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Construction of an Indian Muntjac BAC Library and Production of the Most Highly Dense FISH Map of the Species

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Chyi-Chyang Lin, Pei-Ching Hsu, Tzai-Shiuan Li, Shu-Ju Liao, Ya-Ming Cheng, Lie-Jiau Hsieh, and Yueh-Chun Li (2008) Construction of an Indian muntjac BAC library and production of the most highly dense FISH map of the species. *Zoological Studies* **47**(3): 282-292. Following completion of the genome sequences of some mammalian species, comparative genomic studies in mammals have been actively conducted to assess gene changes or to identify syntenic conservation during evolution. The Indian muntjac (*Muntiacus muntjak vaginalis*) (2*n* = 6 in the female and 7 in the male) may have evolved from an ancient deer species with a karyotype 2*n* = 70 through extensive chromosome rearrangements creating the lowest chromosome number of a mammalian species. Therefore, the species has become a good resource for studying syntenic conservation among deer species. An Indian muntjac bacterial artificial chromosome (BAC) library that contains 126,336 individual BAC clones with an average insert size of 80 kilobases was obtained in this study. The frequency of clones with inserts was 88%, and thus this library corresponds to approximately 4x coverage of the Indian muntjac genome. Individual chromosomal locations of 1619 BAC clones on the Indian muntjac metaphase chromosomes were identified by fluorescence *in situ* hybridization (FISH). Among these clones, 1517 BAC clones were mapped onto specific loci, and 102 BAC clones were mapped onto the centromeric region. This provides the most highly dense FISH BAC clone map for the species. This densely ordered map can be used as a blueprint for comparative FISH mapping studies of other deer species in order to investigate the mechanism of genomic rearrangement and karyotypic evolution. Moreover, centromeric BAC clones will provide an excellent resource for studying the structure and function of mammalian centromeres.
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Key words: BAC library, FISH mapping.

With the completion of the sequencing of the human, rat, and mouse genomes (Lander et al. 2001, Venter et al. 2001, Waterston et al. 2002, Gibbs et al. 2004) more-accurate and higher-resolution comparative mapping studies have been achieved among 8 phylogenetically distinct species (human, horse, mouse, rat, cat, dog, pig, and cattle). Those studies found that nearly 20% of chromosome breakpoint regions were reused during mammalian evolution. In addition, it was shown that the reused breakpoint regions were significantly associated with centromeric sequences (Murphy et al. 2005). An early study by Frönicke and Scherthan (1997) revealed that centromeric satellite DNAs cluster at the margins of conserved syntenic segments based on human-Indian muntjac Zoo-fluorescent *in situ* hybridization (FISH) experiments. Other FISH analyses also shown that centromeric satellite DNA and telomeric DNA are located at interstitial chromosomal sites on Indian muntjac (*Muntiacus muntjak vaginalis*) chromosomes, representing a remnant of chromosome break and fusion sites during restructuring of the Indian muntjac karyotype (Lin et al. 1991, Lee et al. 1993, Scherthan 1995, Li et al. 2000). Thus it is possible that evolutionary

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breakages preferentially occur at sites of ancestral centromeres or telomeres. However, eukaryotic centromeric DNA sequences are rapidly evolving (Sullivan et al. 2001). Therefore, in order to further illuminate the molecular mechanism of chromosome evolution, whole-genome sequence-based maps from phylogenetically different species are needed (Murphy et al. 2005). Since only a small number of mammalian genomes have been completely sequenced, a bacterial artificial chromosome (BAC) library would facilitate more-detailed comparative analyses of centromere and karyotype evolution at present (Wind et al. 2005).

The Cervidae, with 41 known species, is an ideal family for chromosomal evolution studies because of the unusually diverse karyotypes among species (ranging from 2*n* = 6/7 in the Indian muntjac to 2*n* = 80 in Siberian roe deer; *Capreolus capreolus pygargus*) (Wurster and Benirschke 1970, Neitzel 1987, Fontana and Rubini 1990, Whitehead 1993). Based on the number of interstitial centromeric and telomeric sites located on Indian muntjac chromosomes, it is believed that the Indian muntjac may have evolved from an ancient deer species with a karyotype 2*n* = 70 through extensive tandem fusions and several centric fusions (Lee et al. 1993, Scherthan 1995, Yang et al. 1995, Li et al. 2000). Recently, Chi et al. (2005) established an Indian muntjac BAC library and mapped 223 BAC clones onto Chinese muntjac (*M. reevesi*) chromosomes. Seventy of those clones were also mapped onto Indian muntjac chromosomes. Later, Huang et al. (2006) mapped 304 Indian muntjac BAC clones onto the chromosomes of the Chinese muntjac and black muntjac (*M. crinifrons*). Both comparative FISH mapping studies confirmed the orientation of centromere-telomere (head-tail) tandem fusions during the evolution of the Indian muntjac and black muntjac karyotypes. However, the molecular mechanism by which fusion sites were formed has yet to be determined. A BAC library of the Indian muntjac with denser FISH mapping would not only be valuable for delineating the comparative genomes among species, but would also be particularly useful in providing informative BAC clones for studying the fusion mechanism in the Cervidae family.

Centromere biology still remains an enigma. It is unclear how a functionally conserved centromere can be composed of both diverse centromeric DNAs and conserved centromeric proteins among higher eukaryotic species. Detailed analysis of the organization of the centromere among divergent species would help clarify this puzzle. Indian muntjac BAC clones mapped onto centromeric/pericentric regions can be used to address specific questions pertaining to centromeric and pericentromeric organization and evolution, and the mechanisms involving such drastic karyotypic changes between closely related species.

In the present study, we produced a highly dense FISH map of Indian muntjac chromosomes with 1619 BAC clones from a BAC library (~4x coverage of the Indian muntjac genome) constructed by our laboratory. Among these BAC clones, 1517 BAC clones were mapped onto specific loci, and 102 BAC clones were mapped onto centromere regions. The BAC clones mapped in this study will be a valuable resource for studying centromere biology and karyotypic evolution.

