MMV-1.0 sequence (see Materials and Methods). Sufficiently more 1 kb PCR products were

obtained from the black tailed deer and the woodland caribou with a new primer pair. The resulting clones were designated as OHH-1.0 and RTC-1.0 respectively. DNA sequencing showed that the OHH-1.0 (892 bp) and the RTC-1.0 (891 bp) were also 60% AT rich, were void of any apparent internal subrepeats and shared a very high degree of sequence homology between each other (93%) and between the 1 kb clones obtained from the muntjacs (Fig.3). Therefore, all four 1 kb clones appear to belong to the same cervid satellite IV DNA family. Copy number estimation showed that about 0.06% of the black tailed genome and about 0.03% of the caribou genome contains this satellite DNA sequences.

Chromosomal mapping cervid satellite IV DNA sequences

The fluorescence in situ hybridization (FISH) experiment initially localized cervid satellite IV (MMV-1.0) DNA exclusively at the centromeres of all chromosomes in the complement of Indian muntjac (data not shown). Succeeding FISH study was conducted by simultaneously hybridizing digoxigenin-labeled cervid satellite II (Mmv-0.7) probe with biotinylated cervid satellite IV (MMV-1.0) probe onto metaphase chromosomes of male Indian muntjac. The satellite II signals (green fluorescence) were located at the centromeric regions and at specific interstitial regions of the chromosome arms as reported by Li et al. (2000b). The hybridization signals of satellite IV (red fluorescence) co-localized with the satellite II signals at the centromeric regions of all chromosomes (Fig.5a). In addition, sequential centromeric protein (CENP) immunofluorescence and FISH experiments with satellite IV DNA probe (MMV-1.0) were performed. The satellite IV DNA was found to be closely associated with the CENPs (green fluorescence) in the kinetochore domains (Fig. 5b). Furthermore, FISH studies localized the Chinese muntjac cervid satellite IV clone (MR-1.0) to the centromeric regions of all Chinese muntjac chromosomes with the exception of the Y chromosome that appeared to be lacking in detectable hybridization signals (Fig. 5c). In the black tailed deer, the hybridization signals of biotin-labeled black tailed deer satellite IV clone (OHH-1.0) were also exclusively observed at the centromeric regions of all chromosomes of the species (Fig. 5d).

Discussions

In the course of amplifying cervid satellite II DNA from the genome of two muntjac species using a pair of primer sequences derived from white tailed deer satellite clone (OvDII) (Qureshi and Blake 1995), a prominent ~1 kb product was obtained in addition to the expected 0.7 kb satellite II DNA elements from the muntjac species as well as from black tailed deer and Canadian woodland caribou. Southern blot analysis, DNA sequencing and FISH studies indicated that the ~1 kb elements belong to a new family of cervid centromeric satellite DNA which is 60% AT-rich in contrast to the three previously identified cervid satellite DNAs (I, II and III) which are GC-rich. To our knowledge, it has not been reported that two different centromeric satellite DNA sequences could be simultaneously amplified with a single primer pairs. Extremely high sequence homology (over 90%) was found among those ~1 kb clones isolated from the four deer species studied. Repeated DNA found in the centromere, such as satellite DNA is thought to have resulting in the lack of sequence conservation among a wide range of species (Henikoff et al. 2001). However, sequence homology of certain satellite DNA clones can be observed among related species. For example, cervid satellite I DNA clones with monomeric sequences sharing reasonably high sequence similarity were found among a number of deer species (Lee et al. 1997a). These cervid satellite I DNA monomers showed to contain internal subrepeats organized in a higher-order fashion (Lee and Lin 1996). In the present study, no internal subrepeats were observed in the sequenced ~1kb satellite clones.

Although a regular A-type banding pattern often characteristic of satellite DNA (Horz and Zachau 1977) was not observed for the Southern blot hybridization of ~1 kb satellite DNA probe (MMV-1.0) to differentially-digested Indian muntjac genomic DNA, the *Pst*I digestion did exhibit a 1 kb register pattern, along with other hybridization bands. This suggests that a portion of this repeated DNA family is organized as 1 kb tandem repeats in the genome of this deer species. Since the repeated sequence is localized at the centromeres of all deer species examined, it should be considered as a new cervid centromeric satellite DNA that was probably not detected previously due to its comparatively reduced abundance in these genomes (0.03% - 0.16%). In general, some predominant DNA centromeirc satellite DNA families may predominate in a given species because of dynamic evolution processes involving amplification and homogenization mechanisms (e.g.human α-satellite DNA, bovine satellite II and mouse major satellite) (Willard and Waye 1987; Nijman and Lenstra 2001; Wong et al. 1990). In the mean time, less prominent, or newly created satellite DNAs may coexist with the more prevalent and older satellite DNAs with some of the new sequences potentially being derived from elements of pre-existing satellite sequences (Buntjer et al. 1998). Whether this newfound repetitive DNA is derived from element of other cervid satellite DNA sequences, particularly the satellite II family remains to be explored. This newly discovered centromeric repeated DNA could be a relatively "new satellite" only present in a small number of closely related species. Further studies should be carried out to examine whether this newly found repeated DNA is also present in the genomes of other deer species. On the other hand, FISH studies revealed that hybridization signals of this satellite DNA are co-localized with the CENPs in the centromeres and due to the fact of its sequence conservation, one could speculate that the newfound repeated sequence could be a candidate for the functional "core sequence" of the cervid centromere.

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Legends to the figures

Figure 1. Electrophoretic analysis of PCR products. 100ng of PCR products from each of the four deer species (Mmv, *Muntiacus muntjak vaginalis*; Mr, *M. reevesi*; Ohh, *Odocoileus hemionus hemionus* and Rtc, *Rangifer tarandus caribou*) amplified with a primer pair derived from cervid satellite II clone (OvDII) of white tailed deer were electrophoretic separated in a 1.2% agarose gel. A prominent 1.1 kb band and a 1.9 kb band are observed in Mmv and Mr lanes. In addition, there are three bands sized 0.7-, 1.4- and 2.8-kb shown in Mmv lane and a 1.4 kb light band found in Mr

lane. Three bands of 0.7 kb register (0.7-, 1.4- and 2.1 kb) are seen in both Ohh lane and Rtc lane.

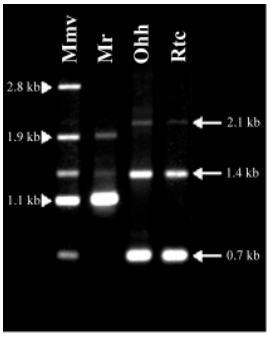


Figure 2. Southern blot hybridization of Indian muntjac genomic DNA digested with restriction enzymes: *Apa*I, *Eco*RI, *Nco*I, *Pst*I, *Pvu*II and *Nhe*I, fractionated by conventional gel electrophoresis, and probed with ^{32}P-labeled MMV-1.0 DNA. All digests showed multiple hybridization bands of repeated DNA nature but a typical ladder pattern often seen cervid satellite DNA was not observed in any digestion. However, 1 kb, 2 kb, 3 kb and 4 kb hybridization bands are observed in *Pst*I digested lane suggesting that a significant portion of the repeated sequence is having a 1 kb register.

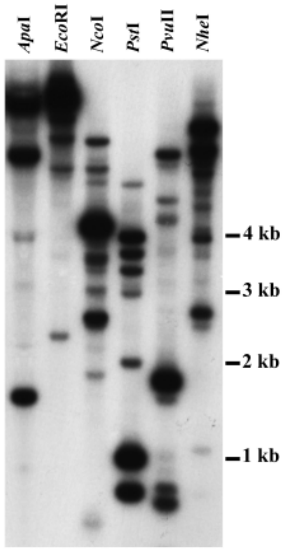
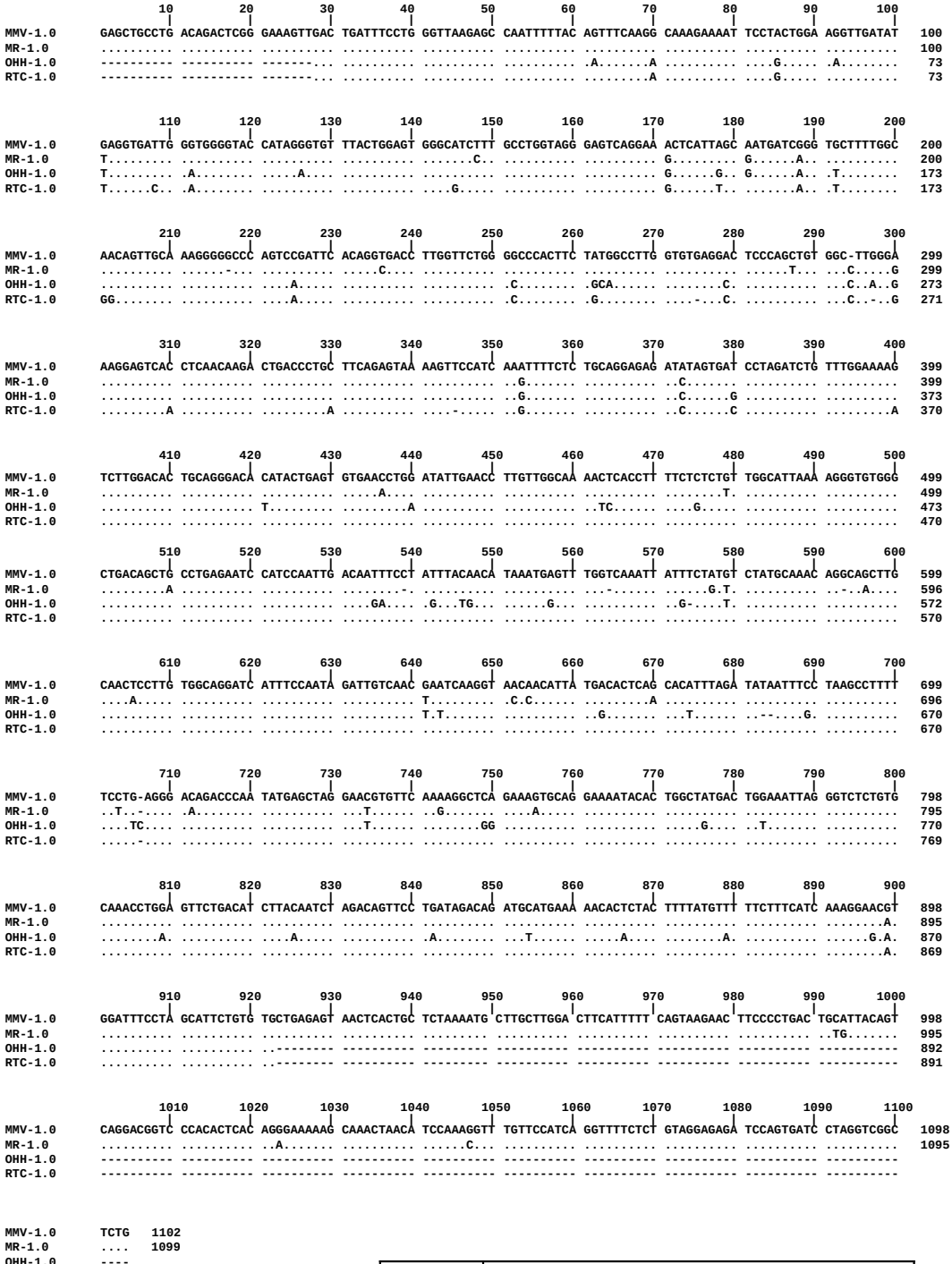


Figure 3. DNA sequences comparison of ~1 kb PCR clones MMV-1.0, MR-1.0, OHH-1.0 and RTC-1.0. The sequence of MMV-1.0 is shown at the top panel of each set. Only unmatched bases (in comparison with MMV-1.0 sequence) of MR-1.0, OHH-1.0 and RTC-1.0 are indicated and the matched nucleotides are indicated by dots. Occasional gaps (-) are introduced to improve the alignment. The percentage of sequence homology between any two clones of the four ~1 kb clones examined are also presented (in inset).



	MR-1.0	OHH-1.0	RTC-1.0
MMV-1.0	96%	93%	96%
MR-1.0	-	91%	94%
OHH-1.0		-	93%

Figure 4. PCR-Southern blot analysis. a.The eletrophoretic separation of PCR products (10ng each for Mmv and Mr, and 150ng each for Ohh and Rtc) shown in right panel were transferred and hybridized with the ^{32}P-labeled MMV-1.0 clone DNA probe. The corresponding Southern blot is shown in the left panel. A prominent hybridization band sized 1.1 kb is observed in Mmv lane. A

very strong 1.1 kb hybridization band plus two faint bands, 0.4 kb and 2.1 kb in size, can be seen in Mr lane. A hybridization band of 1.1 kb can also be observed in both Ohh and Rtc lanes. This confirms the presence of ~1 kb repeated DNA. b. The electrophoretic seperation of PCR products (Mmv:60ng, Mr: 40ng, Ohh: 30ng and Rtc: 20ng) is shown in the right panel. The corresponding Southern blot shown in the left panel was obtained after hybridization with a mixture of Mmv-0.7, Mr-0.7, Ohh-0.7 and Rt-0.7 cervid satellite II DNAs as the probe. Mr-0.7 and Ohh-0.7 are cervid satellite II clones from Chinese muntjac and black tailed deer respectively (Li et al. unpublished data). Four bands of 0.7 kb register (0.7-, 1.4-, 2.1- and 2.8-kb) are shown in Mmv lane and a three band pattern of 0.7 kb register (0.7-, 1.4- and 2.1-kb) is seen in Mr, Ohh and Rtc lanes confirming the presence of satellite II DNA products. M in the right panels denotes the standardized DNA marker.

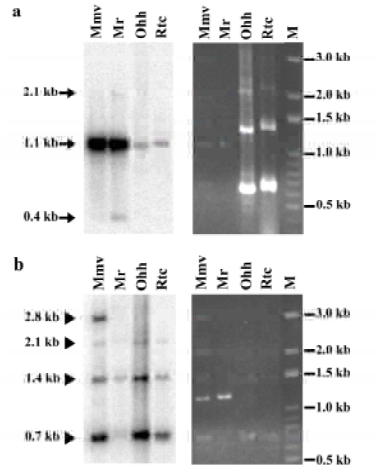


Figure 5. Chromosomal localization of satellite IV DNA clones and satellite II DNA clone as well as immunofluorescence detection of kinetochore with human CREST serum. **a** Co-localization of satellite IV clone MMV-0.1 (red signals) and satellite II clone Mmv-0.7 (green signals) on centromeric regions of chromosomes from a male Indian muntjac. **b** Simultaneously detection of MMV-1.0 (red signals) and immunofluorescent signals of anti-centromere antibodies (green signals) on the centromeric region of the male muntjac chromosomes. Both fluorescent signals appear to be located together at the kinetochore sites. Bar in **b** represents 10μM in **a** and **b**. **c** Localization of cervid satellite IV clone (MR-1.0) DNA to all the centromeric regions (except the Y-chromosome indicated by an arrow) and certain interstitial sites of the male Chinese muntjac chromosomes. **d** Localization of cervid satellite IV clone (OHH-1.0) DNA to all the centromeric regions of female black tailed deer chromosomes. Bar in **d** represents 10μM for **c** and **d**.

