BAC Libraries from Closely Related Species: Studying Chromosome Biology

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Importance: 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 shed light on the temporal relationships of extant species, with each species chosen to represent a branch and to serve as an anchor for framing evolutionary hypotheses. Karyotypic differences between distantly related species indicate the dynamic nature of the genome, evolving through rearrangement, invasion, duplication, and loss (Wienberg and Stanyon 1997; Koonin 2000; Eichler 2001; Murphy 2001; Ostertag and Kazazian 2001; Pevzner and Tesler 2003). It is tantalizing to hypothesize that such changes result in reproductively incompatible genomes and to speciation events (Searle 1998; Rieseberg 2001). Indeed, there is evidence from several studies that karyotypes of even very closely related species can vary in fundamental ways (Liming 1980; Garagna 1997; Nickerson and Nelson 1998; Carbone 2002; Coghlan and Wolfe 2002; de Pontbriand 2002; Samonte and Eichler 2002).

We propose the construction of BAC libraries for the black-and-white colobus monkey, the black lemur, the tarsier, and two species of muntjac deer. Libraries for these species were chosen for their potential to inform specific questions pertaining to centromeric and pericentromeric evolution, primate genome evolution, and the mechanisms and consequences of karyotypic change between closely related species. Such studies are best served by creation of BAC libraries that sample more deeply within individual branches of the evolutionary tree. The primary uses of these libraries will be comparative sequence analysis and cytogenetic mapping of genome rearrangement, expansion, and loss, both to delineate the full complement of these changes and the mechanisms by which they occur. The molecular analyses of such changes should shed light on the mechanisms mediating evolutionary events that separate species and on the events leading to chromosome rearrangement and segmental loss or duplication within a species.

Usage:

1. Centromeric regions of eukaryotic chromosomes present an enigmatic example of conserved function in the face of rapidly evolving DNA sequence (Sullivan 2001). The centromere is required in cis for the proper segregation of chromosomes at both mitosis and meiosis. This function is mediated by the kinetochore, a proteineceous structure that assembles onto centromeric DNA (Sullivan 2001). While the proteins of the kinetochore have largely been conserved through evolution, the DNA that comprises the site of kinetochore formation— the centromere— has not (Sullivan 2001). Each species studied to

date, from yeast to worms to flies to humans, apparently uses a different DNA sequence to accomplish centromere function. Recent mapping and sequencing of the pericentromeric region of the human X chromosome has revealed the sequences capable of acting as the functional centromere (Schueler 2001), allowing the X chromosome to emerge as a model for the study of centromere evolution. The primate species included in this proposal were chosen to complement existing primate BAC libraries (CHORI); these were identified in previous studies to reflect discrete evolutionary points in the evolution of the primate X centromere. Comparative sequence analysis of this centromere locus will provide rare insight into the evolutionary steps leading to the currently active human X centromere and to the currently active centromeres of several non-human primate species. This dense data set, consisting of multiple data points from a single lineage over a very brief evolutionary time, will be unprecedented in its potential to elucidate the mechanisms of centromere formation and maintenance.

2. 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 sequences will be evaluated for their role in centromere function using an artificial-chromosome assay, in which BACs are transfected into cultured cells and analyzed for their ability to form de novo centromeres (Harrington 1997; Ikeno 1998). The resulting mitotically stable microchromosomes have been used to evaluate the necessary elements of a functional human chromosome, with the hope that one day such vectors might prove useful for gene therapy (Calos 1996; Warburton 1999). The artificial-chromosome assay will be conducted using candidate non-human primate centromere sequences in both human and primate cell lines. 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.

3. Like the centromere locus, pericentromeric regions of primate chromosomes are quite dynamic. Bombarded by satellite DNA families (Lee 1997; Sun 1997; Copenhaver 1999), transposable elements (Korenberg and Rykowski 1988; Sun 1997; Copenhaver 1999), and paralogous duplications (Eichler 2001; Bailey 2002), these regions are highly repetitive and reveal great diversity even amongst closely related species (Jackson 1999; Horvath 2000; Samonte and Eichler 2002). Comparative sequence analysis of regions where there is variation in the presence or absence of segmental duplications can be used to determine the timing and molecular basis of these changes. In a similar fashion, analysis of the various satellite families populating pericentromeric regions of primate chromosomes can be used to investigate the relative impact of unequal crossover, sequence conversion, and transposition on the expansion and contraction of pericentromeric regions.