MATERIALS AND METHODS

Construction of an Indian muntjac genomic BAC library

Fibroblast cells from a male Indian muntjac cell line CCL-157 (American Type Culture Collection, Manassas, VA, USA) were used to prepare high-molecular-weight (HMW) genomic DNA. Briefly, about 8 x 10^6 cells (corresponding to 40 μg of DNA) were embedded in 0.5% low-melting temperature agarose (Sea Plaque GTG agarose, Cambrex Bio Science, Rockland, ME, USA) in phosphate-buffered saline (PBS) for each plug. Cells in the plug were treated with proteinase K and lysis solution to extract the genomic DNA. After extraction, the genomic DNA in the plug was first run through a 1% Pulsed Field Certified Agarose gel (Bio-Rad, Hercules, CA, USA) using a pulsed-field gel electrophoresis (PFGE) apparatus (Gene Navigator Pulsed Field System, Amersham Biosciences, Uppsala, Sweden) in 0.4x TBE buffer at 12°C and 120 V/cm for 10 h with a 5 s pulse time to remove the mitochondrial DNA. Subsequently, the genomic DNAs in the plugs were partially digested with *Eco*RI/*Eco*RI methylase (New England Biolabs, Ipswith, MA, USA) in a 37°C water bath for 16 h. The partially digested DNAs were double-size fractionized by PFGE in low-melting-temperature agarose to obtain HMW genomic DNA. Gel slices containing the DNA fragments of 60-100 and 100-150 kilobases (kb) were excised. The HMW

genomic DNAs were eluted and then ligated with a pCC1BAC™ *Eco*RI cloning-ready vector (Epicentre Biotechnologies, Madison, WI, USA) by Fast-Link™ DNA Ligase (Epicentre Biotechnologies). Ligation was drop-dialyzed against 5% PEG or TE buffer with Contains 0.025 μM nitrocellulose membranes (Millipore, Bedford, MA, USA) for 1 h. The dialyzed ligated DNA was electroporated into 33 μl of EPI300 competent cells (EpiCentre Biotechnologies). After electroporation, cells were incubated in 600 μl 2x LB medium containing 1 mM $MgCl_2$ and 20 mM glucose at 37°C with gentle shaking for 1 h and then spread on 2x LB plates containing chloramphenicol (12.5 μg/ml), X-gal (40 μg/ml), and IPTG (100 μg/ml). The plates were incubated at 37°C overnight. The recombinants were identified by blue and white colony selection. Approximately 20 transformations were carried out to obtain > 6000 BAC clones. White, positive BAC clones were manually picked out and placed in 96-well microtiter plates containing 100 μl freezing media (0.5% w/v NaCl, 1% w/v Bacto-Tryptone, 0.5% w/v Bacto-extract, 13 mM KH_2PO_4, 36 mM K_2HPO_4, 1.7 mM sodium citrate, 6.8 mM $(NH_4)SO_4$, 4.4% v/v glycerol, 0.4 mM $MgSO_4 \cdot 7H_2O$, and 12.5 μg/ml chloramphenicol). The microtiter plates were incubated at 37°C and shaken at 350 rpm for 18-20 h. Two copies of each 96 well microtiter plate were prepared and stored at -80°C in separate refrigerators. The detailed protocol was described by Peterson et al. (2000).

Estimation of the average insert size

Randomly picked recombinant clones were pre-grown in 2 ml of 2xLB medium containing 12.5 μg/ml chloramphenicol for 5 h; then, 2 μl of 1000x CopyControl Induction Solution (EpiCentre Biotechnologies) was added and the culture was continuously grown at 37°C with 250 rpm shaking for another 18-20 h. The bacterial broth was collected in order to isolate the BAC DNA. The BAC DNA was isolated following the standard alkaline lysis protocol. The purified BAC DNA obtained was digested by *Not*I to excise the insert. The insert was fractionated by running 0.8% agarose through the PFGE in 0.4x TBE buffer at 12°C and 6.0 V/cm for 15.5 h with 1- to 15 s pulse times.

Fluorescence *in situ* hybridization (FISH)

The degree of chimerism in this library and the physical mapping of BAC clones were evaluated by FISH. Metaphase chromosomes were prepared from the male Indian muntjac cell line, and the BAC DNA probes were labeled with either digoxigenin-11-dUTP or biotin-16-dUTP (Roche, Basel, Switzerland) by nick-translation. The procedures for denaturation, hybridization, post-hybridization washing, and signal detection are described in detail elsewhere (Li et al. 2000). Fluorescent signals were captured with a Leica ALM fluorescence microscope (Leica Microsystems, Wetzlar, Germany) 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, Migdal HaEmek, Israel).

RESULTS

Characterization of the BAC library

We constructed a BAC library of the Indian muntjac with a pCC1BAC vector (8.1 kb). The complete library consists of 126,336 clones, which were stored in 1316 x 96 well microtiter plates. In total, 545 randomly chosen BAC clones were sized using *Not*I digestion followed by PFGE. Among the BAC clones analyzed, 480 contained inserts and 65 did not. Thus, we estimated that the frequency of BAC clones with inserts was 88%. A part of the PFGE results of BAC clones showed that the average size of a BAC clone with an insert was 80 kb (Fig. 1). Assuming that the Indian muntjac genome contains 2.2 x 10^9 bp (http://www.genomesize.com), the total library constructed corresponds to about 4-fold coverage of the Indian muntjac (MMV) genome.

Chromosomal mapping of BAC clones by FISH

In total, 1619 BACs were mapped onto specific regions of the Indian muntjac metaphase chromosome by FISH. None of the FISH signals of BAC clones with specific loci was observed at more than 1 site, indicating that the chimeric frequency was roughly 0%. The densely ordered BAC clone map was constructed based on the assignment of FISH signals on specific chromosome bands according to a published high-resolution G-banded karyotype of the Indian muntjac (Li et al. 2000). The mapping result is summarized in supplementary table 1. Among these mapped clones, 693 clones were mapped

Table 1. Summary of 1619 bacterial artificial chromosome (BAC) clones mapped onto the chromosomal regions of the Indian muntjac by fluorescent *in situ* hybridization