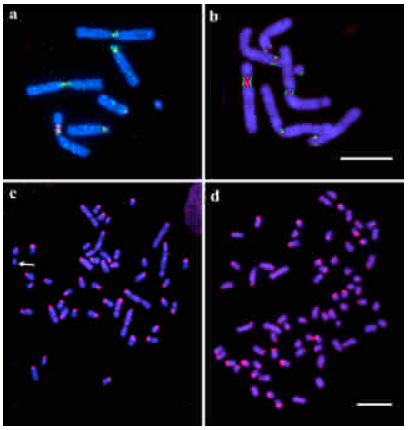


Table 1. Cervid satellite DNA families

Family	Species		Satellite DNA clone	Reference
Satellite I	Indian muntjac	*Muntiacus muntjak vaginalis*	MMVsatIA	Bogenberger et al. (1982)
	Chinese muntjac	*Muntiacus reevesi*	C5	Lin et al. (1991)
	Roe deer	*Capreolus capreolus*	CCsatI	Scherthan (1991)
	Caribou	*Rangifer tarandus caribou*	Rt-Pst3	Lee et al. (1994)
	Red deer	*Cervus elaphus*	Ce-Pst1	Lee and Lin (1996)
	Moose	*Alces alces*	Aa-Msp	Lee et al. (1997a)
	White tailed deer	*Odocoileus virginianus*	Ov-Msp	Lee et al. (1997a)
	Mule deer	*Odocoileus hemionus*	Oh-Msp	Lee et al. (1997a)
	Fallow deer	*Dama dama*	Dd-Pst1	Lee et al. (1997a)
Satellite II	White tailed deer	*Odocoileus virginianus*	OvDII	Qureshi and Blake (1995)
	Caribou	*Rangifer tarandus caribou*	Rt-0.7	Li et al. (2000a)
	Indian muntjac	*Muntiacus muntjak vaginalis*	Mmv-0.7	Li et al. (2000b)
Satellite III	Roe deer	*Capreolus capreolus*	CCsatIII	Buntjer et al. (1998)
	Chinese water deer	*Hydropotes inermis*	HI-III	Li et al. (unpublished)

Appendix 2:
Cloning, characterization and physical mapping of three cervid satellite DNA families in the genome of Formosan muntjac *(Muntiacus reevesi micrurus)*

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Abstract. *Background and aims:* Due to the high sequence diversity even among closely related species, satellite DNA sequences can be a useful molecular marker for phylogenetic and taxonomic analyses. To characterize the satellite DNAs in the genome of a native muntjac species of Taiwan, the Formosan muntjac, satellite DNA clones representing three different cervide satellite DNA families from this species were isolated and analyzed. *Methods:* PCR amplifications for cloning of cervid satellite DNAs from Formosan muntjac genome using primer pairs specific for cervid satellite DNA families were performed, and the obtained DNA clones were subjected to sequences analysis and comparison, Southern blot hybridization analysis and copy number estimation. Multi-color FISH experiment was performed to simultaneously map the different satellite DNA families in the Formosan muntjac centromeric region. *Results:* Three resulted Formosa muntjac satellite DNA clones representing cervid satellite family I, II and IV were designated as FM-satI (1,391 bp), FM-satII(1,143 bp) and FM-satIV(1,103 bp), and found to share ~82%, 81% and 98% sequence homology with Chinese muntjac satellite I clone (C5), Indian muntjac satellite II clone (Mmv-0.7) and Chinese muntjac satellite IV clone (MR-1.0), respectively. The three satellite DNA families are organized in a pter <– FM-satII – FM-satIV – FM-satI –> qter orientation in the centromeric region. *Conclusion:* Satellite DNA sequence comparisons, in combination with FISH data concludes that Formosan muntjac is another subspecies of *M. reevesi*, closely related to Chinese muntjac. With the kinetochore satellite DNA terminally located, the Formosan muntjac chromosomes could be truly telocentric.

It remains intriguing that large amount and multiple families of satellite DNAs distribute mainly, if not exclusively, in the centrmeric/pericentromeric heterochomatin regions of mammalian chromosomes. Although the functional role of most satellite DNAs has yet to be defined, their particular mode of evolution, species specific and organization in the genome by itself is valuable for phylogenetic and karyotypic evolution studies (Garrido-Ramos et al., 1999; Li et al., 2000a). Satellite DNA is also the most dynamic part of the genome, as such new satellite DNA with few simple subunits could evolve through replication slippage (Levinson and Gutman, 1987). More complex satellite DNA repeats were then derived by duplication of subunits, inversions and insertions (Charlesworth et al, 1994), and finally, high copy number of repeats was achieved by unequal crossing over, or more efficiently, by the rolling circle amplification mechanism (Walsh, 1987). New satellite sequences could be derived from pre-existing satellite sequences or from a new sequence (Buntjer et al., 1998) and may coexist with the old satellite DNAs. It is not uncommon that several satellite families were found to cluster around certain chromosomal regions, e.g. pericentric region (Modi et al., 1993) or in close proximity with each other (Li et al., 2000b). Detailed characterization of the genomic organization of these satellite DNA families may shed more light on the structural and functional aspects of satellite DNA.

The closely related Asian muntjac deers have great variation in chromosome number from 2n=6/7 of Indian muntjac (*Muntjacus muntjak*) to 2n=46 of Chinese muntjac (*M. reevesi reevesi*), and satellite DNAs were thought to play a pivotal role in such extensive karyotypic evolution (Lin et al., 1991;Yang et al., 1997; Li et al., 2000a). The Fomosan muntjac (*M. reevesi micrurus*) is considered as a native endangered subspecies of *M. reevesi* in Taiwan (Wilson and Reeder, 1993). However, unlike the other subspecies of *M. reevesi*, the Chinese muntjac, no karyotypic analysis and/or satellite DNA characterization have been reported. In this study, we detected a 2n=46 chromosome complement in Formosan muntjac with DAPI/G-banding patterns resembling to those

of Chinese muntjac (Yang et al., 1995). Furthermore, sequence comparisons were made between Formosan muntjac satellite DNA families I, II and IV with their counterparts of Chinese muntjac and Indian muntjac satellite DNAs. A very high degree of sequence homologies were found. Together, we support the notion that Formosa muntjac is another subspecies of *M. reevesi* and is closely related to another subspecies; the Chinese muntjac. In addition, we simultaneously mapped these three satellite DNA families in centromeric regions of the Formosan muntjac chromosomes using multi-color FISH technology to delineate their centromeric/ pericentric orientation.

Materials and methods

Primary culture and establishing skin fibroblast cell line

The skin biopsies of a male Formosan muntjac (*Muntiacus reevesi micrurus*) kindly provided by Taipei-Zoo, Taipei, Taiwan were initially grown at 37^{o}C in Dulbecco's modified Eagle medium (DMEM) (Gibco/BRL) supplemented with 15% fetal calf serum, 1% glutamine, 1% penicillin-streptomycin-neomycin. After the fifth passage subculture of skin fibroblast, the cell cultures were maintained in the same DMEM (Gibco/BRL) supplemented with 10% fetal calf serum, 1% glutamine, and 1% penicillin-streptomycin-neomycin.

PCR amplifications and cloning of Formosa muntjac satellite DNA elements

Genomic DNA was isolated from the above established skin fibroblast cell line of Formosan muntjac according to the procedure described elsewhere (Li et al., 2000a,b). PCRs were performed in 25ul reaction volumes, each with 10 mM Tris-HCl, pH 9.0, 50 mM KCl, 0.1% Triton X-100, deoxynucleotide triphosphates (200uM each), 200 nM of satellite DNA primer pairs [for cervid satellite I, primer pairs: 5'-ACCAGAAACAGCTTCGTG-3' and 5'-GGTTATATTCTCGAGTTAACG-3' (Lin et al., 1991); for satellite II, primer pairs: 5'-GGCCTGGGAGTGTGG-3'and 5-'TGTGAAGAGAGCCCC TG-3'; for satellite IV, primer pairs: 5'- GAGCTGCCTGACAGACTCG - 3' and 5'- CAGAGCCGACCTAGGATCAC - 3'(Li et al., 2002)], 100 ng of genomic DNA template and 1.25 units of Taq DNA polymerase (Promega). The PCR reactions were carried out with an initial denaturation at 94^{o}C for 5 min, followed by 30 cycles of 94^{o}C for 30 s, 50^{o} C for 30s, 72^{o}C for 1 min and a final extension at 72^{o}C for 10 min. The PCR products were electrophoretically fractionated on a 1.5% agarose gel and prominent DNA fragments were excised, purified, and ligated into pGEM-T easy vector (Promega). The ligated DNA was then used to transform XL1-Blue E. coli competent cells according to the standard protocol (Maniatis et al. 1982) and insert-containing clones were randomly chosen for further characterization.

DNA sequencing, Southern blot analyses, and copy number estimations

Several PCR clones from the genome of Formosan muntjac were obtained of which a 1.4-kb clone of satellite I DNA, a 1.14-kb clone of satellite II DNA and a 1.1-kb clone of satellite IV DNA were designated as FM-satI, FM-satII and FM-satIV, respectively, and sequenced from both ends using a dideoxy chain termination kit (United States Biochemical) and read on a Perkin-Elmer ABI DNA sequencer. The complete DNA sequences of these three clones were deposited into the GenBank database (accession numbers AY380827, AY380828 and AY380829). The three sequences were also subjected to single base shift self-comparisons (Pluciennicak et al. 1982) to investigate for the presence of internal subrepeats.

For Southern blot experiments, 10µg aliquots of Formosan muntjac genomic DNA were incubated with one of six different restriction endonucleases. The digested DNA samples were electrophoretically fractionated on 0.8% agarose gel, transferred to a nylon membrane (Biodyne) and hybridized with ^{32}P-dCTP-labeled insert satellite I DNA of FM-satI clone. The conditions used for hybridization, filter washing and autoradiography were described previously (Lee et al. 1994). The same nylon membrane was used to hybridize with ^{32}P-dCTP-labeled insert satellite II DNA and insert satellite IV DNA of FM-satII clone and FM-satIV clone respectively after the previous probe was washed off by means of an alkali solution. Slot-blot hybridization procedure for copy number

estimation of the three cervid satellite monomers in the Formosan muntjac genome is also described earlier (Li et al. 2000b).

Fluorescence in situ hybridization

Chromosome preparations were performed from the Formosa muntjac fibroblast cell line following standard protocols which include a 30 min. treatment in 0.1µg/ml Colcemid (Gibco/BRL), 15 min. hypotonic treatment in 0.075M KCl, three fixations in 3:1 methanol: glacial acetic acid and air-dried slide preparation. The insert DNA fragments of FMsatI, FM-satII and FM-satIV were labeled with SpectraRed-dUTP (Vysis), biotin-dUTP (Roche) and digoxigenin-dUTP (Roche) by nick translation, respectively. For multi-color FISH experiments, the three different satellite DNA probes were simultaneously hybridized to Formosan muntjac metaphase spreads. Biotin-labeled probes were detected with cy5-streptavidin while digoxigenin-labeled probes were detected with FITC-conjugated antibodies and chromosomes were counter stained with DAPI. The procedures for denaturation, hybridization, post hybridization washing and signal detection are described in detail elsewhere (Lee et al. 1999). Fluorescent signals were captured on an Olympus BX60 fluorescence microscope equipped with appropriate filter sets and a cooled charge-coupled device (CCD) camera (Photometrics KAF 1400). Images were normalized and enhanced using the MacProbe v4.0 software (Perceptive Scientific).

Results

Isolation and characterization of three satellite DNA clones from Formosan muntjac genome

Satellite I DNA elements were cloned from PCR-amplification with the genomic DNA template of Formosan muntjac and a primer pair derived from the Chinese muntjac satellite I clone (C5 clone) (Lin et al. 1991). One of the PCR clones was sequenced with 1391 bp in length and 52.7 % GC-rich. This clone was designated as FM-satI. An *Eco*RI-digested complete monomer, with 796 bp in length from the nucleotides 202-997 of the FM-satI clone, has 81.7% sequence similarity to the nucleotides of the C5 clone (Fig.1A). Interestingly, there is a 92% sequence identity between the nucleotides 1-201 of the FM-satI clone and the nucleotides 583-783 of C5 clone. The nucleotides 998-1391 of FM-satI clone also share an 87% sequence homology with the nucleotides 1-399 of C5 clone (Fig.1B). The satellite II DNA elements were amplified from the genomic DNA template of Formosan muntjac using a pair of primers derived from the Indian muntjac satellite II clone (Mmv-0.7 clone) (Li et al. 2000a) and cloned into pGEM-T vector. One of those satellite II DNA transformants, designated as FM-satII, was characterized as 1143 bp in length, 61% GC-rich and showed 81% sequence similarity to Mmv-0.7, confirming that it is a Formosan muntjac satellite II DNA clone (Fig. 2). The FM-satIV clone was generated from PCR-amplification with a primer pair derived from the white tailed deer satellite II clone, OvDII (Qureshi and Blake 1995). The FM-satII clone has 1103 bp in length, 56.4 % AT-rich, and 98% sequence homology with satellite IV DNA clone of Chinese muntjac, MR-1.0 (Li et al. 2002) (Fig. 3).

The single base shift and self-comparisons of a monomer from FM-satI clone showed the presence of 31 bp-subrepeats in the monomer (data not shown). In average, there are about 30% sequence similarities among subrepeats. This indicated that the 31-bp subrepeats were also organized in a high-order fashion as ~0.8 kb monomer in Formosan muntajc genome. In comparison, there was no internal repeat found in neither FM-satII clone nor FM-satIV clone.

The Southern blot hybridization of the newly isolated FM-satI clone to *Apa*I-, *Bam*HI-, *Eco*RI-, *Nco*I-,*Pst*I-, and *Pvu*I-digested genomic DNA from Formosan muntjac revealed a type A ladder pattern typical for satellite DNA in a 0.75 kb register (see PvuI digestion in Fig. 4A). Such a 0.75 kb register was also found in *Bam*HI, *Eco*RI and *Pst*I digestions. This indicates that the cervid satellite I DNA in the Formosan muntjac genome is organized primarily as 0.75 kb tandem repeats. The Southern blot hybridization of the FM-satII DNA probe to restriction enzyme-digested Formosan muntjac genomic DNA produced atypical patterns of hybridization bands for the satellite II DNA (Fig. 4B). Five of six restriction digestions did however reveal certain bands in a 0.75 kb register

(e.g. *Apa*I-, *Nco*I- and *Pvu*II- digested DNAs) suggesting some portion of cervid satellite II DNA in the Formosan muntjac genome is organized as 0.75 kb tandem repeats. An irregular hybridization pattern was observed in the Southern blot hybridization with the FM-satIV probe to the restriction digested Formosan DNA. Nevertheless, prominent hybridization bands of 1 kb, 2 kb, 3 kb, 4 kb and 7 kb were observed in *Pst*I-, *Pvu*II-, *Bam*HI-, *Nco*I-, and *Eco*RI-digested DNA, respectively (Fig. 4C). This indicated that the monomer of cervid satellite IV DNA is likely 1 kb in size in the Formosan muntjac genome.

Quantitative slot-blot hybridization experiment estimated that approximately 3.8%, 2.1% and 0.16% of the Formosan muntjac genome consists of FM-satI, FM-satII and FM-1V-like DNA sequence, respectively.

Physical mapping of cervid satellites I, II and IV in the Formosan muntjac chromosomes

The fluorescent in situ hybridization experiment was carried out by simultaneously using a rhodamin labeled satellite I probe (FM-satI), a biotinylated satellite II probe (FM-satII) and a digoxigenin-labeled satellite IV probe (FM-satIV) to hybridize with male Formosan muntjac chromosome spreads. The FISH results revealed satellite I signals (appeared as red fluorescence) at the pericentromeric region of all chromosomes except for a large pair of autosomes (identified as chromosome 3s) and the Y chromosome (Fig. 5A,B). Hybridization signals were also observed at specific interstitial sites on five pairs of autosomes. Among those, two satellite I interstitial sites were found in each chromosome 1 and 3 homologue and one satellite I interstitial site was observed in each of the remaining 3 autosomal pairs. The hybridization signals of biotinylated satellite II probes detected with streptavidin-Cy5 (appeared as a yellow pseudocolor) are present at the pericentromeric region of all Formosan muntjac chromosomes with the exception of the Y chromosome (Fig. 5C). These satellite II hybridization signals were often observed as two smaller doublets located on the lateral sides of the pericentric/centromeric regions. No detectable interstitial satellite II signals were observed. The green FISH signals produced by satellite IV DNA probe were detected with FITC-conjugated antibodies (Fig. 5D). The hybridization signals of satellite IV were observed at all centromeric regions of Formosan muntjac chromosomes except chromosome 2s, 3s and Y. Multicolor-FISH image can be achieved to show the hybridization signals of three satellite DNAs simultaneously and their order in the centromeric/pericentromeric regions can also be determined (Fig. 5E). In a given centromeric/periccentromeric region where all three satellites are present, the doublets-like satellite II signal was found at the terminal of the region. This is closely followed by a singlet-like (or a block) satellite IV signal in the middle of the pericentromeric (primary constriction) region. The satellite I signal is locates after the satellite IV towards the other end of the chromosome (Fig. 5E,F).