4. Currently, BAC libraries for 18 non-human primate species have been constructed or approved (Figure 1), sampling both semi-orders— Strepsirhini and Haplorhini. While this is a substantial collection, the three primate species requested here fill specific niches in that collection. While the close phylogenetic relationship of the proposed species might at first blush be considered a negative aspect of our proposal, we believe this is an asset. The chimpanzee sequencing effort has been undertaken specifically to address questions of human uniqueness (McConkey 2000; Varki 2000; Olson and Varki 2003) by comparing the highly similar genomes of human and chimpanzee. The mounting primate BAC resource (Eichler and DeJong 2002) will have an unprecedented impact on our interpretation of chimpanzee-human comparisons and will allow for other comparisons of the human genome and its nearest neighbors on the evolutionary tree.

5. BAC libraries from two species of muntjac deer are requested in this proposal. The Chinese muntjac and the Indian muntjac together provide an example of highly variant chromosomal constitution between closely related species (Figure 2) (Liming 1980). The six large chromosomes of the Indian muntjac mark the smallest diploid chromosome number of any mammalian species studied to date (Levy 1993). The Chinese muntjac, with a diploid chromosome number of 46 (Wurster and Benirschke 1967), is morphologically very similar to the Indian muntjac and, indeed, these two species can mate to produce viable (albeit sterile) offspring (Gray 1954). Genomic resources from both species are necessary to study the reductional events that culminated in the Indian muntjac genome (Wang and Lan 2000) and the barriers to reproduction that exist in the F1 hybrid. Comparative sequence analysis between these two species will be used to investigate the mechanisms, timing, and consequence of such global genomic upheaval. Specifically, this resource will be used to study centric fusion, centromere attrition or inactivation, telomere-mediated translocation, and other mechanisms of genome rearrangement.