chromosomal region	BAC clone
1p45	0096E6, 0111E6, 0122H12, 0124H12, 0137H12, 0139A1, 0876E6, 0994E6, 0996E6, 1031A1, 1048E6, 1161H12
1p44	0118H12, 0281A7, 0382H12, 0412E6, 0433E6, 0495H12, 0670H12, 0688E6, 0735A1, 0768H12, 0797A1, 0839H12, 0855H12, 0908A1, 0908E6, 0973E6, 0987H12, 1003H12, 1010E6, 1106H12, 1130A1, 1218H12, 1260H12, 1276A1
1p43	0097E6, 0430A1, 0743A1, 0773H12, 0787A1, 1134A1,1144E6, 1165H12, 1198E6
1p42	0092A1, 0406E6, 0485E6, 0625H12, 0664H12, 0675E6, 0760A1, 0780A1, 0811A1, 0821E6, 0875H12, 0889H12, 0906E6, 1015H12, 1052E6, 1071H12, 1092E6, 1099E6, 1127E6, 1147H12, 1181E6, 1218A1, 1218H12, 1238A1
1p41	0110A1, 0135E6, 0158E6, 0746E6, 0794A1, 0822E6, 0926A1, 01041E6, 1142A1, 1196E6, 1259H12
1p34	0113H12, 0121H12, 0125E6, 0335H12 , 0380A1, 0585A1, 0631E6, 0776H12, 0805E6, 0915E6, 0941H12, 0967E6, 0983A1, 1017A1, 1036H12, 1082H12, 1108A1, 1109H12, 1196A1
1p33	0012H12, 0031E6, 0038A1, 0116A1, 0145E6, 0404A1, 0486H12, 0734A1, 0774H12, 0852E6, 1056E6, 1113E6
1p32	0089A1, 0101H12, 0677E6, 0750A1, 0758H12, 0760E6, 0845E6, 0849E6, 0911E6, 0950E6, 0956E6, 0994H12, 1019H12, 1023A1, 1075A1, 1077A1, 1102E6, 1115E6, 1116E6, 1142E6, 1151A1, 1176H12, 1216E6, 1225H12
1p31	0040H12, 0152A1, 0390H12, 0487A1, 0737H12, 0749E6, 0752A1, 0752H12, 0759A1, 0793H12, 0809E6, 0822H12, 0885E6, 0889A1, 0968E6, 1065H12
1p26	0084A1, 0118A1, 0160A1, 0305E6, 0327E6, 0579H12, 0672E6, 0730A1, 0774E6, 0791E6, 0834E6, 0904E6, 0994A1, 1057H12, 1094H12, 1135A1, 1155A1
1p25	0325E6, 0666H12, 0768E6, 1073H12, 1074A1, 1141H12, 1184H12
1p24	0144E6, 0419A1, 0497E6, 0726E6, 0786E6, 0818E6, 0827E6, 0831A1, 0961H12, 1122H12, 1236H12, 1254E6
1p23	0281A3, 0419E6, 0599A1, 0732E6, 0772E6, 1131H12, 1138E6, 1174H12,
1p22	0005A1, 0115H12, 0313A1, 0388E6, 0492E6, 0496A1, 0513H12, 0675H12, 0794H12, 0804A1, 0816H12, 0837A1, 0887H12, 0937H12, 1046H12, 1073A1, 1096E6, 1115A1, 1139A1, 1170E6, 1212H12, 1226A1
1p21	0016E6, 0506A1, 0595A1, 0744A1, 0747H12, 0765H12, 0782H12, 0826E6, 0907E6, 0922A1, 0997H12, 1005H12, 1071A1, 1072A1, 1110E6, 1169H1
1p17	0352A1, 0690A1, 0691E6, 0785E6, 0825E6, 0943H12, 1044H12, 1052H12, 1113H12, 1126A1, 1191A1, 1195A1, 1238H12
1p16	0060H12, 0939E6, 0999E6, 1144A1, 1152E6, 1259E6
1p15	0006H12, 0065E6, 0281A4, 0338A1, 0398E6, 0587E6, 0592A1, 0725H12, 0779H12, 0927H12, 0965H12, 0980E6, 1030H12, 1049H12, 1062E6, 1129H12, 1143H12, 1165A1, 1195A1
1p14	0051A1, 0095A1, 0122A1, 0129H12, 0157H12, 0739H12, 0824E6, 0850H12
1p13	0003H12, 0012A1, 0502A1, 0636H12, 0674E6, 0729H12, 0739A1, 0754H12, 0768A1, 0901H12, 0915H12, 0920H12, 0924H12, 0940A1, 0970E6, 0974H12, 0997E6, 1012A1, 1037A1, 1037E6, 1039E6, 1113A1, 1134H12, 1141E6, 1161E6, 1258A1
1p12	0008H12, 0041E6, 0093E6, 0394H12, 0738A1, 0833E6, 1014H12, 1024E6, 1085H12, 1184A1, 1190A1, 1227E6
1p11	0111A1, 0147E6, 0352E6, 0367A1, 0374H12, 0579E6, 0596H12, 0665E6, 0686H12, 0740E6, 0803E6, 0820E6, 0843E6, 0881A1, 0900A1, 0913H12, 0968A1, 1080E6, 1085A1, 1145E6, 1234E6, 1254A1
1q11	0003A1, 0041H12, 0281B4, 0281B5, 0331H12, 0389E6, 0390A1 , 0488A1, 0903A1, 0998H12, 1090A1, 1097E6, 1148H12, 1171A1
1q12	0012H12, 0033H12, 0687H12, 0998A1, 1008E6, 1097H12, 1121A1, 1125A1, 1183E6, 1267H12, 1278A1
1q13	0124E6, 0281A12, 0384A1, 0487H12, 0493E6, 0592E6, 0765A1, 0766H12, 0770H12, 0789E6, 0799H12, 0856A1, 0986E6, 1017E6, 1039A1, 1093A1, 1119A1, 1200H12, 1222E6, 1270H12
1q14	0038A1, 0489 E6, 0586H12, 0724H12, 0856H12, 0858H12, 1118H12
1q15	0333A1, 0414H12, 0487E6, 0829A1, 0902H12, 1013A1, 1041H12, 1116H12, 1124A1
1q16	0281A5, 0585E6, 0600H12, 0766A1, 0955A1, 0985H12, 1061E6, 1063A1, 1101A1, 1156H12, 1219A1, 1256H12, 1280H12, 1302A1
1q17	0002E6, 0004A1, 0051H12, 0151A1, 0368E6, 0542A1, 0568A1, 0589H12, 0675A1, 0728H12, 0734E6, 0741A1, 0775H12, 0805H12, 0837E6, 0842H12, 0869A1, 0995H12, 1032E6, 1047H12, 1049E6, 1089E6, 1136E6, 1258E6
1q18	0116E6, 0118E6, 0586E6, 0645E6, 0857E6, 0877A1, 1025A1, 1028A1, 1046E6
1q19	0047E6, 0088H12, 0126A1, 0136E6, 0144H12, 0159E6, 0281A8, 0355H12, 0408H12, 0506E6, 0616E6, 0639A1, 0679A1, 0685A1, 0725E6, 0763E6, 0797H12, 0826H12, 0845A1, 0855A1, 0969A1, 1006E6, 1013H12, 1051A1, 1069E6, 1144H12, 1152A1, 1179E6, 1182A1, 1261E6
1q21	0127E6, 0500H12, 0502H12, 0584H12, 0727A1, 0762E6, 0829E6, 0903E6, 0906A1, 0909A1, 1042E6, 1095E6, 1155H12, 1200A1, 1240E6, 1262A1
1q22	0002H12, 0005E6, 0007H12, 0101E6, 0108E6, 0140A1, 0387H12, 0581E6, 0847H12, 0892H12, 0900H12, 0936H12, 0937E6, 0950A1, 1018E6, 1064A1, 1102A1, 1108E6, 1134A1, 1194A1, 1217A1

(to be continued)