Discussion

Highly repetitive and tandemly arranged DNA sequences in eukaryotic genome is known as satellite DNAs. In vertebrates, satellite DNA is commonly located in the centromeric/pericentric regions of the chromosomes. Due to the apparent lack of functional constraint, the nucleotide sequences of many satellite DNAs are highly variable among species. However, through the process of concerted evolution (Elder and Turner, 1995), intra-specific sequence homogenization of satellite DNA families could occur, and can be presented as useful molecular markers for phylogenetic analysis (Garrido-Ramos et al., 1999; Kato, 2003). The Formosan muntjac (*M. reevesi micrurus*) and Chinese muntjac (M. reevesi reevesi) are both subspecies of Reeves' muntjac (*Muntiacus reevesi*) (Wilson and Reeder, 1993). Formosan muntjac and Chinese muntjac appeared to have similar G-banded karyotypes. In the present study, at least 82% nucleotide sequence similarity between Formosan muntjac satellite I DNA (FM-satI) and Chinese muntjac satellite I DNAs (C5) was observed suggesting that the two species are closely related (Lee et al., 1997). Moreover, sequence comparisons the satellite IV DNA clone of Formosan muntjac (FM-satIV) and Chinese muntjac satellite IV clone (MR-1.0) revealed a 98% sequence homology. Although satellite IV DNA was known to be well conserved with very high sequence homology between several cervid species

studied (Li et al., 2002), such high sequence similarity of satellite IV DNA sequences between these two species is somewhat surprising. Furthermore, both species have the same amount of satellite IV in their respective genome (~0.16%). These findings together indicate both species are very closely related and thus support the above taxonomic classification.

Satellite II DNA initially isolated from the white tailed deer (Qureshi and Blake, 1995) is the only cervid satellite DNA known to co-precipitate with centromere protein A (CENP-A) in the Indian muntjac (Vafa et al., 1999). CENP-A located at the inner kinetochore plate is specifically associated with functional centromere (Warburton et al., 1997). The association of satellite II and CENP-A suggests that this satellite could have structural /functional role for the cervid centromere. We have identified a satellite II DNA clone (FM-satII) from Formosan muntjac in this study and compared the nucleotide sequences of this clone with the Indian muntjac satellite II clone (Mmv-0.7) obtained previously (Li et al., 2000). An 81% sequence similarity between these two clones was observed. This finding shows that Formsan muntjac is also closely related with Indian muntjac.

Study of genomic organization of various centromeric satellite DNA families is also important for understanding the role played by these satellite DNAs in centromere structure and function. Simultaneous mapping of three cervid satellite DNA families, I, II and IV in the centromeric/perientromeric regions of a deer species was achieved for the first time. The orientation of these three satellite DNA sequences on a given Formosan muntjac chromosome can be delineated as: pter <– satellite II –satellite IV – satellite I –> qter (Fig.5E,F). The satellite II DNA is located at the terminal of centromeric /pericentric region on the chromosome. Since this satellite DNA is a kinetochore DNA which co-purifies with CENP-A (Vafa et al., 1999), most if not all the Formosan muntjac chromosomes could be truly telocentric with terminals located kinetochore/centromere. This telocentric nature could also facilitate the tandem centric process for karyotypic evolution among muntjac species, since no short arm chromosome material is there to be lost. The Cervid satellite IV is a newly evolved satellite DNA family due to its highly conservative nature. Most likely, it could be derived from a section of satellite II DNA since satellite IV DNA clones were generated using primer pairs from satellite II DNA of other deer species (Li et al., 2002 and present study). It is therefore not unexpected to see that the satellite IV DNA is located in close proximity with satellite II DNA in the pericenric regions of Formasan muntjac chromosomes. Satellite I DNA is the most abundant cervid satellite DNA that occupies ~4% of the Formosan muntjac genome and is the only cervid satellite DNA studied so far (including the present study) to be identified as having internal subrepeats in it's monomers (Lee et al., 1997 and this study). It is located in the pericentric region toward the center portion of the Formosan muntjac chromosome. This cervid satellite DNA was also found in some interstitial chromosome sites as in its Chinese muntjac counter parts (Yang et al., 1997). These interstitial satellite I DNA sequences are considered as remnants of ancestral centromeric satellite DNA after the course of restructuring *M. reevesi* karyotypes.

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Legends of figures

Fig. 1. DNA sequence comparison of Formosn muntjac satellite I clone (FM-satI) with satellite I clone, C5 derived from the Chinese muntjac. (**A**) DNA sequence comparison of the FM-satI

clone (nucleotides 202-997) with the C5 clone. Nucleotides of C5 clone, which are identical to the FM-satI, are indicated by dots. Occasional gaps (-) were introduced to improve the alignment. (**B**) Schematic illustration of the homology between sections with nucleotides 1-201 of FM-satI and 583-782 of C5 showing a 91.6% sequence similarity, between sections in (**A)**, (shaded, the FM-satI section is marked by two EcoRI.sites incated by E) showing 81.7% homology, and between sections with nucleotides 998-1391 of FM-satI and nucleotides 1-399 of C5 showing an 85.9% sequence similarity.

A

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FM-satI  202 ---------C CTGCAGTGAC TTGAGTGCAA GCTGGATTTC ATCTCACAAG ATGAAGGGAT GTCTGAAGCC CCTGGGGAGG CACTAGAGAA TGCCCTAGCA 292
C5         1 CTTCAGTGA- ..T..... .-........ .....--A... .......... ..A..... .......... .........A .......... A........T 96

                  110       120       130       140       150       160       170       180       190       200
                    |         |         |         |         |         |         |         |         |         |
FM-satI  293 CACTGCCCTT TCTCGACAGT CGGCCTCCCA TCCATTGGAC AGCTCGAGAG G-AACACGGA GTTCAATGAT GCCAAAGGAG AAGATGCCTG ACTCCTCTGG 391
C5        97 .C.....T.A ...A..G..G .......... ..A......G .......... .G........ .......... T......... .C........ .......... 196

                  210       220       230       240       250       260       270       280       290       300
                    |         |         |         |         |         |         |         |         |         |
FM-satI  392 AAAATGGACA GGAATCCCAG GATCCAAGTG GCAACAGGAA CGGGAACCTG GGTCTTCAGC CTCACCTC-G AGAGGAGTTC CTATGGACCT TTCAAGCCGC 490
C5       197 .......... .......... .....C.... .........T A....TAA.. .....GTC.. .......TT. ...A.TT... ......C... ........C. 296

                  310       320       330       340       350       360       370       380       390       400
                    |         |         |         |         |         |         |         |         |         |
FM-satI  491 GAGGAGACTC CCGAGGGGTC CCTCGCAAAC TAGACAGGAG TCCTGACGTC GCTGAACACA CCC-GTGTAT GGAAGGGCCA TCCCCGACGT AACTCGAGAA 589
C5       297 .......... .......-.. ....A..-- C.A..... .......C.. .......... ...C....-. T......... .....TCGT. .......... 392

                  410       420       430       440       450       460       470       480       490       500
                    |         |         |         |         |         |         |         |         |         |
FM-satI  590 TATACCCCAG GTTCCCTCCT CAACTCGAGA AAAAACCATG AGACTCCCGC CTCGCCGCGA GATTAAGCCT GATTCCCCTG CACTGCGAGC AAAGAAATAC 689
C5       393 ....A..-.. --.T..G..G .......... ....--.G.. ..T....AC. ......A... ...A.G...C .......... ...A..AT.. .G..C...TG 487

                  510       520       530       540       550       560       570       580       590       600
                    |         |         |         |         |         |         |         |         |         |
FM-satI  690 TGTGCTCCCC GTAAAACC-- GAAAGGAGCC TTGATTTTCT TGATGGCACT CCAGAGAAAG TCAAGGAACA CTCTCTCAAG TATAGAGGGA TCCTGAGCTC 787
C5       488 C...T....A A.C.....CG .....A.... .......C.. -....---.. .AC....--- -----.C.G. .CG.-..C.. -.C.TGT... ....A.AG.. 573

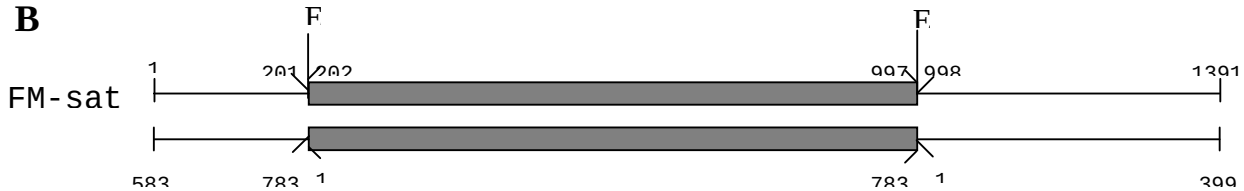
                  610       620       630       640       650       660       670       680       690       700
                    |         |         |         |         |         |         |         |         |         |
FM-satI  788 ACTGCAGGAA CCAGAAAGAG CTCCGTGTAC CCCCAATCAT CTCGAGGTGA GAGCGGATTT ACTGGCTTCA AATCAAGAGG AATGCCAACT GTCCACAAGC 887
C5       574 ...T..TC.. .......C.. ..T......T TT.A..A.T. ......C... ...AT..... C......... .......... ....A..... .......... 673

                  710       720       730       740       750       760       770       780       790       800
                    |         |         |         |         |         |         |         |         |         |
FM-satI  888 ACCACAAGAG GAGGCCTCTC GCAGCTGTAC GTTTGGGAGA AGGACGTTGA ATTTATGGCT TCCACTGGAA TCGACCCCGA GATGCCCTGA CTCGAAATCA 987
C5       674 .......... .....T.... .......... ..C...TT.. G...G..... ....GC...C ....G..... ....A..... .......... .......... 773

                 810
                   |
FM-satI  988 GGCCGGAATT 997

C5       774 .......... 783
```

B



Identity 91.6% 81.7% 85.9%

Fig. 2. Comparison of DNA sequences of Formosa muntjac satellite II clone (FM-satII) with Indian muntjac satellite II DNA clone Mmv-0.7. Comparisons were made between nucleotides 1-580 of FM-satII and 84-665 of Mmv-0.7 showing 81.3% sequence homology (**A**), and between nucleotides 620-1143 of FM-satII and 1-526 of Mmv-0.7 showing 80.1% sequence homology (**B**). Nucleotides of Mmv-0.7 identical to FM-satII are indicated by dots. Gaps (-) were used to improve the alignment.

```
                10        20        30        40        50        60        70        80        90       100
                 |         |         |         |         |         |         |         |         |         |
FM-satII     1 GGCCTGGGAG TGTGGAGCCC ATAGCTGGCC CCAAGACAAT TGGCCAAGGG CC-TGAGAGC GGACAAACCC GATGCGTTAT TGTCCTGGGC TTGGGAGCAG  99
Mmv-0.7     84 .......... .......... CC..G...T. ..C..G.TGA G..T....T. ..C......A ....C.-... ..A...A.T. .C...C...G ..........  182

               110       120       130       140       150       160       170       180       190       200
                 |         |         |         |         |         |         |         |         |         |
FM-satII    100 GGTGCGTGGC TTGCAGGAAA TGTGGGTCTC CCTCGGGTAG AGTGCTTGCA AACTGGCCTT TGGAGGCAGC CGGGGA-GGT CTTCCAGCGC CAGAGTGCTG  198
Mmv-0.7     183 A....AG... ....G..... C.....C... ....T..G.. .......... ..TG.A.... .......... ......A... .......... T..C....A.  282

               210       220       230       240       250       260       270       280       290       300
                 |         |         |         |         |         |         |         |         |         |
FM-satII    199 GATTTTCTCT GAGGACCCAC AGGCCTGGGG CAGGTCCCAG CATGAATACA GCTCCAGACC AGACTAGCCC AGACCTTCCT GGCCTCCGGG GGTACTGCCC  298
Mmv-0.7     283 .T.C.G..AC ........T. G....A.T.A ....G..G.. ....G.CCTT ..A....G.. ..C..T.... ....T..... .......... ..........  382

               310       320       330       340       350       360       370       380       390       400
                 |         |         |         |         |         |         |         |         |         |
FM-satII    299 CATGAAAGAG CAGTCTGTGG GGTGTGTGTC CATACATGCC AAGGCGCCTC CCCAGCCTCA TTTCATTAGC GCCTTTTCCC TGGTGCCTAG GCCTCATTTG  398
Mmv-0.7     383 ..C...G.G. ...G...C.. ..A....A.. ..A....... C.A..C...G TA...GG..C ..C.C.A... ......C..T .......... ..........  482

               410       420       430       440       450       460       470       480       490       500
                 |         |         |         |         |         |         |         |         |         |
FM-satII    399 AGGCCTACTT GCAGGGGAGG TTTCTGGCAG GGGCTCTCTT CACACTGCAG GGGACTGGAG CCCGGACGGT CTGGTCAGAG GGAAGAGGCC AGCAAGGGGT  498
Mmv-0.7     483 .......... .G.T..T... A......... .......... .........A .AAC...... TT.......A .......... .......... ....T.....  582

               510       520       530       540       550       560       570       580
                 |         |         |         |         |         |         |         |
FM-satII    499 TTGATCCCCA AGGCCTCTTT GGCCCAGCAG CCTCAGGGCA GCAGGAAGAG CAAGTGG-CC ACTGTGCCCC AAGGTTGGCC CTG 580
Mmv-0.7     583 .CT..G.... .......... T.T....... ....C.T... .T...G.A.. .......C.. ......AT.. T....C...T ... 665
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FM-satII    620 GAGCTGCCTG ACATACTCTG GATCACCTGA GCTCTCTGAC GGTGCAGAGG GCCGGAAGAG ACGTGGGCAT GTGGTTCTGC CTTGGCCTGG GAGTGTGGAT  719
Mmv-0.7       1 .......... ...G....G. .C...G...G .......... .......GA. T.A....... .....T.... ....C...C. .......... .........G  100

FM-satII    720 CCCCTAGTTG TACCCAAGGC AATTGGCCAA GGGCC-TGAG AGCGGACGAA CCTGATGCGC TATTGTCCTT GGCTTGGGAG CAGAGTGCGT GGCTTGCGGG  818
Mmv-0.7     101 ....C..G.. GT...C.... TGAG..T... .T...C.... ..A....C.C ..-..A...A .T..C...CG ..G....... ........AG ..........  199

FM-satII    819 AAATGCGGGT CTCCCTCGGG TAGAGTGCTT GAAAACTGGC CTTTGGAGGC AGCCGGGGT- GGTCTTCCAG CGCCAGAGTG CTGGATTTGC TCTGTGGCCC  917
Mmv-0.7     200 ...C.T...C .......T.. G......... .C...TG.A. .......... ........AA .......... ...T..C... .A..T.C... .AC.A..A..  299

FM-satII    918 CACAGTCCTG GGGCAGGTCC CAGCATGGAT ACTGCTCCAG ACCAGCCTTG CCGAGACCTA CCTGGCTTCC GGGGGTACTG CCCCATGAAG GAGCTGGCTG 1017
Mmv-0.7     300 .T.G.G..A. T.A....G.. G........C CT...A.... G......... ..C....T.T ......C... .......... .....C.... .G..A.....  399

FM-satII   1018 TGGGGTGTGT GTCCATACAT AACCAGGCGC CTCCCCAGGC TCATTTTATT AGCACCTTTT CCCTGGTGCC TAGGCCTCAT TTGAGG-CAA ATTGCAGGGG 1116
Mmv-0.7     400 C....A.... A....A.... GC...A..C. ..GTA....G ..C..CCC.A ...G.....C ..T....... .......... ......C.T. C...G.T..T  499