Research Community: The resources proposed are relevant to a broad range of studies in addition to those discussed in this white paper. Several researchers have been contacted for their input and interest in the requested species. Specifically, Evan Eichler (Case Western Reserve University, mechanisms of recent primate genome evolution), Mariano Rocchi (University of Bari, comparative primate cytogenetics), Hunt Willard (University Hospitals Research Institute, centromere structure and function, X-inactivation), Caro-Beth Stewart (University at Albany, SUNY, primate molecular evolution), Barb Trask (Fred Hutchinson Cancer Research Center, genome organization and evolution), Anne Yoder (Yale University, prosimian evolution), Oliver Ryder (Center for Reproduction of Endangered Species, primate species conservation), Roscoe Stanyon (National Cancer Institute, primate genome evolution), Steve O'Brien (National Cancer Institute, comparative genomics and molecular phylogenetics), Pieter De Jong (Children's Hospital Oakland Research Institute), Morris Goodman (Wayne State University, primate genome evolution and molecular phylogenetics), and Eddy Rubin (Lawrence Berkeley National Laboratory, mouse genetics and comparative genomics)..
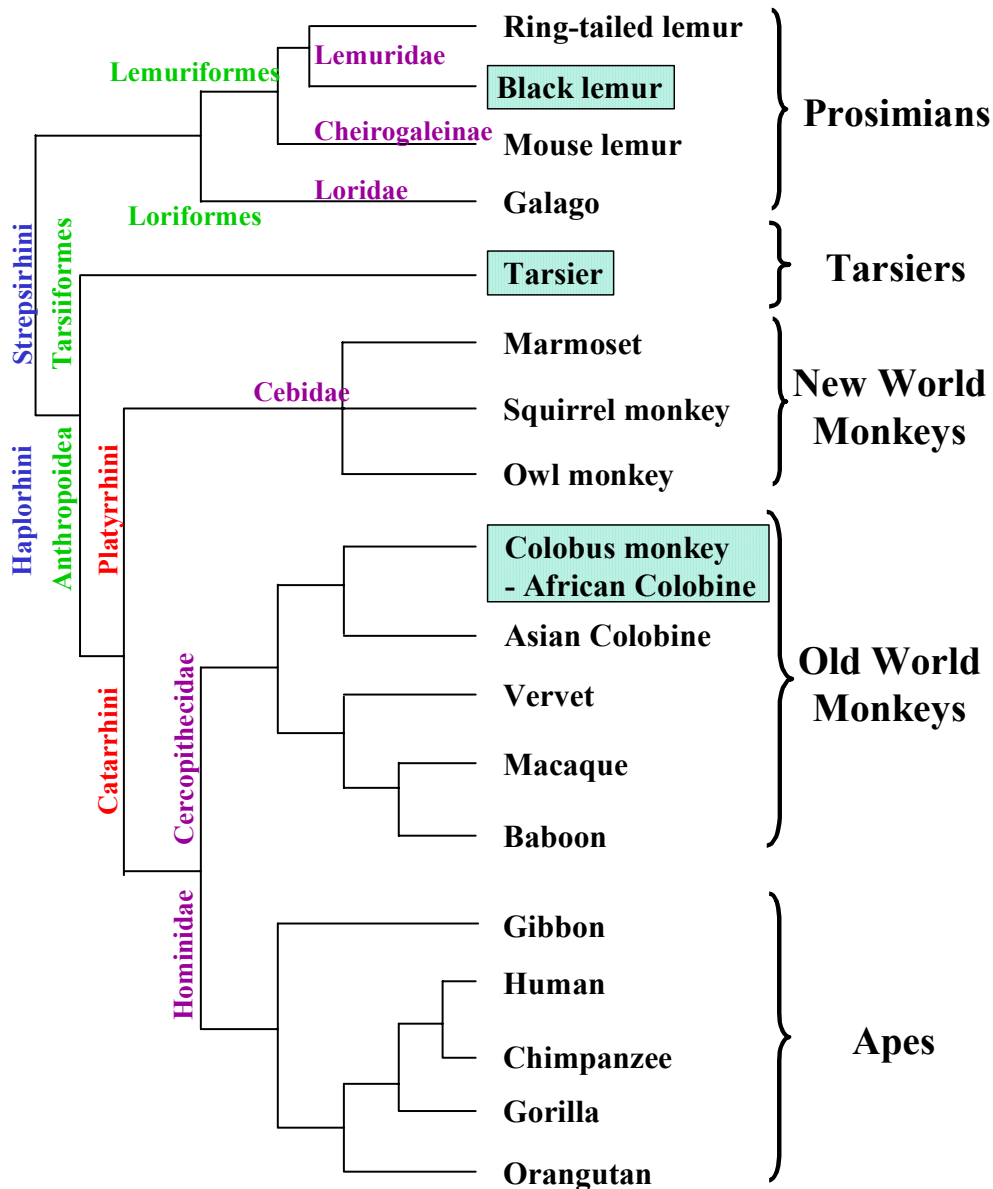
Figure 1: Status of primate BAC library resource. The species included on the tree have been approved (plain text) or proposed (green boxes) for BAC library construction. The tree is drawn to depict the relative position of each species in the primate tree to demonstrate the phylogenetic rationale for each. The two major primate semiorders, Strepsirhini and Haplorhini, are shown divided into suborders, infraorders and families. The tree is not intended to be a complete account of primate diversity. Branch lengths do not approximate divergence times
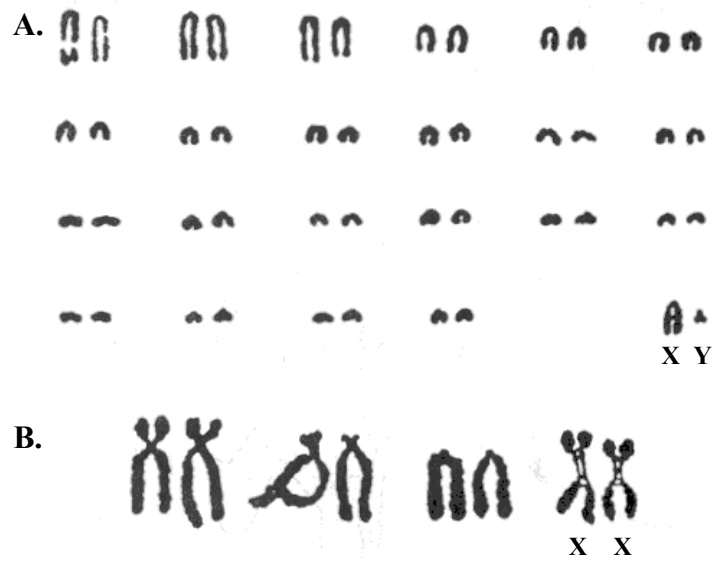
Figure 2: Muntjac deer karyotypes A. Diploid karyotype of a male Chinese muntjac (*Muntiacus reevesi reevesi*, 2n=46) **B.** Diploid karyotype of a female Indian muntjac deer (*Muntiacus muntjac vaginalis*, 2n=6).