1q23	0029A1, 0087H12, 0119A1, 0307H12, 0827A1, 1033E6, 1089A1, 1124H12
1q24	0039A1, 0154E6, 0493H12, 0510A1, 0669E6, 0680A1, 0762H12, 0791A1, 0845H12, 0882H12, 0938A1, 0948E6, 1007A1, 1008H12, 1055E6, 1091A1, 1150E6, 1259A1
1q25	0130E6, 0302E6, 0669A1,0736E6, 1007E6
1q26	0036E6, 0141H12, 0599A1, 0678H12, 0723E6, 0769A1, 0773A1, 0850A1, 0871 E6, 0927E6, 1030E6, 1058A1, 1074H12, 1104E6, 1175H12, 1191E6
1q27	0001E6, 0013E6, 0072H12, 0330A1, 0382A1, 0601E6, 0759H12, 0766A1, 0840H12, 0864A1, 0903H12, 0940E6, 0956A1, 1049E6, 1087E6, 1090E6, 1190E6, 1199H12, 1255H12
1q28	0038E6, 0100H12, 0106H12, 0122E6, 0151H12, 0155H12, 0290H12, 0311H12 , 0382E6, 0485A1, 0488E6, 0580A1, 0679E6, 0684E6, 0726H12, 0732A1, 0743E6, 0744A1, 0751A1, 0767H12, 0770A1, 0783H12, 0801H12, 0804E6, 0808A1, 0809H12, 0813E6, 0833H12, 0838E6, 0874H12, 0889A1, 0902A1, 0906H12, 0912H12, 0950H12, 0954H12, 0976H12, 0997A1, 1015E6, 1042A1, 1055A1, 1065E6, 1066E6, 1078A1, 1154H12, 1157A1, 1166H12, 1169A1,1183A1
1q31	0144A1, 0145H12, 0157A1, 0354E6, 0409H12, 0678A1, 0727H12, 0790E6, 0803H12, 0905E6, 0972A1, 1001E6, 1013E6, 1043A1, 1051H12, 1084E6, 1300H12
1q32	0347H12, 0778H12, 0939A1, 0990H12, 1122A1, 1260A1, 1300E6
1q33	0069A1, 0094H12, 0362A1, 0807H12, 0844E6, 0984H12, 1117A1
1q34	0001H12, 0028H12, 0040E6, 0150E6, 0329H12, 0356E6, 0417E6, 0506H12, 0590A1, 0595E6, 0635E6, 0685H12, 0690E6, 0737E6, 0740A1, 0757A1, 0758E6, 0771A1, 0800A1, 0801A1, 0828A1, 0828E6, 0889E6, 0917A1, 0917H12, 0931A1, 0984A1, 1011H12, 1086A1, 1101H12, 1109E6, 1195E6, 1216A1
2p12	0013H12, 0281A2, 0488H12, 0774A1, 1000H12, 1174E6, 1316A1
2p11	0003E6, 0155E6, 0357A1, 0419A1, 0421A1, 0799E6, 0836E6, 0966A1, 1054E6, 1100E6, 1103A1, 1148E6
2q11	0064E6, 0510E6, 0596E6, 0634H12, 0683H12, 0687A1, 0757E6, 0788E6, 0802E6, 0821H12, 0952H12, 1036E6, 1102H12, 1132E6, 1232A1, 1262H12, 1269E6
2q12	0041A1, 0130A1, 0498A1, 0784A1, 0812H12, 1069A1, 1075E6, 1220H12
2q13	0016H12, 0058A1, 0084E6, 0089E6, 0109H12, 0154E6, 0427H12, 0731E6, 0770E6, 0880A1, 0895E6, 0904H12, 0921E6, 0930H12, 0980A1, 1018H12, 1020A1, 1074H12, 1075H12, 1104H12, 1117H12, 1153E6, 1280A1
2q14	0064H12, 0590E6, 0733H12, 0738H12, 1059A1
2q15	0089H12, 0151E6, 0310E6, 0399H12, 0412A1, 0582E6, 0589E6, 0802H12, 0870H12, 0898E6, 1030A1, 1040E6, 1049A1
2q16	0593E6, 0779E6, 0797E6, 0947A1, 1031E6, 1114H12, 1260E6
2q17	0030A1, 0033A1, 0136H12, 0497A1, 0730H12, 0736H12, 0762A1, 0763H12, 0798A1, 0842A1, 1025H12, 1083H12, 1126E6, 1126H12, 1198A1, 1261A1
2q18	0082A1, 0098H12, 0119H12, 0365H12, 0490A1, 0725A1, 0736A1, 0744E6, 0843A1, 0857A1, 0896E6, 0898H12, 0899H12, 0914E6, 0945E6, 0974A1, 0991H12, 1096A1, 1100H12, 1129A1
2q21	0008E6, 0024A1, 0094A1, 0099E6, 0121E6, 0136H12, 0490H12, 0580H12, 0587H12, 0598E6, 0676A1, 0691A1, 0741H12, 0773E6, 0823A1, 0893H12, 0894H12, 0933E6, 0949E6, 1002A1, 1006A1, 1021A1, 1042H12, 1057E6, 1078E6, 1098A1, 1164E6, 1301A1, 1301E6
2q22	0499A1, 0584E6, 0593E6, 0687E6, 0874A1, 0877A1, 0907A1, 0912E6, 1084A1
2q23	0102A1, 0399E6, 0494A1, 0808H12, 0841E6, 0959E6, 0963E6, 0982H12, 1177E6, 1187H12, 1190H12, 1224H12
2q24	0356A1, 0662H12, 0841H12, 0899E6, 1256E6
2q25	0324A1, 0395A1, 0490E6, 0666E6, 0691H12, 0815E6, 0996A1, 1027E6, 1101E6, 1187A1, 1194E6, 1278H12
2q26	0091A1, 0104A1, 0594E6, 0726A1, 0766A1, 0861E6, 0910E6, 0971E6, 0985A1, 1006H12, 1131A, 1140A1, 1227A1
2q27	0015E6, 0112A1, 0134H12, 0504A1, 1045A1, 1134E6, 1139E6, 1184E6, 1188E6
2q28	0578A1, 1092A1, 1193A1
2q29	0104E6, 0106A1, 0111H12, 0361H12, 0416H12, 0491H12, 0500A1, 0588H12, 0621E6, 0638H12, 0663E6, 0735H12, 0767E6, 0772A1, 0777E6, 0846H12, 0971H12, 0979A1, 1017H12, 1035A1, 1076H12, 1201H12, 1257H12, 1273H12
2q210	0008A1, 0104A1, 0123E6, 0385E6, 0400A1, 0429A1, 0493A1, 0600A1, 0723H12, 0756E6, 0851E6, 0914A1,1139H12, 1220E6, 1225E6
2q31	0062H12, 0123H12, 0281A11, 0324H12, 0358A1, 0360A1, 0406H12, 0420H12, 0735E6, 0763A1, 0818A1, 0886A1, 0968H12, 0975A1, 0978A1, 1048A1, 1129E6, 1135H12, 1302H12
2q32	0678E6, 0959A1, 0972E6, 1001H12, 1149A1
2q33	0086H12, 0110E6, 0677H12, 0745H12, 0767A1, 0781A1, 0922E6, 1043H12, 1114A1, 1117E6, 1161A1, 1173A1, 1177H12
2q34	0352H12, 0391E6, 0974E6, 0975A1, 0976E6, 0987A1, 1031H12, 1091E6, 1167H12, 1254H12
2q35	0016A1, 0034H12, 0048H12, 0065A1, 0084H12, 0119E6, 0154H12, 0328E6, 0343E6, 0427E6, 0491E6, 0676H12, 0791H12, 0817E6, 0821A1, 0966E6, 1128E6
2q36	0007A1, 0147A1, 0163E6, 0361E6, 0366H12, 0502E6, 0598A1, 0802E6, 0969E6, 1158H12, 1186E6, 1193H12, 1305A1
2q37	0005H12, 0015A1, 0015H12, 0127A1, 0290A1, 0395E6, 0486A1, 0494H12, 0550A1, 0585H12, 0599E6, 0744H12, 0776E6, 0792H12, 0805A1, 0836A1, 0869E6, 0893A1, 0908H12, 0919H12, 0973H12, 1020H12, 1027H12, 1063H12, 1072E6, 1100H12, 1143A1, 1188H12, 1197E6, 1242E6, 1281A1