FM-satII   1117 AGGTTTCTGG CAGGGGCTCT CTTCACA  1143
Mmv-0.7     500 ...A...... .......... .......  526
```

Fig. 3. DNA sequence comparison of Formosa muntjac satellite IV clone (FM-sat-IV) with Chinese muntjac satellite IV clone (MR-1.0) and shows a 98.1% sequence similarity.

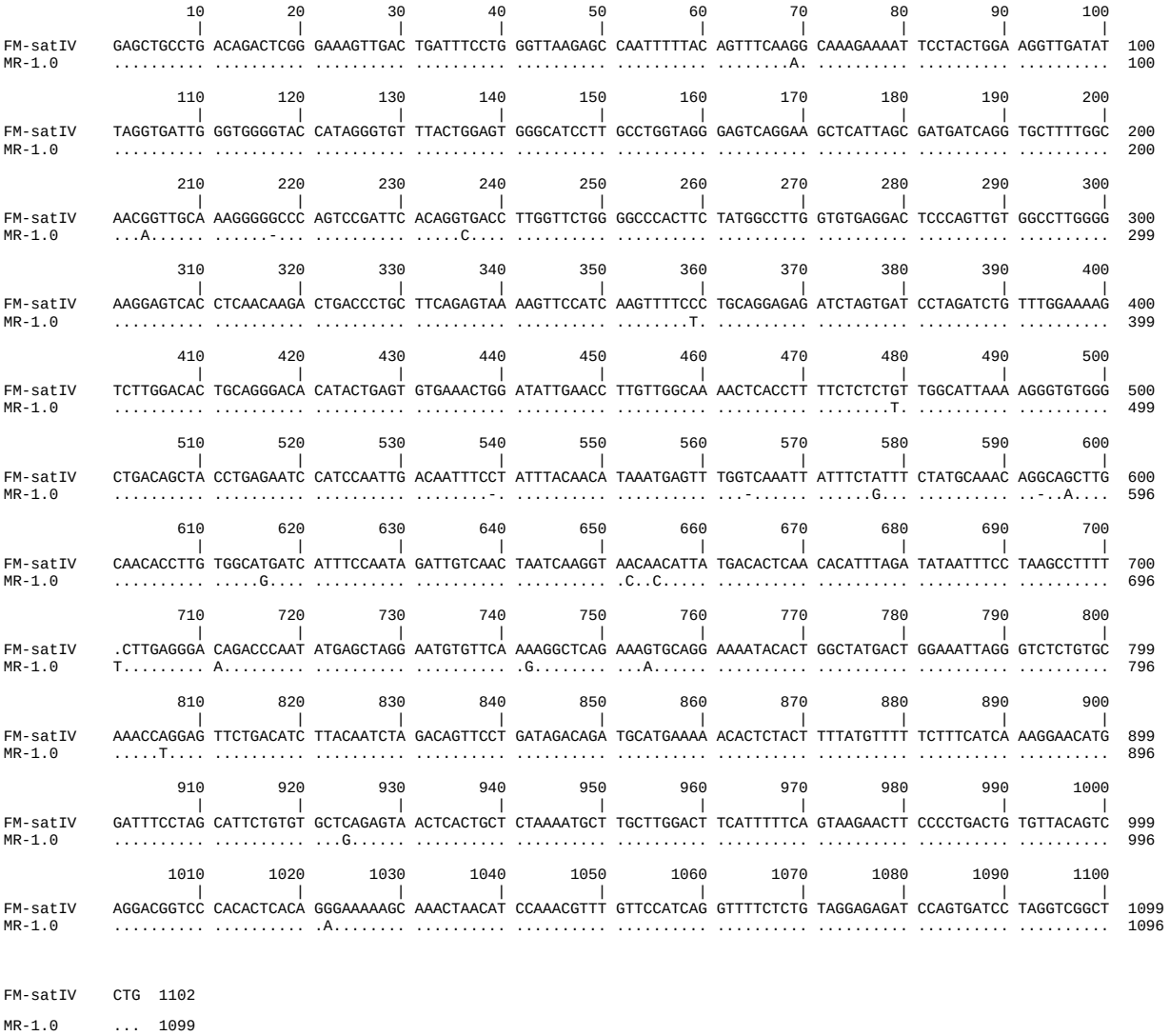


Fig. 4. Southern blots of *Apa*I-, *Bam*HI-, *Eco*RI-, *Nco*I-, *Pst*I- and *Pvu*I-digested genomic DNA of a male Formosa muntjac and hybridized to ^{32}P-labeled satellite DNA probes of FM-satI (**A**), FM-satII (**B**) and FM-satIV (**C**). Fragment sizes are indicated on the left hand side of each blot. (**A**) Typical A-type ladder pattern with 0.7 kb register is seen in *Pvu*I digested, probed with FM-satI DNA. Such 0.7 kb register is also observed in *Bam*HI, *Eco*RI and *Pst*I digestions. (**B**) Multiple hybridization bands showing atypical ladder pattern is observed in digests probed with FM-satII. However, hybridization bands in a 0.7 kb register can be observed in *Apa*I, *Nco*I and *Pvu*II digestions. (**C**) Digests hybridized with FM-sat IV DNA probe show multiple hybridization bands of repetitive DNA but lack of typical A type ladder pattern. Nevertheless hybridization bands of 1 kb register can be detected in *BamHI*, *EcoRI*, *NotI*, *PstI*, and *PvuII* digestions.

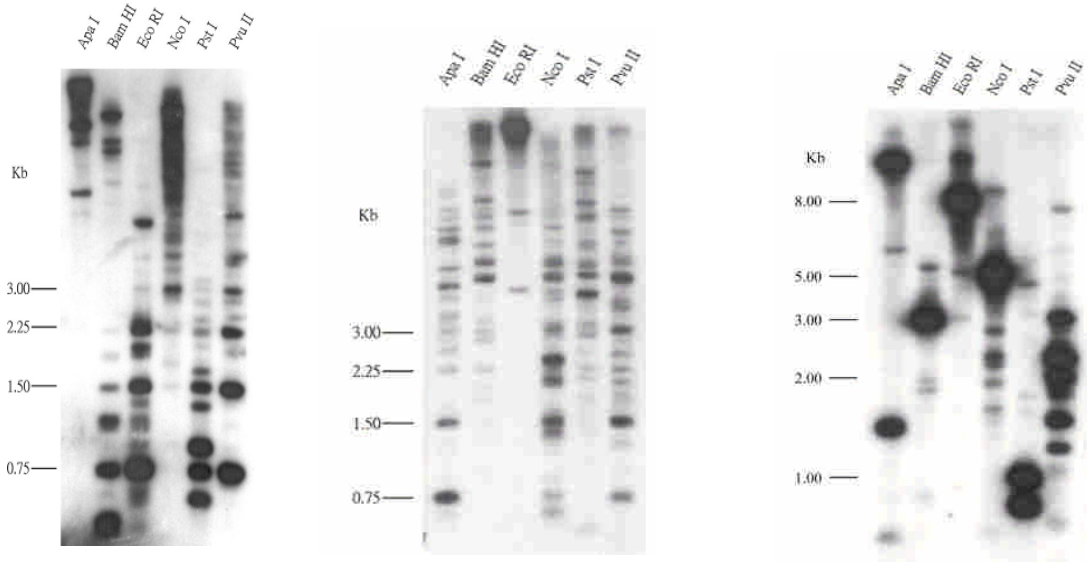
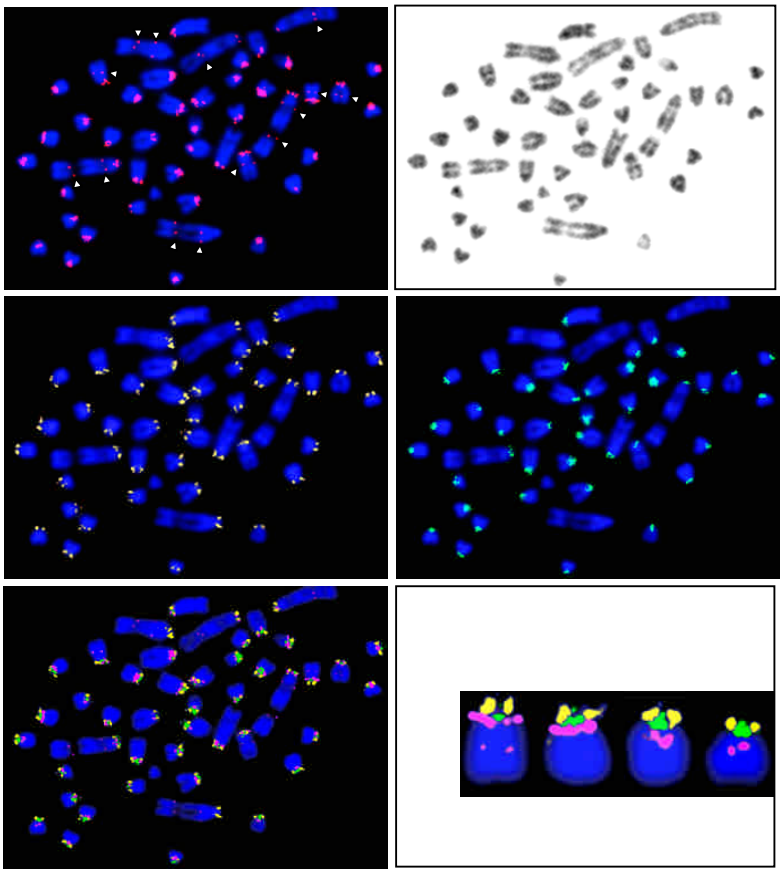


Fig. 5. Physical mapping of satellite I, satellite II and satellite IV DNA clones in the male Formosa muntjac chromosomes. (**A**) Localization of satellite I clone (FM-satI) (red signals) on all centromeric/pericentric regions, except one large pair of autosome 3 and the Y chromosome (indicated in panel B). Hybridization signals also observed at specific interstitial sites on 5 pairs of autosomes, among those, two interstitial hybridization signals are seen in two large pairs of autosomes, and one interstitial site is seen in the other three pairs of autosomes. All interstitial sites are indicated by arrow heads and chromosomes identified are indicated in panel B. (**B**) Same metaphase spread seen in panel A, C, D and E with inverse DAPI-banding shows a 2n=46 chromosome complement. The chromosomes 1, 2, 3, 5,X, and Y are indicated. (**C**) Localization of satellite II DNA clone (FM-satII) in the same metaphase spread of (**A**) showing hybridization signals (yellow fluorescent) in all centromeric/pericentric regions except the Y chromosome. Almost all the signals appear as doublets located at the lateral sides of the primary constriction. (**D**) Localization of satellite IV DNA clone (FM-satIV) (green signals) on the same metaphase showing hybridization signals at the centromeric regions of all chromosomes with the exception of chromosomes 2, 3 and Y. (**E**) Simultaneous localization of three satellite DNA clones in the same metaphase spread shown above. Satellite II signals are most terminal located, jointed by the satellite IV signals and then followed by the satellite I signal. The orientation of these the satellite DNA families in the centromeric/pericentic regions can be clearly demonstrated in the enlarged chromosomes shown in (**F)** as pter <- satII(yellow) – satIV(green) – sati(red) -> qter.



Appendix 3:
Genetic Analysis of Two Subspecies of Reeves' Muntjac (Cervidae: *Muntiacus reevesi*) by Karyotyping and Satellite DNA Analyses

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***Zoological Studies* 43(4):** 749-758 (2004)**.**

We analyzed the karyotypes of the Formosan muntjac (*Muntiacus reevesi micrurus*) including G-banding, C-banding, and NOR-staining analyses. The results showed the species has a 2n=46 chromosome complement. The G-banding patterns as well as the localizations of rRNA gene clusters and constitutive heterochromatins were similar to those of Chinese muntjac (*M. reevesi reevesi*). In addition, satellite DNA analysis was also carried out. The restriction periodicity of FM-satI revealed a 0.75-kb register indicating that this deer species belongs to the plesiometacarpalia division. Finally, the FISH study demonstrated that the Formosan and Chinese muntjacs have similar localizations of satellite I DNA in their respective genomes. Although the Formosan and Chinese muntjacs share almost identical results of cytogenetic analyses, Southern blot and FISH studies revealed some sequence divergence of satellite I DNA between these 2 species supporting the classification of the Formosan muntjac as a subspecies of, not the same species as, the Chinese muntjac. Furthermore, the data suggest that satellite I DNA of the Formosan muntjac and that of the Chinese muntjac may have originated from different ancestral sequences or that they may have experienced different homogenization patterns in the course of evolution.

INTRODUCTION

Muntjac deer (Muntiacinae, Cervidae) are classified into 9 known species: *Muntiacus crinifrons, M. feae, M. gongshanensis, M. muntjak, M. putaoensis, M. reevesi, M. rooseveltorum, M. truongsonensis,* and *M. vuquangensis* (Shi and Ma 1988, Amato et al. 1991, Nowak 1991, Evans and Timmins 1994, Timmins et al. 1998, Giao et al. 1998, Wang and Lan 2000). Based on the morphological and anatomical studies, these species of the genus *Muntiacus* demonstrate quite-similar appearances, and a sterile hybrid was produced from 2 closely related species, *M. muntjak* and *M. reevesi* (Shi et al. 1980). However, these morphologically similar and closely related species have significant diversity in diploid chromosome numbers and karyotypes from 2n=6 (female Indian muntjac; *Muntiacus muntjak vaginalis*) to 2n=46 (Chinese muntjac, *M. reevesi reevesi*) (Fontana and Rubini 1990). Such chromosomal divergences are not uncommon within species, such as in lemurs (Dutrillaux 1979), mole rats (Nevo et al. 1994), and gibbons (2n=38, 44, 50, and 52) (Jauch et al. 1992) or within races, such as in the house mouse (*Mus musculus domesticus*) (Nachman et al.1994). Those studies suggest that karyology might be an excellent model for investigating speciation. More recently, molecular phylogenetic studies were performed to identify species, which include a total DNA homology study (Schmidtke et al. 1981), restriction fragment length polymorphism (RFLP) analysis of mitochondrial DNA (mtDNA) (Lan et al. 1993 1995, Lan and Shi 1994), RFLP analysis of highly repetitive DNA (Lima-de-Faria et al. 1984, Bogenberger 1985 1987, Grechko et al. 1997, Nijman and Lenstra 2001), and a genomic organization study of satellite DNA (Lee et al. 1997). However, actual phylogenetic distances between 2 populations (interspecific divergence) could not be determined based on the above-mentioned morphological, anatomical, karyological, and even molecular phylogenetic studies alone. It was reported that gene analysis of mtDNA can be utilized to assess evolutionary distances (Cronin 1991, Wang and Lan 2000). Moreover, sequence divergences of centromeric satellite DNA have also been useful in delineating phylogenetic relationships, thanks to its rapid evolutionary rate among species and concerted evolution within species (Hatch et al. 1976, Lin et al. 1991, Wijers et al. 1993, Kato et al. 1999, Li et al. 2000, Kato 2003). Cervid satellite I DNA is

prominently localized in the cervid pericentromeric region. This satellite DNA is organized in hierarchical higher-order repeats (HORs) of 31-bp subrepeats (Bongenberger et al. 1985, Yu et al. 1986, Lee and Lin 1996). Interestingly, cervid satellite I DNA is organized primarily as 1-kb monomers in telemetacarpalia-division cervids; however, it is organized as a 0.8-kb monomer in plesiometacarpalia-division cervids (Lee at al. 1997). Comparisons of sequences of this given satellite DNA monomer showed over 95% identities within species, but lower sequence similarities between species (Lee at al. 1997). Moreover, it was reported that the interstitial distribution of satellite I DNA corresponds to the chromosomal fusion site (Lin et al. 1991, Lee et al. 1993, Yang et al. 1997, Fronicke and Scherthan 1997, Li et al. 2000). Therefore, chromosomal distribution of satellite DNA can serve as an indicator in mapping the course of karyotypic evolution.
Muntiacus reevesi (Reeves' muntjac) includes 2 subspecies: *M. reevesi reevesi* (Chinese muntjac) and *M. reevesi micruru* (Formosan muntjac) (Whitehead 1972, Wilson and Reeder 1993). While the Chinese muntjac is widely distributed throughout southeastern China, the Formosan muntjac is endemic to the island of Taiwan. The appearances of the 2 subspecies are alike except that the Formosan muntjac has a darker coat. Some aspects of the natural history of the Formosan muntjac have been reported (Chen 1992, Pei and Liu 1994), but almost no genetic information is available except for its diploid chromosome number 2n=46 (Wang 1987). Herein, we report on detailed cytogenetic and satellite DNA analyses of the Formosan muntjac and compare results with those of the Chinese muntjac.

MATERIALS AND METHODS

Primary culture and establishment of a skin fibroblast cell line
Skin biopsies of male Formosan muntjacs, which were kindly provided by the Taipei Zoo, Taipei, Taiwan, were primarily grown at 37 °C in Dulbecco's modified Eagle medium (DMEM) (Gibco/BRL, N.Y. U.S.A) supplemented with 15% fetal calf serum, 1% glutamine, and 1% penicillin-streptomycin-neomycin. After the 5th passage during subculture of the skin fibroblasts, skin fibroblast cells were maintained in DMEM supplemented with 10% fetal calf serum, 1% glutamine, and 1% penicillin-streptomycin-neomycin.

G-banding, C-banding, and NOR-silver staining

Chromosome preparations were obtained from an established male Formosan muntjac cell line according to standard protocols (Dracopoli et al. 2001). *G-banding*: A slide aged for 2 wk was treated with 0.05% trypsin/EDTA for 10~15 s at room temperature and stained with Wright's dye for 60~80 s. *C-banding*: The aged slide was pretreated in 0.2 N HCl at ambient temperature for 1 h, rinsed with ddH_2O, treated in an alkali solution containing 5% $Ba(OH)_2$ at 50 °C for 10 min, then washed with a large amount of ddH_2O, and finally incubated in 2X SSC at 60 °C for 1 h before staining with Wright's dye. *Silver-NOR staining*: The aged slide was treated with 3 volumes of 2% gelatin and 4 volumes of 50% silver nitrate solution at 65 °C for 2~4 min. Subsequently, the slide was washed with 3% acetic acid to terminate the reaction of the silver nitrate. Finally, the slide was washed with ddH_2O, air dried, and stained with Wright's dye.

Southern blot analysis, subrepeat analysis, and copy number estimation

For Southern blot experiments, 10-μg aliquots of muntjac genomic DNA were incubated with one of 6 different restriction endonucleases. The digested DNA samples were electrophoretically fractionated on a 0.8% agarose gel, transferred to a nylon membrane (Biodyne), and hybridized with a ^{32}P-dCTP-labeled satellite I DNA clone. The conditions used for hybridization, filter washing, and autoradiography were described previously (Lee et al. 1994). In the subrepeat analysis, a monomer of the FM-satI clone (GenBank accession no.: AY380827) (Lin et al. 2004)

was subjected to single-base-shift self-comparisons based on the method of Pluciennicza k et al. (1982) to investigate the presence of internal unidirectional subrepeats. This self-comparison method is described in greater detail elsewhere (Lee and Lin 1966). Briefly, cervid satellite DNA monomer A was compared with a DNA sequence comprising 2 adjacent copies of the same monomer, AA. Monomer A was then shifted to the right in 1-base increments, with respect to AA. After each shift, the overall number of identical nucleotides detected between 2 aligned DNA sequence A's was plotted on a line graph using the CA-Cricket Graph III program (Computer Associates, CA). If monomer A contains 31-bp subrepeats, a peak in the line graph is observed every 31 base shifts due to a significantly high number of identical nucleotides between A and AA in that "in-frame" aligned position.
Copy number estimation of cervid satellite I monomers in the Formosan muntjac genome was also based on an earlier described procedure (Lee et al. 1994).

Fluorescence in situ hybridization