Has the organism been previously proposed? To our knowledge, no other proposals are under review for any of the species requested in this proposal.

Are there other genomic resources that will complement the organisms proposed? Cell lines and/or frozen samples of *Colobus guereza* and *Eulemur macaco* exist at either the Frozen Zoo (San Diego) or at Duke University Primate Center. Cell lines for both muntjac species are held by various laboratories (Lee 1993; Yang 1997; Li 2000) and are being pursued by Oliver Ryder's laboratory for the individuals from whom the libraries will be made. Dr. Ryder will investigate immortalizing fibroblast cell lines from both *M. muntjac vaginalis* and *M. reevesi reevesi*. Generation of a tarsier cell line for DNA isolation for BAC construction and for future comparative cytogenetics is being pursued by the authors.

Species, genome size, and source:

Black lemur *(Eulemur macaco)*

Rationale: Insights about the earliest events in the evolution of the primate centromere have been revealed by comparative cytogenetic studies of the X chromosome from two different prosimians (Ventura 2001), that of the ring-tailed lemur (*Lemur catta*, 2n = 56, prosimian) and that of the black lemur (*Eulemur macaco*, 2n = 44, prosimian).

Strikingly, while both species' X chromosomes are completely co-linear with the human X chromosome, the position of the X chromosome centromere varies between the lemur genera and between each lemur and human (Ventura 2001). The most parsimonious explanation for this arrangement is the independent emergence of a centromere on each of these lemur X chromosomes (Ventura 2001; Wong and Choo 2001). Comparative sequence analysis and cytogenetic mapping of BACs from each of these lemurs would reveal the underlying molecular events that led to this fundamental difference. It is not known if the lemuridae centromere consists of alpha satellite DNA, as is the case for the remaining primates studied to date (Alexandrov 2001), nor is it known if both the ring-tailed lemur and the black lemur share the same type of centromeric DNA. Events leading to the active prosimian centromere may mark an alternative path to that taken by the anthropoid lineage. Alternatively, these events may be early steps in the cascade that resulted in the currently active human centromere. A Ring-tailed lemur library (5.8X coverage) is already in existence (CHORI).

Source: The Duke University Primate Center (DUPC) has agreed to provide the sample required for *Eulemur macaco*. DUPC has on hand the largest colony of prosimian species in the United States and supports its live colony assets with banked blood samples as well as frozen tissue and cadaver resources. The center director, Bill Hylander, approved our request and awaits approval of this white paper and contact from a library producer.