(to be continued)

2q38	0113E6, 0119A1, 0578E6, 0742A1, 1016A1, 1060H12, 1133E6, 1220A1
2q39	0034E6, 0064A1, 0145A1, 0157E6, 0392E6, 0577H12, 0582A1, 0584A1, 0591E6, 0592H12 ,0788A1, 0810A1, 0819A1, 0854H12, 0879A1, 0905A1, 0952E6, 1022E6, 1047E6, 1068E6, 1105H12, 1118A1, 1125E6, 1127A1, 1146A1, 1164H12, 1230A1
Xp15	0050A1, 0121A1, 0160E6 , 0374E6, 0383H12, 0579A1, 0591H12, 0751E6, 0792E6, 0978E6, 0986A1, 1046A1, 1047A1, 1058E6, 1064A1, 1122E6, 1181A1, 1269A1
Xp14	0086A1, 0281A1
Xp13	0297A1, 0343H12, 0742H12, 0750E6, 0753E6, 0857H12, 0887A1, 0952A1, 1009A1, 1087A1, 1156A1, 1224A1, 1279E6
Xp12	0067E6, 0130H12, 0366E6 , 0583H12, 0686E6, 1028E6, 1086H12, 1130H12
Xp11	0364E6 , 0739E6, 0746A1, 0830E6, 0882A1, 0928H12, 1060E6, 1099E6, 1219H12, 1249A1
3q11	0017A1, 0027H12, 0409E6, 0500E6, 0589A1, 0673A1, 0732H12, 0761E6, 0793E6, 0815A1, 0835A1, 0841A1, 0880H12, 0962E6, 0987E6, 0988E6, 0988H12, 1008A1, 1089H12, 1137A1, 1141A1, 1197H12, 1214H12, 1219E6, 1251A1, 1302E6
3q12	0035A1, 0369H12, 0432A1, 0769H12, 0810H12, 0843H12, 0866H12, 0885H12, 0993E6, 0995H12, 1115H12, 1183H12
3q13	0006E6, 0007E6, 0010A1, 0037A1, 0093H12, 0104H12, 0115H12, 0384E6, 0397H12, 0576H12, 0586A1, 0802A1, 0803A1, 0924E6, 0955H12, 0962H12, 0977H12, 0993H12, 1044A1, 1095A1, 1096H12, 1192A1, 1284H12, 1313H12
3q14	0495A1, 0740H12, 0810E6, 0938E6, 1084A1, 1251H12, 1273A1
3q15	0091H12, 0146A1, 0155A1, 0316H12, 0339A1, 0339H12, 0341A1, 0341H12, 0343A1, 0343H12, 0345E6, 0345H12, 0414E6, 0492H12, 0495E6, 0597E6, 0600E6, 0671E6, 0690H12, 0736A1, 0765E6, 0771E6, 0781H12, 0788A1, 0793A1, 0823H12, 0865E6, 0983H12, 0988A1, 0989E6, 0994H12, 1000A1, 1024H12, 1039H12, 1044E6, 1059H12, 1073E6, 1090A1,1090H12, 1114E6, 1559H12, 1216H12
3q21	0082H12, 0341E6, 0366H12, 0420E6, 0792A1, 0831E6, 0927A1, 0933H12, 1163H12, 1173E6, 1181H12,
3q31	0004E6, 0021H12, 0057E6, 0063H12, 0092H12, 0146H12, 0723A1, 0733E6, 0749A1, 0749H12, 0861A1, 0888E6, 0890E6, 0933A1, 0940H12, 1026H12, 1081H12, 1156E6, 1247H12
3q32	0367H12, 0572A1, 0598H12, 0754A1, 0786H12, 0811E6, 0873A1, 0971A1, 1050E6, 1118E6, 1128H12, 1159A1, 1222A1, 1263E6
3q33	0006A1, 0067A1, 0090E6, 0117A1, 0141E6, 0148E6, 0281A1, 0281A9, 0365E6, 0369A1, 0385A1, 0390E6, 0489A1, 0667A1, 0738 E6, 0752 E6, 0766E6, 0772H12, 0795H12, 0804H12, 0825A1, 0825H12, 0844A1, 0893E6, 0902E6, 1021H12, 1026A1, 1153H12, 1188A1, 1238E6, 1257E6
3q34	0038A1, 0085H12, 0091E6, 0096H12, 0141A1, 0688A1, 0844H12, 0886E6, 0894A1, 0909E6, 0969H12, 0990E6, 1070E6, 1074E6, 1108H12, 1162H12
3q35	0058H12, 0112E6, 0140E6, 0741E6, 0748H12, 0753H12, 0838A1, 0839A1, 0839E6, 0871H12, 0929H12, 1011E6, 1038A1, 1070H12, 1132H12
3q36	0032H12, 0160H12, 0686A1, 0730E6, 0782E6, 0795A1, 0813A1, 0877E6, 0895H12, 0983E6, 0991A1, 1125H12, 1162A1, 1163E6
3q37	0140H12, 0297H12, 0367E6, 0486E6, 0580E6, 0734H12, 0779A1, 0880E6, 0897H12, 1045H12, 1095H12, 1172H12 , 1255A1
3q38	0123H12, 0332H12, 0411H12, 0491A1, 0820H12, 0883A1
3q39	0059H12, 0087E6, 0107H12, 0386E6, 0401A1, 0405E6, 0417H12, 0485H12, 0683E6, 0728A1, 0729A1, 0742E6, 0760H12, 0764E6, 0783E6, 0785H12, 0806A1, 0891H12, 0948A1, 1013A1, 1054A1, 1057A1, 1059E6, 1153A1, 1154A1, 1164A1, 1186H12, 1197A1, 1271H12
3q41	0055A1, 0124A1, 0349E6, 0402E6, 0581H12, 0665A1, 0682A1, 0684A1, 0884H12, 0910H12, 0934H12, 1012H12, 1094E6, 1109E6, 1169E6, 1225A1, 1307A1
3q42	0099H12, 0489H12, 0492A1, 0498E6, 0582H12, 0588E6, 0591A1, 0662A1, 0751H12, 0753A1, 0848E6, 0897E6, 0920A1, 0944A1, 0964A1, 0980H12, 0982E6, 1035E6, 1068A1, 1095H12
3q43	0084E6, 0297A1, 0667E6, 0671A1, 0747E6, 0761A1, 1140H12, 1199A1
3q44	0061A1, 0082A1, 0088A1, 0088E6, 0105H12, 0115E6, 0137A1, 0316E6, 0318A1, 0334A1, 0583E6, 0666A1, 0685E6, 0727E6, 0748E6, 0754E6, 0771H12, 0859H12, 0860H12, 0932E6, 0941A1, 0958E6, 0964E6, 0977E6, 0978H12, 1016E6, 1043E6, 1063E6, 1086E6, 1088E6, 1120H12, 1130E6, 1143E6, 1145H12, 1152H12, 1169A1, 1170A1, 1283H12
Pseudo-autosomal region of X and Y	0599H12, 0672H12, 1012E6, 1121H12, 1154E6
p-ter or q-ter of Y	0497H12, 0881E6, 0946E6, 1088H12
Centromeric clones	0025H12, 0037A1, 0037E6, 0039E6, 0051E6, 0058E6, 0060A1, 0077A1, 0085E6, 0098A1, 0097H12, 0116H12, 0117H12, 0150A1, 0154A1, 0159A1, 0159H12, 0189E6, 0237A1, 0293A1, 0293E6, 0293H12, 0294A1, 0294E6, 0294H12, 0295A1, 0295E6, 0295H12, 0296A1, 0296H12, 0297E6, 0301H12, 0331A1, 0359E6, 0359H12, 0370E6, 0378E6, 0402E6, 0411A1, 0415E6, 0418E6, 0430E6, 0433H12, 0434A1, 0435H12, 0494E6, 0496H12, 0499E6, 0504 E6, 0504H12, 0590H12, 0594A1, 0667A1, 0673H12, 0674A1, 0676E6, 0684H12, 0747A1,0748A1, 0750H12, 0816E6, 0836H12, 0846E6, 0866E6, 0868A1, 0876A1, 0926H12, 0976A1, 0985E6, 0990A1, 0992A1, 1014E6, 1015A1, 1034A1, 1040A1, 1051E6, 1053E6, 1056H12, 1104A1, 1105E6, 1106E6, 1110A1, 1111H12, 1128A1, 1132A1, 1136A1, 1136H12, 1140E6, 1142H12, 1150H12, 1158A1, 1177H12, 1179H12, 1189A1, 1189E6, 1198H12, 1228E6, 1228H12, 1240H12, 1241H12, 1276E6, 1296A1