Metaphase chromosomes were prepared from an established male Formosan muntjac cell line and a male Chinese muntjac cell line (kindly provided by Dr. F. Yang, University of Cambridge, England, UK). The FM-satI and C5 were labeled with SpectraRed-dUTP (Vysis) by nick translation. The procedures for denaturation, hybridization, post-hybridization washing, and signal detection are described in detail elsewhere (Lee et al. 1999). Fluorescent signals were captured on an Olympus (Tokyo, Japan) BX60 fluorescence microscope equipped with appropriate filter sets and a cooled charge-coupled device (CCD) camera (Photometrics KAF 1400,USA). Images were normalized and enhanced using the MacProbe v4.0 software (Perceptive Scientific Instruments, USA).

RESULTS

Karyological studies

Each of 20 chromosome spreads of a female and male Formosan muntjac was analyzed. The G-banding analysis showed 22 pairs of autosomes and 1 pair of sex chromosomes in the complement of Formosan muntjac chromosomes. A karyotype and ideogram were constructed based on the chromosome size and G-banding patterns (Fig. 1). Three pairs of larger autosomes were designated numbers 1, 2, and 3. The X chromosome has a similar size to chromosome number 4, and the Y chromosome is the smallest one in the complement. All chromosomes were shown to be telocentric/acrocentric except the Y chromosome, which was acrocentric/submetacentric. There is a secondary constriction in the middle of chromosome 1. The G-banded karyotype of the Formosan muntjac is generally the same as that of the Chinese muntjac (Fig. 1a, b). Constitutive heterochromatin banding (C-banding) was carried out to further identify the chromosomal morphology. The result showed that the constitutive heterochromatin is terminally located in every Formosan muntjac chromosome except the Y, in which the heterochromatin is located at the sub-middle region of that chromosome (Fig. 2a). Chromosomes 1, 2, and 3 appeared to have less heterochromatin, whereas the X chromosome has a larger amount of heterochromatin. The silver-NOR staining showed that there are 4 nucleolar organizer regions (NORs), two of which are located in the secondary constriction of chromosome 1 homologs at band 1q28, and the others are located in the terminal end of the q arm of chromosome 5 homologs at band 5q26 (Fig. 2b).

Satellite I DNA analysis

An *Eco*RI-digested complete monomer of FM-satI DNA (one of the Formosan muntjac's satellite I DNA clones), occupying 796 bp in length from nucleotides 202~997 of the FM-satI clone (Lin et al. 2004), was used to detect the existence of any internal subrepeats. The single-base-shift self-comparison analysis (Pluciennicza k et al. 1982) showed in-frame peaks approximately every 31

single-base shifts. This indicates the presence of internal 31-bp subrepeats in the monomer examined (Fig. 3). Therefore, these 31-bp subrepeats were organized into a higher-order repeated hierarchical structure as ~0.8-kb monomers in the Formosan muntjac genome.
Southern blot hybridization with FM-satI DNA as a probe produced a typical type A ladder pattern with a 0.75-kb register in *Pvu*II-, *Bam*HI-, *Eco*RI-, and *Pst*I-digested fragments (Fig. 4a). All of these results suggest that the cervid satellite I DNA in the Formosan muntjac genome is organized primarily as 0.75-kb tandem repeats. The pattern of hybridization bands is almost the same between the Formosan and Chinese muntjacs (Fig. 4a, b) with the exception of a stronger 3.2-kb band found in *Eco*RI-digested Formosan muntjac DNA.

Chromosomal distribution of satellite I DNA

The SpectraRed-labeled satellite I DNA probe (FM-satI) was hybridized to metaphase chromosome spreads from a male Formosan muntjac. The FISH study was carried out and revealed satellite I DNA signals (which appeared as red fluorescence) at the pericentromeric region of all chromosomes, except for a large pair of autosomes (identified as chromosome 3s) and the Y chromosome (Fig. 5a). Hybridization signals were also observed at 7 specific interstitial sites on 5 different autosomes per haploid genome. Among those, 2 satellite I interstitial sites were found each on chromosomes 1 and 3, and 1 satellite I DNA interstitial site was observed on each of the other 3 autosomes (identified as chromosomes 2, 5, and 10, see Fig. 4b). In a comparison of chromosomal localization of Chinese muntjac satellite I DNA (C5) (Fig. 5c, d), there was a stronger satellite I DNA signal present at the pericentromeric region of Formosan muntjac chromosome 4s; in addition, only 1 interstitial signal was detected in each chromosome 2 of the Formosan muntjac, whereas 2 interstitial signals were observed in Chinese muntjac chromosome 2. No interstitial signal was found in chromosome 4 of the Formosan muntjac, whereas, an interstitial signal was observed in the counterpart of the Chinese muntjac chromosome.

DISCUSSION

In the present karyological study, we observed that the Formosan and Chinese muntjacs share similar G-banded karyotypes. The location of the NOR and the C-banding pattern of the Formosan muntjac are also the same as those of the Chinese muntjac (Shi et al. 1980). The G-banded ideogram of the Formosan muntjac showed some minor differences with the enhanced DAPI-banded ideogram of the Chinese muntjac as reported by Yang et al. (1995). We exchanged chromosome 11 of the Chinese muntjac, identified by DAPI-enhanced banding (Yang et al. 1995), with chromosome 10, based on the high resolution of G-banding and the chromosome size. Even so, the karyotypes of these 2 subspecies of *M. reevesi* are highly conserved. This differs from a subspecies of the house mouse (*Mus musculus domesticus*) which shows a wide range of variations of karyotypes (Nachman et al. 1994). Furthermore, it was reported that the greater the similarity of a given satellite DNA family among species, the closer the phylogenetic distances are among those species, by analyses of RFLP patterns, monomer size, sequence divergence, and chromosomal localization of satellite DNA among species (Lin et al. 1991, Wichman et al. 1991, Lee et al. 1997, Kato et al. 1999, Li et al. 2000, Slamovits et al. 2001, Kato 2003). In the present study, we found an almost identical restriction periodic pattern of satellite I DNA arrays between the Formosan and Chinese muntjacs with the exception of a stronger 3.2-kb band in *Eco*RI-digested Formosan muntjac genomic DNA. The genomic organization of this satellite I DNA in the Formosan muntjac was characterized by a ~0.8-kb higher-order repeat (HOR) monomer which in turn is comprised of degenerate 31-bp subrepeats. Such a hierarchical pattern of HORs of satellite I DNA further implies that the Formosan muntjac should also be classified as a plesiometacarpalia deer (Lee et al. 1997). In comparison to an earlier FISH study of satellite I DNA (C5) distribution in the Chinese muntjac (Li et al. 2000), the Formosan muntjac has the same chromosomal localization

of satellite I as the Chinese muntjac with the exception of 2 interstitial satellite I DNA signals that were undetectable in the haploid genome of the Formosan muntjac compared to its Chinese muntjac counterparts. The 2 interstitial signals being undetectable may have been due to lesser amounts of satellite I DNA or to degradation of that particular satellite DNA in the course of tandem fusion. Previously, by a comparative G-banding study (Fontana and Rubini 1990) as well as FISH with chromosome-specific painting probes and centromeric satellite DNA probes (Yang et al. 1995 1997), it was suggested that the karyotype of the Chinese muntjac had evolved from a 2n=70 ancestor by 12 sequential repeated-tandem fusions without involvement of Robertsonian translocation. In this study, the G-banding karyotype analysis and FISH results obtained together support the notion that the Formosan muntjac is a subspecies of *M. reevesi*, and that its karyotype was also derived from a 2n=70 ancestor. Moreover, fossil records indicate that the Formosan muntjac may have existed in the early Pleistocene as did the Chinese muntjac (Ma et al. 1986, Dong 1993). Furthermore, the sequence divergence of satellite I DNA also draws into questions whether the Formosan muntjac is only a different race of the Chinese muntjac. Indeed, satellite I DNA sequence comparisons show that satellite I of the Formosan muntjac is more similar to that of the Indian muntjac (86% homology) than to the Chinese muntjac (82% homology) (Lin et al. 2004). Based on satellite DNA sequence comparison data alone, one could argue that the Formosan and Indian muntjac ancestors shared very high sequence homology of satellite I DNA. On the other hand, if satellite I DNA of the Formosan and Chinese muntjacs indeed originated from the same ancestral sequence, they might have separately experienced different homogenization patterns in the course of evolution (Nijman and Lenstra 2001).

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FIGURE LENGENDS:

Fig. 1. G–banded chromosome analysis of 2 muntjac subspecies. (a) G-banded karyotype of the male Formosan muntjac (*Muntiacus reevesi micrurus*). (b) G-banded karyotype of the male Chinese muntjac (*Muntiacus reevesi reevesi*). (c) The ideogram was constructed based on the G-banding pattern of the male Formosan muntjac.

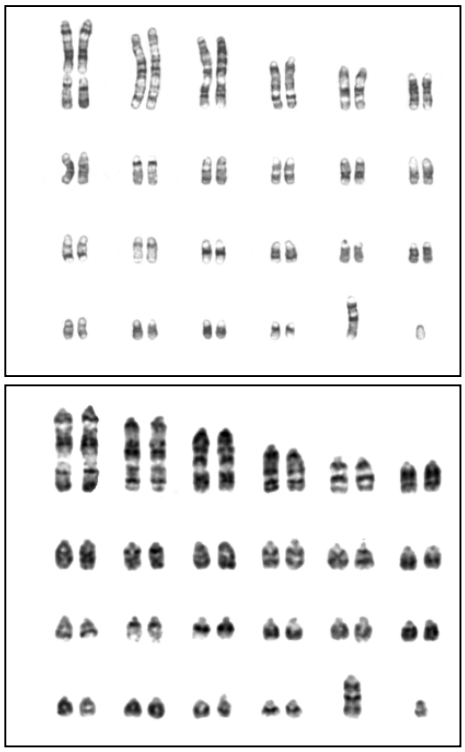


Fig. 2. C-banding and NOR-banding analyses of the Formosan muntjac chromosomes. (a) C-banded metaphase spread of the male Formosan muntjac. Positive C-bands are located on every terminal pericentric heterochromatin region with the exception of the Y chromosome which is mainly heterochromatic (the red arrow indicates the Y chromosome and the black arrow indicates the X chromosome). (b) Localization of NOR sites on male Formosan muntjac chromosomes. There are 4 NOR sites, two of which are located at the secondary constriction region of chromosome 1s and the other two at the terminal end of the q arm of chromosome 5s (as indicated by arrows).

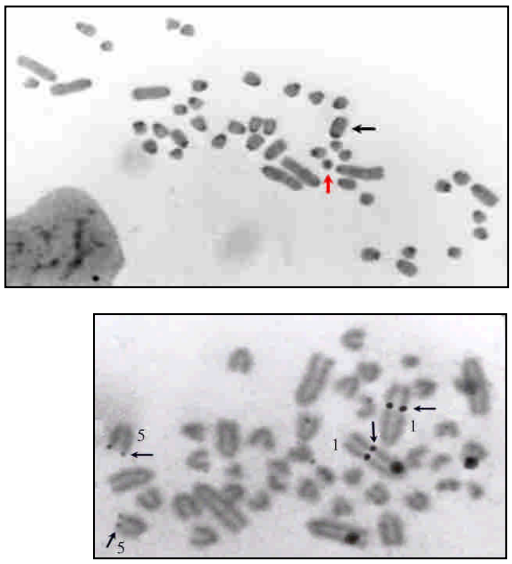


Fig. 3. Presence of 31-bp subrepeats in the FM-satI clone of the Formosan muntjac. The line graph shows increased DNA sequence similarities ("in-frame" peaks) in a 31-bp shift periodicity when monomer A (nucleotides 202~997) of the FM-satI clone is compared with 2 adjacent copies of the identical monomer AA itself and that which is shifted to the right at 1-base intervals. The vertical axis indicates the total number of identical nucleotides between the 2 aligned DNA sequences. The horizontal axis represents the number of base-pair shifts during sequence alignment. As the complete graph is a symmetrical image defined by the vertical axis of symmetry halfway across the graph, only the results of the 1st 398 shifts are presented.

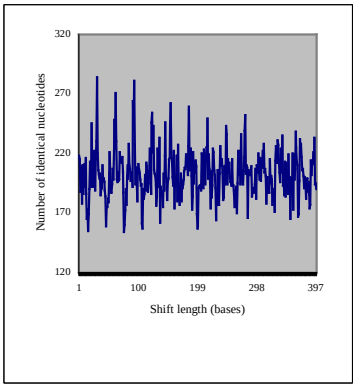


Fig. 4. Restriction periodicity of cervid satellite I DNA in the genomes of Formosan and Chinese muntjacs. (a) Southern blot of Formosan muntjac genomic DNA hybridized to the ^{32}P-labeled FM-satI DNA clone. (b) Southern blot of Chinese muntjac genomic DNA hybridized to ^{32}P-labeled C5 DNA. Fragment sizes are indicated on the left hand side showing a 0.75-kb register for 5 restriction enzymes, *Bam*HI-, *Eco*RI-, *Nco*I-, *Pst*I-, and *Pvu*II-digested genomic DNA, but not for *Apa*I digests.

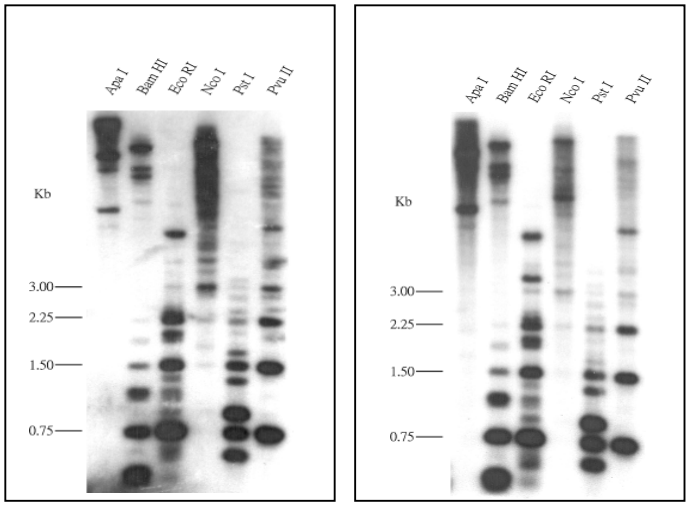
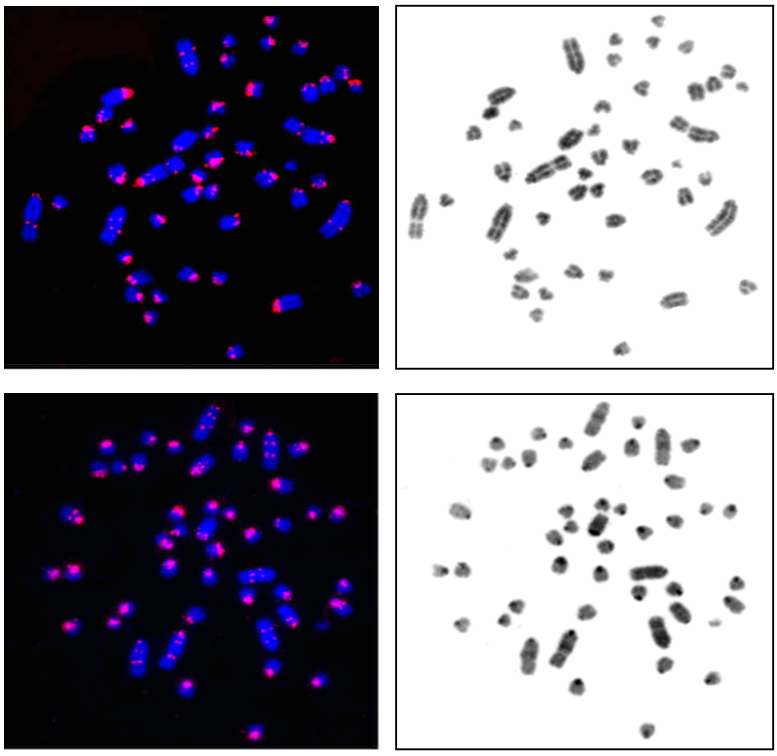


Fig. 5. Chromosomal distribution of cervid satellite I DNA in Formosan and Chinese muntjacs. (a) SpectraRed-labeled FM-satI DNA probe hybridized to the metaphase spread of the Formosan muntjac and hybridization signals (appearing as red fluorescence) localized at all pericentromeric regions except for chromosome 3s and the Y chromosome. Identification of chromosomes by inverse DAPI-banding on the same metaphase in (a) is shown in (b). There were 7 interstitial hybridization signals observed in 5 autosomes (1, 2, 3, 5, and 10), as indicated in panel (b), in a haploid set. (c) Hybridization signals of the SpectraRed-labeled C5 probe observed at all pericentromeric regions of the Chinese muntjac with the exception of chromosome 3s and the Y chromosome. (d) Inverse DAPI-banding of the same metaphase as (c) with the identified chromosomes indicated. There were 9 interstitial signals of C5 observed in 6 autosomes (1, 2, 3, 4, 5, and 10) as indicated in panel (d), in a haploid set.



Appendix 4:

Karyotypic evolution of a novel cervid satellite DNA family isolated by microdissection from the Indian muntjac Y-chromosome

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Chromosoma **114:**28-38 (2005)