Black-and-white colobus monkey *(Colobus guereza)*

Rationale: Comparative cytogenetics of the pericentromeric region of the X chromosome has revealed that gamma satellite present on the human X chromosome is not present on the X chromosome of the black-and-white colobus monkey (*Colobus guereza*, 2n=44, old world monkey), but instead it has an autosomal location (Lee 1999). The same study indicates that this gamma satellite family has an X chromosome location in the great apes and in at least one other old world monkey (*Pygathrix bieti*). In lacking gamma satellite, *Colobus guereza* may represent the ancestral state of the X chromosome or may represent a lineage-specific deletion event. In either case, *Colobus guereza* holds a critical position in a comparative sequence analysis of the origin and maintenance of the gamma satellite family.

Biomedical rationale: The Tribe Colobini of the old world monkey family Cercopithecinae has two major groups, the African colobines and the Asian colobines (Figure 1). African colobines, such as *Colobus guereza*, have been shown to be asymptomatic despite infection by simian immune-deficiency virus (SIV) (Courgnaud 2001). SIV has become a model for studying the transmission and pathogenesis of human immune-deficiency virus (HIV). Dr. Bing Su (Cincinnati) has begun construction of an Asian colobine BAC library (Proboscis monkey). Together, the proboscis and colobus monkeys will serve as phylogenetic anchor species in the primate BAC library resource. Investigation of karyotypic or expression differences between these closely related monkeys may have great impact on our understanding of the pathogenesis of the SIV virus. The relevance of this to ongoing studies of HIV infection is not yet known, but will likely inform this major area of biomedical research.

Source: Multiple sources for a *Colobus guereza* sample have been identified. The Sunset Zoo (Manhattan, Kansas) shepherds the Species Survival Plan for the Black-and-white colobus monkey. The General Curator (Ryan Gulker), the Director of Conservation and Research (Dr. Robert Klemm), and the Chief Veterinary Advisor (Dr. Jim Carpenter) have each been consulted. Dr. Klemm has approved our request and awaits approval of this white paper and contact from a library producer. Pieter De Jong has indicated that a sample could be obtained from the San Francisco Zoo and Oliver Ryder has offered to provide a sample from the San Diego Zoo.

Tarsier

Rationale: The position of this group of nocturnal primates within the primate tree (Figure 1) has been long debated (Napier and Napier 1985; Martin 1993). Sharing morphological features with both prosimians and anthropoids (Fleagle 1999), tarsiiformes are possibly the most unique of the primates, and may represent an evolutionary intermediate bridging the gap between these major groups. Recently, comparative sequence analysis has been employed to place tarsiiformes within the Haplorhini semiorder as a sister group to the anthropoid lineage, splitting off over 55 mya before the radiation of the anthropoidea (Zietkiewicz 1999; Schmitz 2001; Schmitz 2002). Further molecular investigation into the evolution of this unique group is required to better understand the major events leading to the current structure of the primate tree. If, indeed, the tarsier is a more recent relative of the anthropoid lineage than are prosimians, then the tarsier is the proper outgroup for the molecular phylogenetics of the anthropoid lineage, the most recent member of which is Homo sapien. Its role as outgroup should not diminish the fascinating aspects of this primate's habits and morphology. A BAC library from this species will enable a better understanding of its genetics and hopefully facilitate conservation efforts for this threatened species.

Source: A source of *Tarsius bancanus*, *Tarsius syrichta* or *Tarsius spectrum* within the United States is currently being pursued. We have contacted Anne Yoder (Yale), DUPC (frozen samples only), the Cleveland Metroparks Zoo (who apparently have the only live Tarsier in the United States), Steve O'Brien (National Cancer Institute), Roscoe Stanyon (National Cancer Institute), Morris Goodman (Wayne State University), Pieter De Jong (CHORI), Jeanne Beck (Coriell) and Oliver Ryder (Center for Reproduction of Endangered Species). Despite surveying the prominent scientists in primate conservation and research, no tarsier colony or existing cell line has been identified within the United States. The only captive large colony of tarsiers apparently resides on a natural preserve in the Philippine Province of Bohol. If we are unable to locate a fresh blood or tissue sample, we will pursue construction of a cell line from frozen samples provided by DUPC. A cell line will provide a perpetual source of DNA and will serve future comparative cytogenetic analysis. We are requesting provisional approval for constructing a BAC library from a tarsier, such that BAC construction can proceed once a suitable source of DNA is obtained.