onto Indian muntjac chromosome 1; 402 clones were mapped onto chromosome 2; 362 clones were mapped onto chromosome 3; 51 clones were mapped onto the X chromosome; 4 clones were mapped onto the Y chromosome; and 5 clones were mapped onto the pseudo-autosomal regions of X and Y. In total, 1517 clones were mapped onto specific regions on the chromosome arm of the Indian muntjac (Fig. 2). The remaining 102 clones were mapped onto the centromeric regions of the species. The signal distribution pattern observed on the centromere was further classified into 8 different types (Fig. 3). One of the distribution patterns of the centromeric signals was similar to that of the C5 probe (cervid satellite I) showing centromeric and interstitial hybridization signals on all chromosomes, except the Y chromosome. Furthermore, strong fluorescent signals were observed on the pericentromeric blocks of the X+3 chromosome (Lin et al. 1991, Lee et al. 1993, Li et al. 2000) (Fig. 3A). A 2nd type of observed signal pattern was similar to that seen with the Mmv-0.7 probe (cervid satellite II), which appeared in all centromeric and interstitial regions (Li et al. 2000) (Fig. 3B). The hybridization signals of 9 clones were observed only on the pericentromeric regions of the X+3 chromosome; some of them had weaker hybridization intensities on the X-side of the pericentromeric region and stronger signals on the chromosome 3 side of the pericentromeric region of the X+3 chromosome (Fig. 3C), while others showed equal hybridization intensities on both sides of the pericentromeric regions of the X+3 chromosome (Fig. 3D). Twenty-one clones were located on the Yq and centromeric regions of chromosome 3 of the Indian muntjac, resembling cervid satellite V (Li et al. 2005) (Fig. 3E). Four special BAC clones showed unique parallel signals on both lateral sites of the primary constriction where the kinetochores are located (Fig. 3F). Some BAC clones occupied all centromeric heterochromatin regions (Fig. 3G), and some BAC clones were located in the middle region of the centromere, especially X+3 (Fig. 3H).

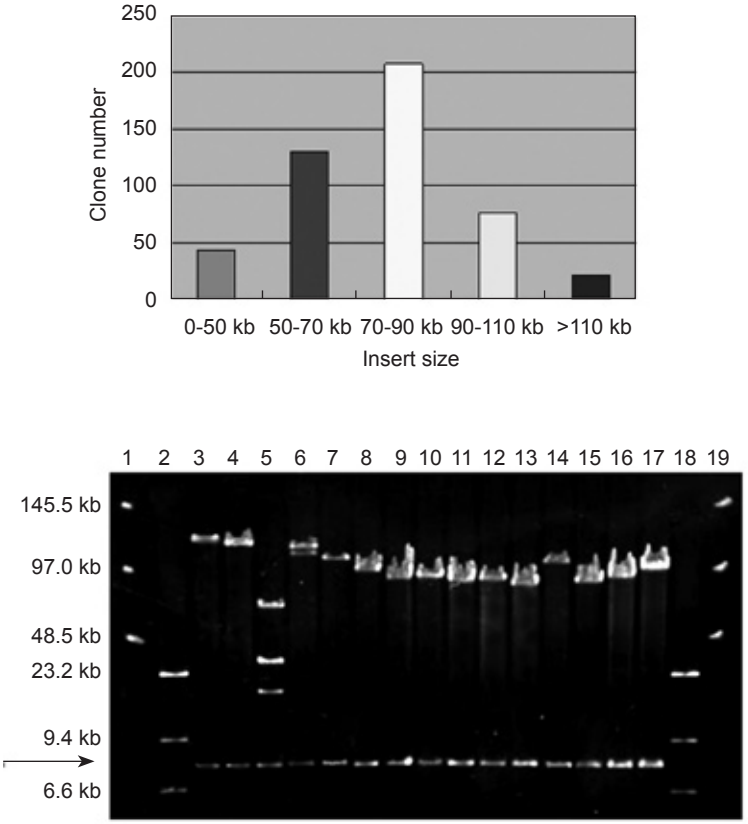

Fig. 1. Characterization of the Indian muntjac bacterial artificial chromosome (BAC) library. (A) Distribution of insert size in the Indian muntjac BAC library. Insert sizes of 545 BAC clones were determined by pulsed-field gel electrophoresis (PFGE) after *Not*I digestion. The horizontal axis refers to the size ranges in kilobases (kb), while the vertical axis indicates the number of clones. (B) Typical examples of *Not*I-restricted BAC clones after the PFGE analysis (lanes 3 to 17). Lanes 1 and 19 denote a PFGE standard DNA marker; lanes 2 and 18 represent the λ-*Hin*dIII marker; each row of vector bands was 7.5 kb (denoted by the arrow).