ABSTRACT A mini-library was constructed from DOP-PCR products using microdissected Y chromosomes of Indian muntjac as DNA templates. Two microclones designated as IM-Y4-52 and IM-Y5-7 were obtained from negative screening of all three cervid satellite DNAs (satellite I, II, and IV). These two microclones were 295- and 382-bp in size respectively, and shared approximately 70 % sequence-homology. Southern blot analysis showed that the IM-Y4-52 clone was repetitive in nature with a ~ 0.32-kb register in *Hae*III digest. Sequence comparison revealed no similarities to DNA sequences deposited in the GenBank database, suggesting that the microclone sequences were from a novel satellite DNA family designated as cervid satellite V. A subclone of an Indian muntjac BAC clone which screened positive for IM-Y4-52 had a 3325-bp insert containing 6 intact monomers, 4 deleted monomers and 2 partial monomers. The consensus sequence of the monomer was 328-bp in length and shared more than 80% sequence homology with every intact monomer. A zoo-blot study using IM-Y4-52 as a probe showed the strong hybridization with *Eco*RI digested male genomic DNA of Indian muntjac, Formosan muntjac, Chinese muntjac, sambar deer and Chinese water deer. Female genomic DNA of Indian muntjac, Chinese water deer and Formosan muntjac also showed positive hybridization patterns. The satellite V was found to specifically localize to the Y heterochromatin region of the muntjacs, sambar deer and Chinese water deer, and to chromosome 3 of Indian muntjac and the X-chromosome of Chinese water deer.

INTRODUTION

Cytogenetically, the Indian muntjac (*Muntiacus muntjac vaginalis*) is a fascinating mammalian species because its small chromosome number and difference in chromosome number between males (2n=7) and females (2n=6). The different chromosome number between male and female of the species is because the X-chromosome is translocated onto autosome 3 by centric fusion forming the X+3 chromosome; therefore, the female has two X+3 chromosomes. The male on the other hand has one X+3 chromosome, an "unfused chromosome 3" and a Y-chromosome, thus having one more chromosome than the female. Initial studies suggested that extensive tandem fusions and a few centric fusions could have occurred in a closely related Chinese muntjac-like ancestor species, with 2n=46, to form the unique karyotype of the Indian muntjac species (Hsu et al. 1975; Shi et al. 1980; Scherthan 1990; Lin et al. 1991). More recent studies have pointed to the possibility that multiple chromosomal rearrangements occurred in an ancestor species, each with 2n=70, resembling the Chinese water deer (*Hydropotes inermis*) or brown- brocket deer (*Mazama gouazoubira*) karyotypes resulting in the present-day Indian muntjac karyotype (Lan et al. 1995; Yang et al. 1997a; Li et al. 2000c). Based on fossil records (Ma et al. 1986), DNA reassociation kinetics (Schmidtke et al. 1981) and analyses of mitochondria DNA RFLP (Lan et al. 1995), it appears that this chromosome restructuring may have occurred rather recently (a few million years ago) and thus provides an ideal model for karyotypic evolution and speciation (Yang et al. 1997a, b). However, the exact mechanism involving the specific chromosomal rearrangements of the species remains elusive, although repetitive DNA sequences such as satellite DNA have been suggested to play an important role in karyotypic evolution of the mammalian species by promoting chromosomal rearrangement (Elder and Hsu 1988; Slamovits and Rossi 2002).

Repetitive sequences of centromeric heterochromatin and telomeric sequences are believed to facilitate multiple inter-chromosomal rearrangements; Ferguson-Smith et al. (1973) postulated that similar centromeric repetitive sequences on different chromosomes may facilitate illegitimate (ectopic) recombination during meiosis. Brinkley et al. (1984) observed non-random aggregation of Chinese muntjac centromeres/kinetochores in the interphase nuclei which could achieve a specific centric fusion event involving multiple chromosomes. Rattner (1986) reported that the"break-off"centromers after fusions were collectively transferred into a specific chromosome site forming a compound centromere as the one seen in the X+3 chromosome of Indian muntjac. Scherthan (1995) presented a model for the evolution of Indian muntjac karyotype based on the close proximity of telomeric and centromeric repeats in one end of every acrocentric chromosome found in the ancestral Chinese muntjac-like species. The association between telomeres from various chromosomes during the bouquet-stage of meiosis also placed the centromeres of different chromosomes close to each other thereby facilitating the multiple chromosome fusion. Co-localization of telomeric sequences with interstitial satellite DNA found in the Indian muntjac chromosomes lends support to his model (Lee et al. 1993; Hartmann and Scherthan 2004). In the present study, we isolated a novel satellite DNA family from the Y-chromosome of Indian muntjac using chromosome microdissection and microcloning techniques. This satellite DNA family is highly specific for the Y-heterochromatin of a small number of the Asian deer; however, it is also found in the X-chromosome of Chinese water deer and chromosome 3 of Indian muntjac. Based on those observations, possible mechanisms were proposed for the evolution of Indian muntjac karyotype from an ancestor species with Chinese water deer-like karyotype.

MATERIALS AND METHODS

Cell lines, chromosome preparations and DNA isolation

Metaphase chromosome spreads and genomic DNA were prepared from the following cell lines: Indian muntjac (*Muntiacus muntjak vaginalis*) (CCL-157, American Type Culture Collection), two subspecies of Reeves' muntjac; Chinese muntjac *(Muntiacus reevesi reevesi)* and Formosan muntjac (*Muntiacus reevesi micrurus*) (Whitehead 1972; Chiang et al. 2004), caribou (*Rangifer tarandus caribou*) (all available in our laboratory, Lin et al., 1991; Lee et al. 1994 and Lin et al. 2004), male black tailed deer (*Odocoileus hemionus hemionus*) (CRL-6193, American Type Culture Collection), male Chinese water deer (*Hydropotes inermis*) (provided by Center for Reproduction of Endangered Species, Zoological Society of San Diego, USA), female Chinese water deer (Center for Veterinary Science, University of Cambridge, UK) and the Formosan sambar deer (2n=62) (*Cervus unicolor swinhoei*) (obtained from the Nan-Tou Deer Farm Association, Nan-Tou County, Taiwan). Genomic DNAs of other male mammals (goat, cow, pig, and rat) were also available in our laboratory for the Zoo-blot analysis. The detailed protocols for chromosome preparation and DNA isolation have been described elsewhere (Li et al. 2000a, c).

Microdissection of the Y chromosome

Y chromosomes of Indian muntjac were scraped from metaphase spreads under an inverted microscope (Olympus X-81) with a siliconized glass needle attached to a mechanical micromanipulator (Narishige). The detailed protocol used for chromosome microdissection was referred to Engelen et al. (1996). Briefly, the glass needle was prepared from a borosilicate glass rod, 1.0 mm in diameter and was drawn by a Shutter puller (model PC-21). The Y chromosomes (scraped off from the metaphase spreads) were collected in 20µl of ddH_2O for the subsequent DOP-PCR experiment.

Degenerate oilgonucleotide primed-polymerase chain reaction (DOP-PCR)

The microdissected DNAs were amplified by DOP-PCR (Telenius et al. 1992) in a thermal cycler (ThermoHybaid, Px2). The DOP-PCRs were performed in 25µl reaction volumes with 20 microdissected Y chromosomes of the Indian muntjac for the DNA template, 2 umole/L of DOP primer (5'-CCGACTCGAGNNNNNNATGTGG-3'), and 12.5 µl of 2X DOP-PCR master mix containing 400 µmole/L of each deoxynucleotide triphosphate, 20 mmole/L Tris-HCl, 100 mmole/L KCl, 3 mmole/L $MgCl_2$ and 5 U *Taq* polymerase (DOP-PCR Master kit purchased from Roche,

Basel, Switzerland). The PCR was carried out with initial denaturation at 95℃ for 5 min, followed by the first round of PCR with five cycles of 94℃ for 1 min, 30℃ for 1.5 min, ramping to 72℃ over a 3min period (3.5℃/15 s) and 72℃ for 3 min, then followed by a second round of PCR with 35 cycles of 94℃ for 1 min, 62℃ for 1 min and 72℃ for 2 min (and increasing 14 s at each cycle), and a final extension step of 72℃ for 7 min.

Construction of microdissection library

The purified DOP-PCR products were ligated into the pBluescript II SK (-) vector (Stratagene). The recombinant plasmids were used to transform XL1-Blue *E. coli* competent cells. Subsequently, the transformation mixture was plated onto Luria-Bertani (LB) broth plates containing 100μg/ml of ampicillin, 40μg/ml of X-Gal and 0.05 mmol/L IPTG to construct a microdissetion library.

Isolation of novel cervid satellite DNA clone

DNA clones from a microdissected DNA library (microclones) were screened by the colony hybridization method using three cervid satellite DNA elements (sat-I, -II and -IV). All duplicated microclones were lifted onto a nylon membrane and denatured with an alkali solution. Three probe mixtures containing ^{32}P-labeled satellite I (C5; Lin et al. 1991), satellite II (Mmv-0.7; Li et al. 2000c) and satellite IV (Mmv-1.0; Li et al. 2002) were used to hybridize the membrane. The conditions for filter hybridization and washing were similar to those mentioned in the zoo-blot analysis section. Two microclones negative for satellite DNA I, II and IV were chosen and designated as IM-Y4-52 and IM-Y5-7, for further characterization. For isolating a large element which may contain several monomers of the Y-originated satellite DNA family, ^{32}P-labeled IM-Y4-52 was used as a probe to screen the BAC library of male Indian muntjac (Li et al. unpublished). Several positive BACs were obtained. One of those BACs was digested with *Eco*RI and the digested fragments were ligated into pBluescript II SK(-) plasmid vector by the shotgun cloning method. The subclone (designated Mmv-0.32#3) which displayed strong hybridization signals with ^{32}P-labeled IM-Y4-52 was selected for sequence analysis.

DNA sequencing and analyses

The inserted fragments of IM-Y4-52, IM-Y5-7, and Mmv-0.32#3 were sequenced by the ABI BigDye v3.1 Sequencing Kit (PE Biosystems, Chiba, Japan) and the ABI 3730 Genetic Analyzer (Applied Biosystems, Tokyo, Japan). Sequences of these clones were deposited into the GenBank database (IM-Y4-52 and IM-Y5-7 were deposited as Mmv-0.32#1 and Mmv-0.32#2, with accession numbers AY684846 and AY684847 respectively; the accession No. for Mmv-0.32#3 is AY684848). We compared the sequences with those in the GenBank database using the nucleotide-nucleotide BLAST tool (http://www.ncbi.nlm.nih.gov/BLAST/); sequence alignments were made by the Multiple-alignment program of DNAMAN (Version 4).

Polymerase chain reaction for sex determination

This study was performed based on the method described by Pomp et al. (1995). PCRs were conducted in a final volume of 25 μl containing 50 mmol/L Tris-HCl pH8.8, 50 mmol/L NaCl, 5mmole/L $MgCl_2$, 200 μmol/L deoxynucleotide triphosphate for each, 400 nmol/L SRY primer pairs (SRYB-3: 5'-GCCAGTAGTCTCTGTGCCTCCT-3'and SRYB-5: 5'-TGAACGCTTTCATTGTGTGGTC-3'), 100ng of genomic DNA and 2.5 units of Taq polymerase. The PCR conditions were carried out with an initial denaturation at 94℃ for 5 min, followed by 30 cycles at 94℃ for 20sec, 55℃ for 10 sec, 72℃ for 20sec, and a final extension at 72℃ for 5 min. PCR products of each reaction were fractionated on a 3% agarose gel.

Southern blot, Zoo-blot, and slot blot analyses

Southern blot and Zoo blot analyses were conducted under similar conditions. In brief, a 10 μg aliquot of genomic DNA from each species was digested with *Eco*RI for Zoo-blot analyses. For Southern blot analyses, 10 μg aliquots of genomic DNA of Indian muntjac were each digested with one of three different restriction endonucleases (*Hind*III, *Eco*RI and *Hae*III). The digests were electrophoretically fractionated on 2% agarose gel, transferred to a nylon membrane (Biodyne) and hybridized with [^{32}P]dCTP- labeled IM-Y4-52. The conditions used for hybridization, filter washing and autoradiography have been described previously (Lee et al. 1994). The slot-blot hybridization procedure for copy number estimation of repeated DNA elements in the genome has

been described elsewhere (Li et al. 2000c).

Fluorescence in situ hybridization and reverse FISH

The direct DOP-PCR products of microdissected Y chromosome DNA and IM-Y4-52 microclone DNA were labeled with digoxigenin-11-dUTP (Roche, Basel, Switzerland) by nick translation. The cervid satellite II DNA (Mmv-0.7) (Li et al. 2000c) was labeled with biotin. For the reverse FISH experiment, the digoxigenin labeled DOP-PCR products were hybridized to metaphase chromosomes of male Indian muntjac. For other single color FISH studies, the digoxigenin labeled IM-Y4-52 DNA was hybridized to metaphase chromosomes of the deer species studied. The hybridization signal of the digoxigenin labeled probe was detected by FITC-conjugated mouse anti-DIG. For dual-color FISH studies, the IM-Y4-52 DNA and satellite II DNA were labeled with digoxigenin and biotin, respectively, and co-hybridized onto metaphase spreads. The digoxigenin-labeled probe was detected by FITC-conjugated mouse anti-DIG (green) and the biotin-labeled probe was detected by AF568-avidin (red). The procedures for denaturation, hybridization, post-hybridization washing and signal detection have been described in detail elsewhere (Lee et al. 1999). Fluorescence 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

Molecular cloning and characterization of microdissected Y-chromosome DNA clones

A microdissected Y-chromosome library was constructed based on DOP-PCR products amplified from 20 microdissected whole Y chromosomes of Indian muntjac (Fig.1A, B). The reverse FISH showed that the positive FITC signals of digoxigenin labeled DOP-PCR products were localized to the major portion of the Y-chromosome as well as to the centromeric regions of all the male Indian muntjac chromosomes (Fig.1C). This indicated that the DOP-PCR products contain a mixture of centromeric DNA (satellite DNA) elements in addition to Y-heterochromatin DNA. Two microclones IM-Y4-52 and IM-Y5-7 (deposited in the GenBank database as Mmv-0.32#1 and Mmv-0.31#2 respectively) lacking all three cervid satellite DNAs (satellite I, II and IV) were investigated. Sequence analysis showed that these two microclones were 295- and 382-bp in size respectively, and shared 71.3% sequences homology (Fig 2). The sequences of these two microclones were compared with the DNA sequences in the NCBI GenBank database and no similarities to any of the sequences deposited in the database were found. Southern blot hybridization with Mmv-0.32#1 probe to male Indian muntjac genomic DNA produced multiple hybridization bands with irregular patterns (Fig. 3). Although no typical A-type pattern was observed in any particular digestion, an approximately 0.32 kb register was detected in the *Hae*III digested DNA sample. Copy number estimations indicated that the Indian muntjac genome contains ~14,750 copies of this particular DNA sequence (Mmv-0.32#1). These observations indicate the insert fragment of the given microclone is likely derived from a new repetitive DNA family.

Isolation of a larger tandem repeated element for monomer size determination

All of the microclones which screened positive with ^{32}P-labeled Mmv-0.32#1 probe had an insert size of less than 400bp (data not shown). Therefore, based on hybridizing patterns of Southern blot and sequence comparison between those microclones, it is hard to verify whether the newly found repetitive DNA sequence is indeed tandemly repeated in nature (satellite DNA). To search for a larger DNA fragment which may contain several monomers, subclones were generated from a male Indian muntjac BAC clone that positively hybridized to the Mmv-0.32#1 clone DNA. A 3325 bp subclone with a strongly positive Mmv-0.32#1 signal was selected for further sequencing and self-comparison analysis. This subclone, then designated as Mmv-0.32#3, was found to contain 6 intact monomers (b, c, f, i, j, and k), 4 deleted monomers (d, e, g, and h) and 2 partial monomers (a and l) (Fig. 4). A 328 bp consensus sequence of the monomers was found to

share more than 80% sequence homology with every intact monomer. The GC content of the consensus monomer is 52%. The microclone Mmv-0.32#1 showed 79.1% sequence similarity with the consensus sequence, whereas sequence homology between microclone Mmv-0.32#2 and the consensus sequence was 77.6%. These findings confirmed that the newly found repetitive DNA sequence is indeed from a novel satellite DNA family which can be designated as MMVsatV (cervid satellite V DNA).

Genomic distribution of cervid satellite V DNA

The Zoo-blot analysis (Fig. 5A) showed that the satellite V predominantly exists in the genomes of male Indian muntjac, male Formosan muntjac, male Chinese muntjac, male Formosan sambar deer and male Chinese water deer. Females of Indian muntjac and Formosan muntjac had weaker hybridization signals in comparison to their male counterparts. Very faint hybridization signals were seen in male animals of roe deer, black tailed deer, caribou and goat, but not seen in other male mammals examined (e.g. cow, pig, rat and human). This observation indicates that the satellite V DNA is quite species-specific. Moreover, the hybridization patterns in the Zoo-blot revealed a ~0.9-kb register in male Formosan muntjac and male Chinese muntjac (lane 3 and 5 of Fig 5). Most likely, this ~0.9-kb register in *Eco*RI digests of Reeves' muntjacs contains three monomers of satellite V.

Chromosomal localization of cervid satellite V DNA sequence

The digoxigenin labeled satellite V probe (IM-Y4-52 clone DNA) was used to hybridize to metaphase spreads of male and female Indian muntjac, Formosan muntjac, Chinese muntjac, Formosa sambar deer and Chinese water deer, as well as to metaphase spreads of male black tailed deer and caribou. The FISH signal on chromosome was detected by FITC conjugated anti-DIG antibody with 3 times amplification. The positive FISH signals of satellite V were present specifically on the Y-chromosomes of Indian muntjac (Fig. 6A), Formosan muntjac (Fig. 6B), Chinese muntjac (Fig. 6C), sambar deer (Fig. 6D) and Chinese water deer (Fig. 6E). In particular, the dual color-FISH study (inset of Fig. 6A) revealed that the hybridization signal was coming from the heterochromatin regions of the Indian muntjac Y-chromosome and appeared to be associated with the satellite II. Since the amount of satellite II far exceeds that of satellite V on the Indian muntjac Y chromosome, the satellite II signal detection conditions were optimized for simultaneously visualizing the distribution of these two DNA sequences on the Indian muntjac Y chromosome. The subsequent reduction of the satellite II DNA signals precluded observation of the interstitial satellite II sites, as observed previously (Li et al. 2000c). Moreover, the satellite V signal was also detected in the pericentric region (associated with the satellite II) of chromosome 3 of male Indian muntjac but not in the X+3 chromosome of the species (Fig. 6A). In addition, the satellite V signal was observed in a region on the X chromosome of the Chinese water deer (Fig. 6E, F). All the centromeric regions of the Chinese water deer chromosomes showed the presence of satellite II DNA with the exception of the Y-centromere. The satellite V signal (green) observed on the X-chromosome was not co-localized with the centromeric satellite II signal (red) of the species (Fig. 6E). No satellite V signal was detected in the chromosome complement neither of female Indian muntjac or female Formosan muntjac (data not shown) nor in the caribou or black tailed deer.

DISCUSSION

On the basis of comparative banding analysis, the hypothetical ancestor of all deer was postulated to have a karyotype of 2n=70 resembling the present day Chinese water deer (*Hydropotes inermis*) or brown-brocket deer (*Mazama gouazoubira*) (Neitzel 1987). The Chinese muntjac (*Muntiacus reevesi*) karyotype (2n=46) was thought to be the result of extensive chromosomal rearrangements from the ancestral deer species (Fontana and Rubini 1990; Yang et al. 1997b). The Indian muntjac (*Muntiacus muntjac vaginalis*) (2n=6/7) was considered to be closely

related to the Chinese muntjac, since viable hybrids can be produced from these two species (Shi and Pathak 1981; Neiztel 1987). Hsu et al. (1975) and Elder and Hsu (1988) postulated that the small number of giant Indian muntjac chromosomes could be the result of multiple tandem chromosome fusion plus a few centric fusions from an ancestral Chinese muntjac-species. Studies based on G-banding patterns (Shi et al. 1980), chromosomal localization of cervid satellite I DNA, telomeric sequence (Lin et al. 1991; Lee et al. 1993; Scherthan 1995; Hartmann and Scherthan 2004) and chromosome painting (Yang et al. 1995) lent support to the above hypothesis. Mitochondrial phylogeny studies further suggested that the reduction of chromosome number in the karyotypic evolution of the muntjac species could be through the route of 2n=70 (e.g. Chinese water deer-like ancestor) → 2n=46 (e.g. *M. reevesi*) → 2n=13/14 (e.g. *M. feae*). Following this lineage, two parallel reduction events may then occurr. One was from 2n=13/14 to 2n=8/9 (e.g. *M. crinifrons*) and the other was from 2n=13/14 to 2n=6/7 (e.g. *M. mumtjak vaginalis*) (Wang and Lan 2000). On the other hand, comparative chromosome painting studies with chromosome specific paint probe of brown-brocket deer, Chinese muntjac and Indian muntjac revealed that tandem and centromeric fusions alone could not account for the reduction of chromosome number from the 2n=46 of Chinese muntjac-like ancestral species to the 2n=6/7 Indian muntjac karyotype (Yang et al. 1997a). These authors proposed an alternative hypothesis which stated that the karyotype of Chinese muntjac and Indian muntjac could have evolved independently from the brown-brocket deer or Chinese water deer-like ancestor with 2n=70 karyotype. This hypothesis was further supported by detailed FISH analysis of interstitial localization of cervid satellite DNAs on the Indian muntjac chromosome complement (Fronicke and Scherthan, 1997; Li et al. 2000c). However, no muntjac species with more then 46 chromosomes have been found; thus more phylogenetic and molecular cytogenetic evidence is needed to sustain this hypothesis.

In this study, we have isolated DNA elements from microclones of microssected Y-chromosome DNA of Indian muntjac and from a BAC library of the species. The sequences and Southern blot analyses revealed that the inserts of microclones or subclones of positively screened BAC clones were derived from a novel cervid satellite DNA family with ~320 bp-sized monomers. Following the traditional nomenclature system (Bogenberger et al.1982; Qureshi and Blake 1995; Buntjer et al. 1998; Li et al. 2000a,c), the novel satellite DNA found in this study have been designated MMVsatV, a cervid satellite V DNA family. Zoo-blot analysis showed that the cervid satellite V is only present among Old World Deer in several Asian deer species, e.g. the muntjac species studied, Formosan sambar deer and Chinese water deer. The cervid satellite V is specifically located on the heterchromatin region of the Y-chromosome in those species examined. In addition, it is also located in the chromosome 3 of the male Indian muntjac and in the X chromosome of the Chinese water deer. All of the above deer species studied have the plesiometacarpal condition except for the Chinese water deer that was classified as the only antlerless telemetacarpalian (Randi et al. 1998). According to the results of the present study, the satellite V profile of the Chinese water deer appears to be more similar to that of Indian muntjac in the sense that both species have their satellite V distributed to chromosomes other than the Y-chromosome. In other words, the Chinese water deer could be more closely related to Indian muntjac then usually thought, since the chromosome distribution of a given satellite DNA family can be used as a tool to measure the species' phylogenetic relationship (Chaves et al. 2000; Chiang et al. 2004).

Unlike other cervid satellite DNA families that are primarily distributed to the centromeric/pericentromeric regions of deer chromosomes (Lin et al. 1991; Lee and Lin 1996; Lee et al. 1997; Li et al. 2000c; Li et al. 2002; Lin et al 2004), the satellite V DNA appeared to be primarily located in the heterochromatin region of the Y-chromosome of the deer species examined. Dual color-FISH study showed satellite V closely associated with satellite II in the Indian muntjac Y-chromosome. However, further analysis by stretched chromatin Fiber-FISH (Li et al. 2000a) would help to determine the organization of satellite V in relation to satellite II DNA in this chromosome. Surprisingly, the satellite V was also found in a region on the Chinese water deer X-chromosome and in the pericentric region (apparently associated with the satellite II, see Fig. 6A) of chromosome 3 of the male Indian muntjac. No satellite V signals were detected in female Indian