DISCUSSION

A BAC library with a total of 126,336 BAC clones was constructed for the Indian muntjac. The average size of the BAC clones was 80 kb, and thus the library corresponds to approximately

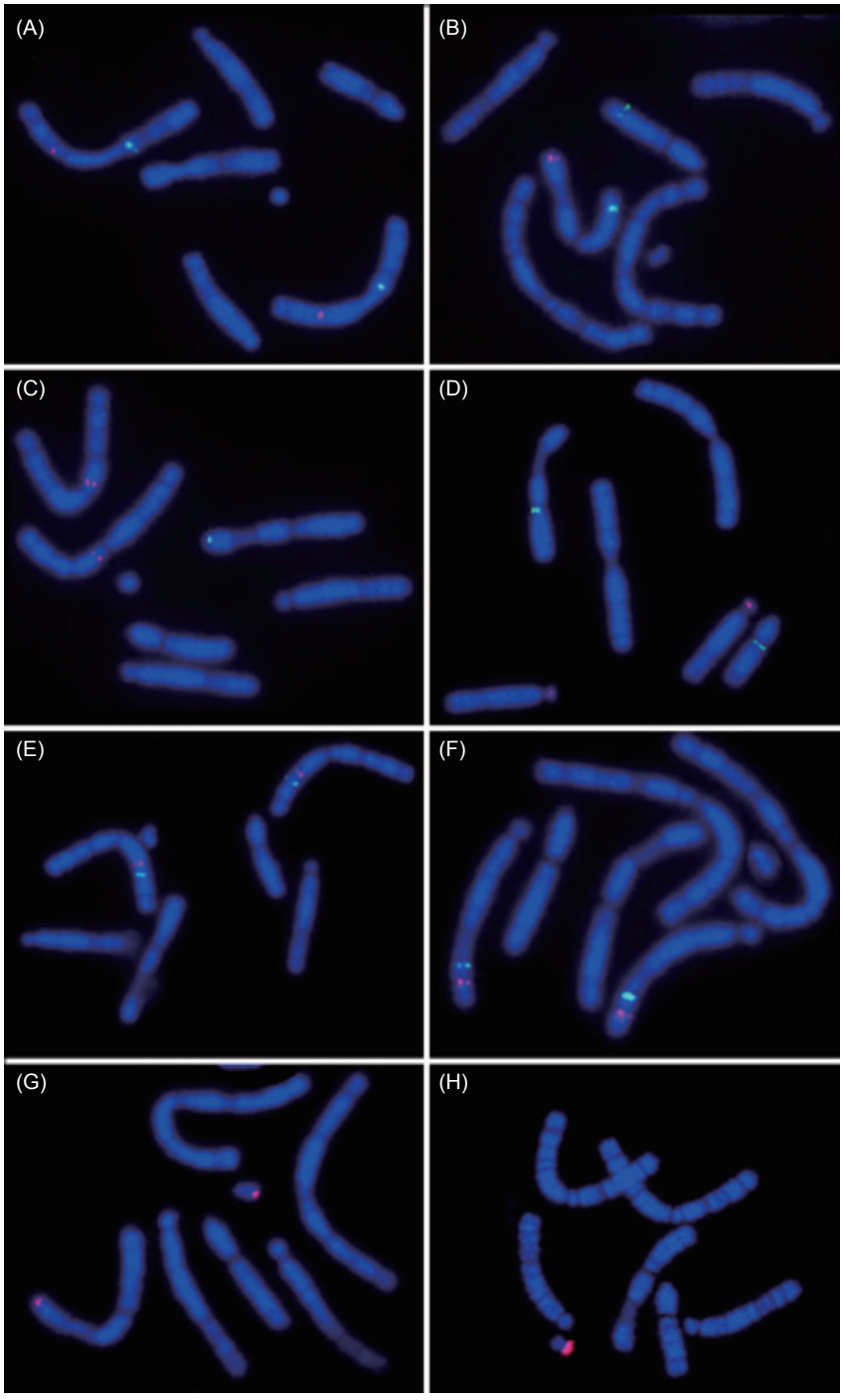

Fig. 2. Bacterial artificial chromosome (BAC) clones mapped onto specific chromosomal sites of the Indian muntjac by fluorescent *in situ* hybridization (FISH). (A) Clone 119A1 was mapped onto 1q23 (green) and clone 122A1 onto 1p14 (red). (B) Clone 121A1 was mapped onto Xp15 (red) and 124A1 onto 3q41 (green). (C) Clone 41E6 was mapped onto 1p12 (red) and 50A1 onto Xp15 (green). (D) Clone 3E6 was mapped onto 2p11 (red) and 4E6 onto 3q31 (green). (E) Clone 127E6 was mapped onto 1q21 (red) and 130E6 onto 1q25 (green). (F) Clone 15A1 was mapped onto 2q37 (red) and 16A1 onto 2q35 (green). (G) Clone 1121H12 was mapped onto the pseudo-autosomal region of the X and Y chromosomes (red). (H) Clone 497H12 was mapped onto the Y chromosome (red).