muntjac and female Formosan muntjac (a subspecies of Reeves' muntjac) by the FISH study; although, the Southern blot experiment revealed substantial hybridization with genomic DNA from these two female species. It is possible that satellite V sequences are also present on the X chromosomes in these muntjac species, but may be in either a small quantity that is below the detection level of FISH or has undergone sequence divergence. The presence of a similar heterochromatin (mainly satellite V DNA) block between the X- and Y-chromosomes suggests it could be the remnant heterochromatin from the ancestral "pre-sex chromosome pairs". If the ancestral X and Y chromosomes in an ancestral species were once homomorphic, one could speculate that the current Y chromosome is derived mainly from an interstitial site on the ancestral homomorphic sex chromosome – where the satellite V sequences can now be observed. Such homology of a heterochromatin segment between the sex- chromosomes also had been reported in the cervid species *Rangifer tarandus* (Lee et al. 1998).

Shuffling satellite DNA between the X- and Y-chromosomes could occur during meiosis at some point of karyotypic evolution; furthermore, satellite DNA has been suggested to play an important role in promoting chromosomal rearrangement between non-homologous chromosomes (Ferguson-Smith 1973; Slamovits and Rossi 2002). Under those scenarios, we postulate the following mechanisms of karyotypic evolution from a Chinese water deer, *Hydropotes inermis*-like ancestral species to the present day Indian muntjac (*Muntiacus muntjac vaginalis*) including the formation of the unique X+3 chromosome of the species (Fig.7). In the course of karyotypic evolution, multiple tandem fusion had occurred in the Chinese water deer-like ancestral species including the formation of the large neo-chromosome 3. At a certain point, an unequal crossing-over occurred between the block of satellite V DNA in the X-chromosome and the block of satellite V DNA in the Y-chromosome during male meiosis (Fig. 6E). This resulted in the transfer of most, if not all satellite V DNA from the X-chromosome to the Y-chromosome. Later, a Robertsonian- type of translocation occurred between a neo-chromosome 3 and the X-chromosome resulting in a loss of most of its satellite V DNA forming the X+ neo-3. This chromosomal restructuring event was likely accomplished with the help of specific homologous repetitive DNA sequences presented in the centromeric/pericentromeric region of these two chromosomes (Ferguson-Smith 1973; Choo et al. 1988; Page et al. 1996). A large amount of satellite I DNA had been found in the pericentric region of all Chinese water deer chromosomes with the exception of the Y-chromosome (Li et al. 2000b). Multivalent association involving the X+neo-3, the neo-3 and the Y- chromosome was formed during subsequent meiosis. Because of the close proximity of neo-3 and the Y-chromosome, a translocation with a breakpoint at the centromeric regions of these two chromosomes could have occurred, resulting in the exchange of satellite DNA between different families. The centromeric region of the Y-chromosome now acquired some satellite II DNA from the neo-chromosome 3 and the neo-chromosome 3 obtained a small amount of satellite V from the Y-chromosome as seen in the present day Indian muntjac Y-chromosome and the chromosome 3 respectively (Fig. 6A). The exact mechanism for satellite V DNA amplification, attribution and movement due to chromosome rearrangement(s), during the evolution of the cervid species still requires more studies.

We could not rule out the possibility that a more complex rearrangement may have occurred after the departure of the Indian muntjac and Chinese muntjac from a common ancestor, based on the mitechondria DNA analysis (Wang and Lan 2000) and chromosome painting results (Yang et al. 1997a).

The interspecific distribution of cervid satellite V DNA thus provides new insight into the karyotypic evolution of the Indian muntjac, a mammalian species with astonishingly few chromosomes. Repetitive sequences such as satellite DNA had been reported to play an important role in the drastic restructuring of chromosomes in a given species within a relatively short evolutionary time frame (Wichman et al. 1991). Discovery of new cervid satellite DNA and studies of genomic organization and distribution among Cervidae species, particularly among species of *Muntiacus*, will shed more light on the karyotypic evolution of Asian muntjacs.

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FIGURE LEGENDS

Figure 1A-C. Microdissection and reverse FISH. (A) A metaphase spread of the male Indian muntjac before the Y-chromosome (indicated by the arrow) was removed. The glass dissecting needle is shown in the left corner. (B) Same cell in (A) after the removal of the Y chromosome by microdissection. (C) The dissected Y chromosome DNA was amplified by DOP-PCR, labeled with digoxigenin and used as a probe for the reverse FISH experiment. The Y chromosome and the centromeric heterochromatin regions (indicated by the arrow and the arrowhead respectively) show positive FITC hybridization signals.

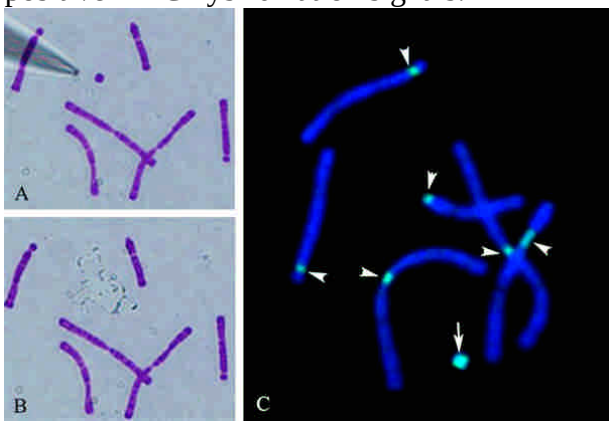

Figure 2. Sequence comparison between microclones IM-4-5 (Mmv-0.32#1) and IM-Y-5-7 (Mmv-0.32#2). Alignment of a corresponding 282 bp region with these two clones shows 72% sequence homology (identical nucleotide sequences from these two clones are shown in black boxes).

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IM-Y-4-52  GGGGTCTGGGTGAAGCTTCAGTGTGAGTTCCAATCTCATCAGATTTCAGAGGACACACGCAGGAGGCAAGCCCCATGTTT    80
IM-Y-5-7   .........GTGAAGGTCCAGTGTGAGGTTCAATCTAATCAGACTCCAGAAGACACACACACAAGAGAAGCCCCATGTTG    71

IM-Y-4-52  GTGGGGAGTGTGGACATTGTTTCTGTCAGACGTCAGTGTTATCAGACACCTGAGACCACACACATCGGGGCGAAACGCTTC    160
IM-Y-5-7   TTGGTGAGTGTGGGCGAAGTCACAGTAAGAAATCCATGTTTTCACACACTAGAATACACAAAGTGGGG.AAAGTCTATTG    150

IM-Y-4-52  TTTGCAGGGAGTGTGGGTGAATCTTCAGGGAGAATTCAGGTGTCAGAGACCAGAAGAAAGGCAGGGGAGAAGCCCTACATT    240
IM-Y-5-7   TTTGCGGAGAGTGTGGGTGAAGGTTCAGGTAGAA....GGTCTCAGATCCCAGAGGACCCAAAGGGGAGAAGCCCTACGTT    226

IM-Y-4-52  TTGTCGGGTGTGTGTGTGAAGAATCAGGTAGAAGTCACTTCTCAGAGGACAGATG.....................    295
IM-Y-5-7   TTTCAGGGAGTGTTGGTTACGACTTCGTTAGAATTCAATTCTCACAAGCCAGAGGACACACAGGGGAGAAGCCCTGTCTG    306

IM-Y-4-52  ............................................................................    295
IM-Y-5-7   TAGCGGTGTCTCTGTGCAGGGTCAGTGTGAGTTCCTATGTCATCAGACTCCAGAAGACACACACAGGGGAGAAGCC      382
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Figure 3. Southern blot hybridization of male Indian muntjac genomic DNA digested with *Hind*III, *Eco*RI and *Hae*III and probed with ^{32}P-labeled Mmv-0.32#1. Multiple hybridization bands with irregular patterns were observed in each digest. However, fragment size with 0.32 kb register (indicated on the right hand side) can be observed in *Hae*III digest. Sizing markers are shown on the left.

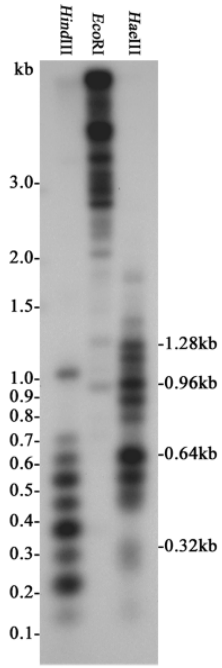

Figure 4A,B. Monomer organization and sequence alignment of the Mmv-0.32 #3 clone. (A) Schematic presentation of 12 tandem organized monomers of Mmv-0.32#3 clone. **a** and **l** are partial monomers. **b, c, f, i, j,** and **k** are complete monomers. **d, e, g,** and **h** are monomers with deletion. (B) Sequence alignment of the 12 monomers of the 3325 bp Mmv-0.32#3 clone. The complete monomer is 328 bp in size. The consensus sequence of these monomers is indicated with bold characters.

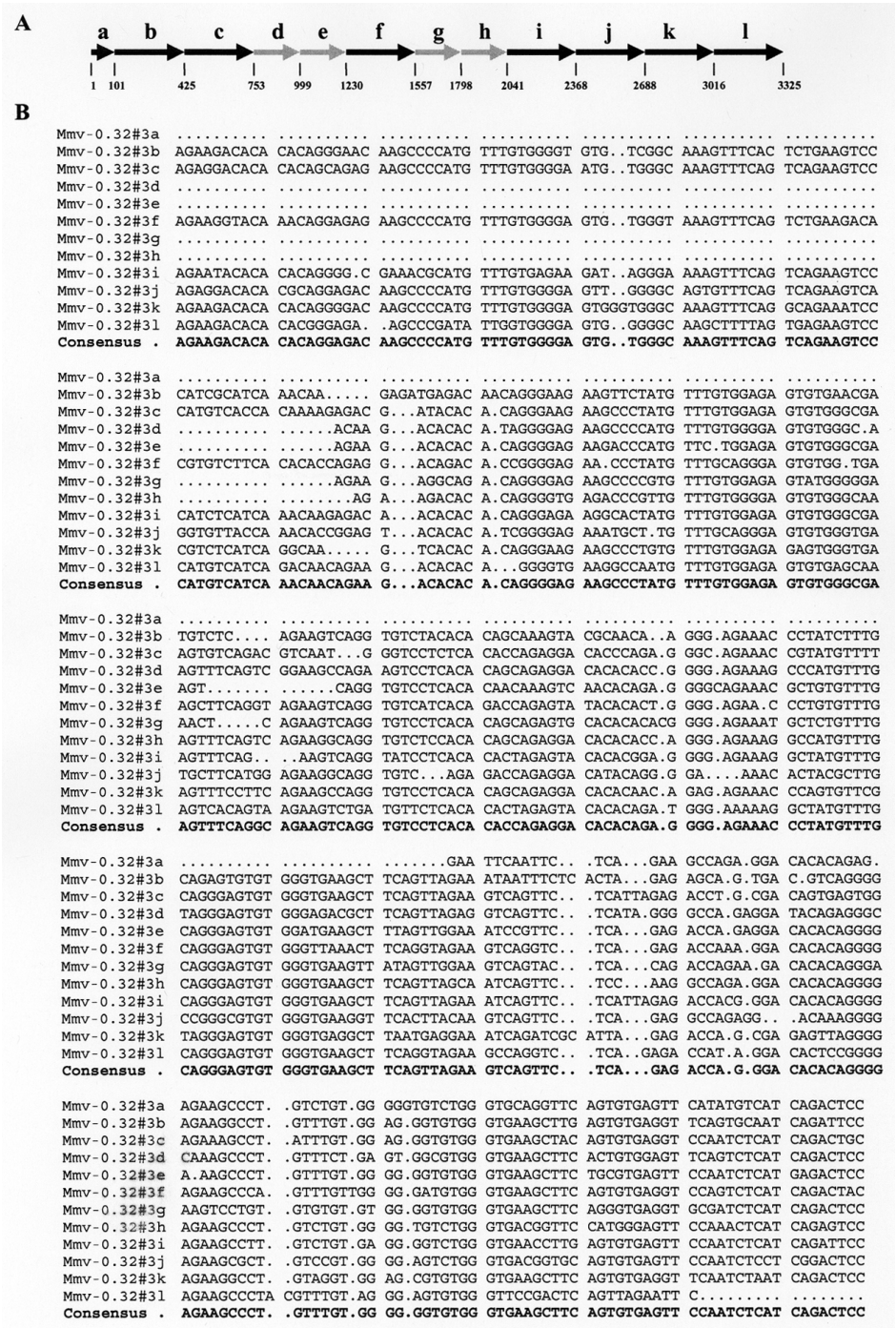

Figure 5A,B. Zoo-blot hybridization of cervid and other mammalian DNA. (A) A Southern blot following *Eco*RI digestion and probed with ^{32}P-labeled Mmv-0.32#1. The male and female Indian muntjac (lanes 1 and 2), male and female Formosan muntjac (lanes 3 and 4), male Chinese muntjac (lane 5), male sambar deer (lane 6), and male Chinese water deer (lane 10) show positive hybridization. Faint cross hybridization signals are observed in the male black tailed deer (lane 8), male caribou (lane 9) and male goat (lane 11). DNA samples from male roe deer (lane 7), bull (lane 12), boar (lane13), male rat (lane 14), man (lane15), and woman (lane 16) show no cross hybridization with the probe. (B) Sex verification of DNA samples by PCR using the SRY primers. The DNA samples tested are in the same order as in Figure 5A. The arrow on the left hand side indicates the PCR product of SRY gene, which is present only in males. (M) Size marker of 100 bp DNA ladder is shown on the left hand side. Lane 17 is a negative control (no DNA) used in the PCR experiment.

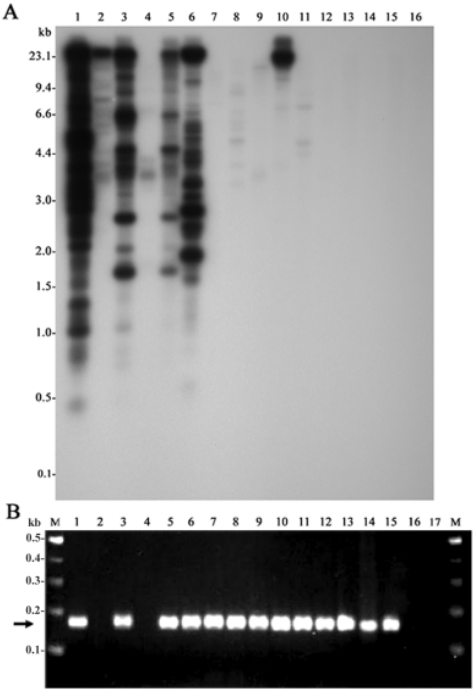

Figure 6A-F. Chromosomal localization of satellite V DNA, IM-Y-4-52 and satellite II DNA, Mmv-0.7 in the metaphase cells of different deer species. (A) A metaphase spread of male Indian muntjac shows the satellite II signal (red) located in the centromeric region of every chromosome, whereas the satellite V signal (green) is specifically located in the Y- chromosome (indicated by arrow) and in the pericentric region of the chromosome 3 (indicated by arrowhead). The bar represents 10µm. Three enlarged Y-chromosomes from different cells of the species show the distribution of satellite II and satellite V (inset). (B, C and D) Metaphase spread of the male Formosan muntjac, the male Chinese muntjac and the male sambar deer respectively show the specific localization of the satellite V (green signals) onto the Y-chromosomes of these deer species (indicated by arrows). The bar in (B) represents 10µm for (B-D). (E) A metaphase spread of the male Chinese water deer with dual color-FISH study shows that the satellite V signal (green) is only located on the Y-chromosome (indicated by the arrow) and on the X-chromosome (indicated by an arrowhead). Satellite V signal appears to be widely distributed on the Y-chromosome but no satellite II signal is detected in this chromosome (inset shows 3 enlarged Y-chromosomes from different cells). The satellite II is distributed onto the centromeric regions of the X-chromosome and of the autosomes. The satellite V is not associated with satellite II in the X-chromosome. (F) A metaphase phase spread of the female Chinese water deer shows the sole localization of satellite V signals (green) on both X-chromosomes (indicated by arrowheads). The bar represents 10µm for (E) and (F).

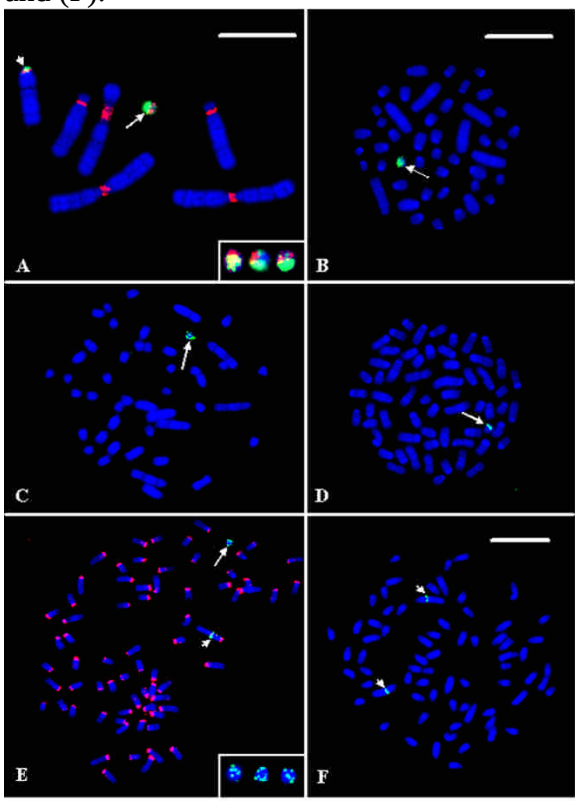

Figure 7. A schematic presentation showing a hypothetical chromosome restructure in a male Chinese water deer-like ancestral species which could have formed the chromosome 3, the X+3 chromosome and the Y-chromosome the present-day male Indian muntjac. The transfer of most if not all satellite V from the X-chromosome to the Y-chromosome of the male *Hydropotes inermis*-like ancestral species (HI) by unequal crossing over was achieved by pairing of the X- and Y-chromosomes and by the special alignment of the satellite V monomers of the sex chromosomes. This resulted in a Y-chromosome with more satellite V, designated as Y(sat.V+). A Robertsonian translocation then occurred between a neo-chromosome-3 and the X-chromosome that was devoid of satellite V (breakpoints on these two chromosomes is indicated by arrows). Satellite DNA (e.g. satellite DNA II) could have likely facilitated the close association between these two non-homologous chromosomes. Multivalent association occurred in the meiosis that brought the remaining neo-chromosome 3 in close proximity to the Y-chromosome. This could have facilitated the reciprocal transfer of some satellite V from the Y-chromosome to the neo-chromosome 3, and some satellite II from centromeric region of neo-chromosome 3 to the centromeric region of the Y-chromosome. Breakpoints of translocation are indicated by arrows. These events could have resulted in the present-day male Indian muntjac (IM) (2n=7) karyotype with the unique X+3 chromosome, the "free" chromosome 3 with satellite II and V in the pericentromeric region and the Y-chromosome with small amounts of centromeric satellite II and relatively abundant satellite V DNA.