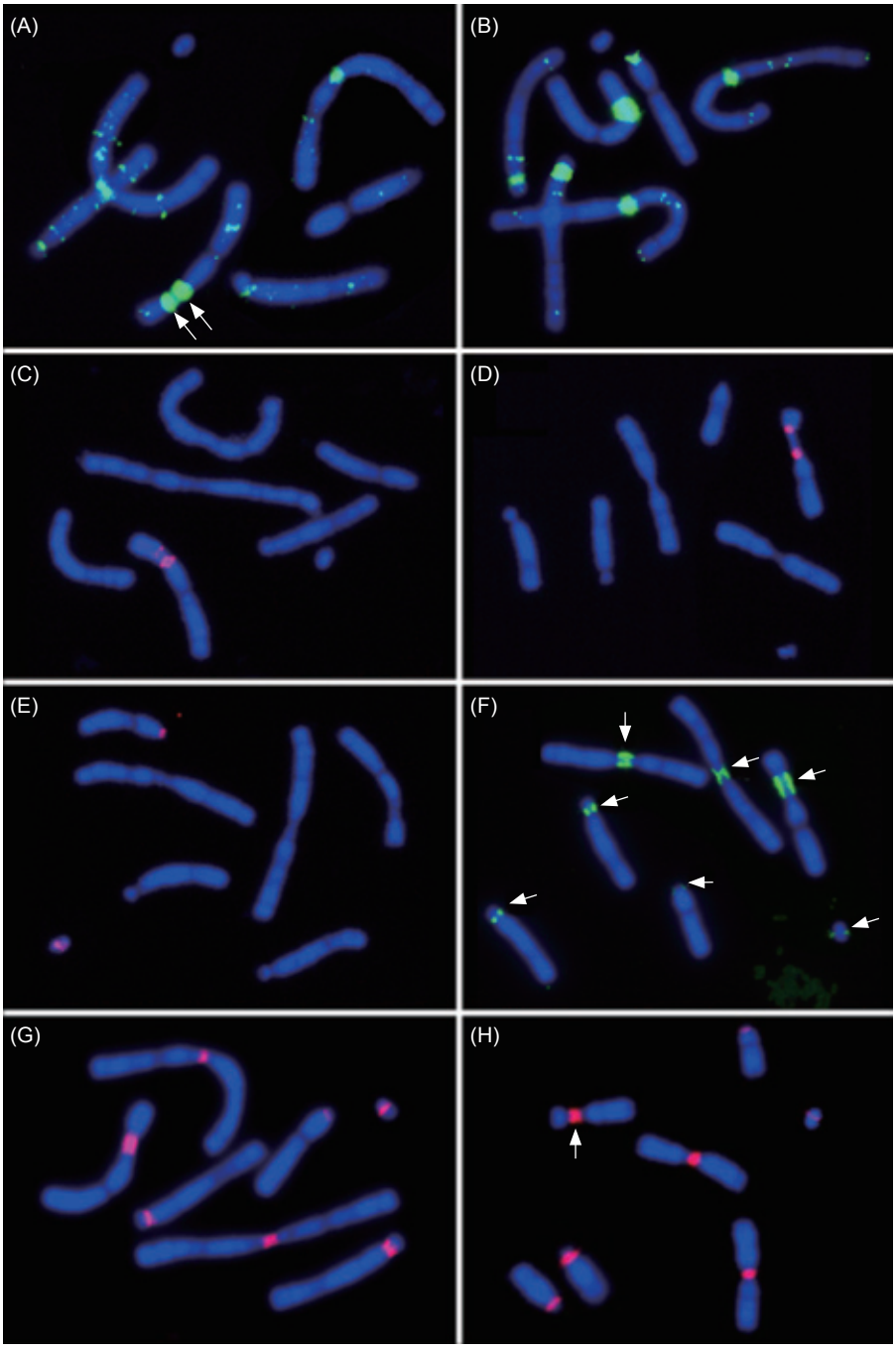

Fig. 3. Bacterial artificial chromosome (BAC) clones mapped onto the centromeric region of the Indian muntjac chromosome by fluorescent *in situ* hybridization (FISH). (A) Clone 1128A1 (green) was mapped onto the centromeric and interstitial regions of the chromosomes. Two blocks of strong fluorescent signals were observed on the pericentromeric regions of the X+3 chromosome (denoted by arrow). (B) Clone 1158A1 (green) showed a signal pattern similar to that of the Mmv-0.7 probe (cervid satellite II) which had centromeric and interstitial signals. (C) and (D) Clones 1189E6 and 1179H12 (both with red signals) were only mapped onto the 2 distal sites of the compound centromere of X+3. (E) Clone 1189A1 (red) was located on the Yq and centromeric region of chromosome 3. (F) Clone 1296A1 (green) was mapped onto both lateral sites of the primary constriction where the kinetochore is located (denoted by arrows). (G) Clone 1241H12 (red) occupied the entire centromeric heterochromatin region of every chromosome in the complement. (H) Clone 747A1 (red) was located in the middle region of the centromere in every chromosome (e.g., X+3 chromosome; denoted by the arrow).

4x coverage of the muntjac genome. If our library is added to a recently constructed Indian muntjac BAC library with 6 genomic equivalents (Chi et al. 2005), the total coverage of the Indian muntjac genome represented by this BAC library is around 10. The larger coverage of genome libraries means fewer gaps in genomic sequence, which will be helpful in completing sequencing of the entire genomic of this species.

Huang et al. (2006) mapped 304 Indian muntjac BAC clones mainly onto the chromosomes of the Chinese muntjac (*M. reevesi*) not onto those of the Indian muntjac. In this study, we provide a high-density FISH map with a total of 1619 clones assigned to specific loci of the Indian muntjac metaphase chromosome by FISH. Among those mapped clones, 1517 were located onto specific loci of the chromosome arms, whereas 102 clones were mapped onto centromeric regions and interstitial sites. The chromosome arm-specific BAC clones can be used for comparative chromosome mapping studies. BAC clones mapped onto interstitial sites (the ancestral chromosomal break and fusion sites) would be most useful for investigating fusion mechanisms during the restructuring of the Indian muntjac karyotype. BAC clones located on the centromeric region also revealed various specific FISH signal patterns. This finding suggests that the genomic organization of the centromere is more complex in this species.

Although the relationship of homologous synteny between the Indian muntjac and other species (e.g., the human, cattle, pig, cat, horse, and seal) was identified by cross-specific chromosome painting (Burkin et al. 1997, Frönicke and Scherthan 1997, Chowdhary et al. 1998), the densely ordered Indian muntjac BAC clones obtained in this study can be used as valuable FISH probes for more precisely delineating the conserved regions of chromosomes between the Indian muntjac and other species. Additionally, comparative mapping studies among phylogenetically related deer species can also be performed using these mapped BAC clones to address questions related to mammalian chromosome evolution.

Chi et al. (2005) and Huang et al. (2006) confirmed that the orientation of tandem fusions occurred as centromere-telomere (head-tail) fusion during the evolution of the Indian muntjac and black muntjac karyotypes. However, the molecular mechanism of these fusions still remains to be determined. The existence of specific DNA motifs at the pericentric heterochromatic regions and subtelomeric regions of the ancestral chromosomes could infer the molecular mechanism of such fusion events. Therefore, the cloning and sequencing of the fusion points would shed new light on the molecular forces that have driven such an exclusive centromere-telomere (head-tail) tandem fusion in the Indian muntjac. A number of the BAC clones mapped in this study showing interstitial signals should be good sources for investigating the mechanism of tandem fusion.

Four BAC clones with unique FISH signals appeared on both lateral sites of the centromeres where the kinetochores are located. The unique location of these BAC clones may indicate that these clones contain specific DNA sequences that are associated with centromeric binding proteins (CENPs). Moreover, these specific DNA sequences might participate in kinetochore assembly of active centromeres. Other centromeric BAC clones produced various unique FISH patterns on the centromeric regions, also suggesting that the genomic organization of the centromere is more complex. These BAC clones are valuable resources for studying the structural organization and function of centromeric DNA. Therefore, further investigation of those centromeric BAC clones should shed more light on the genomic organization, structure, and function of mammalian centromeres. Comparative centromere sequence analysis will provide better insights into the evolution of centromeric DNA that led to the formation of the currently active centromere. This in turn may become an important resource for constructing an artificial mammalian chromosome with the potential for gene therapy.

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