Indian muntjac deer *(Muntiacus muntjac vaginalis)*

Rationale: The Indian (red) muntjac deer (2n=6 female; 2n=7 male) has the lowest diploid chromosome number of any mammal studied to date (Wurster and Benirschke 1967). Moreover, the six very large chromosomes of this species were apparently generated by repeated tandem fusion of the chromosomes of a cervid ancestor (Yang 1997). These fusions are thought to have resulted from telomere-telomere, telomere-centromere, and non-Robertsonian centromere-centromere fusions (accompanied by loss of centromere function at the fusion site) (Liming 1980). Despite these radical changes, the DNA content of the Indian muntjac is highly similar to that of the Chinese muntjac, with the chromosomes of the latter serving as independent 'puzzle pieces' in the assembly of the chromosomes of the former (Liming 1980; Yang 1997). Localization of telomeric and centromeric sequences at the fusion points by cytogenetic mapping (Liming 1980; Lee 1993; Li 2000) has been used to predict the events leading to Indian muntjac chromosome organization; however, comparative molecular analyses are necessary to determine the mechanisms involved in the formation of these fusions.

Source: Pieter de Jong has received an Indian muntjac sample from Oliver Ryder at the San Diego Zoo. High-molecular-weight DNA has been prepared, and initial tests indicate that its quality is excellent and suitable for BAC construction. Upon approval of this white paper, the De Jong lab will proceed with library construction. The laboratory of Oliver Ryder is in the process of establishing fibroblast cell lines and is investigating immortalizing cell cultures.

Chinese muntjac deer *(Muntiacus reevesi reevesi)*

Rationale: The Chinese muntjac deer (2n=46) is commonly used in comparative cytogenetic studies of the highly active cervid genome (Liming 1980; Lee 1993; Yang 1997; Wang and Lan 2000). With a diploid chromosome number of forty-six, this species represents only one of several different cervid chromosomal constitutions. Variation of chromosome number within this lineage is unprecedented, including species with as few as six (see above) and as many as seventy chromosomes (Yang 1997). It has been demonstrated that the full compliment of acrocentric Chinese muntjac chromosomes can be assembled into the six large chromosomes of the Indian muntjac (Liming 1980; Yang 1997). A better understanding of the differences between the chromosomes of the Chinese and Indian muntjacs would provide an excellent foundation for the study of their reproductive isolation.

Source: Pieter de Jong is expecting a Chinese muntjac blood sample at any time from Oliver Ryder at the San Diego Zoo. Upon approval of this white paper, the De Jong lab will proceed with library construction. The laboratory of Oliver Ryder is in the process of establishing fibroblast cell lines and is investigating immortalizing cell cultures.

***Library specifications*:** Preparation of the libraries requested should proceed by standard protocols and with standard vectors (e.g., pBACe3.6 or pTARBAC2.1). Libraries should contain approximately 10X coverage of the genome to ensure the ability to construct highly redundant and accurate BAC contig maps. An average insert size greater than 150 kb is preferable.

Timeframe: Sample collection is likely to be the rate-limiting step in the preparation of these libraries. Blood samples from smaller primates must be collected in small quantities (~3 - 5 mls) over long periods of time (4 - 12 months) to reach the ~25 ml required for library construction. Alternatively, a tissue sample may become available in the normal course of colony maintenance, at which time immediate action is required on the part of the library producer. Approval of the primate libraries is of high priority so that a plan for such response can be set in place. Acquiring the muntjac samples seems to be less of a problem, with one of them now in hand and the other expected at any time.

Other support: To our knowledge, no other support has been applied for or secured for making BAC from any of the species requested in this proposal.

The need for additional libraries if others already exist: None of the libraries we propose would duplicate existing resources. The Black lemur would be the second genus representing the family lemuridae (Figure 1) and complements the existence of BAC libraries for Ring-tailed lemur (Lemuridae) and Mouse lemur (Cheirogaleinae). The Black lemur and Ring-tailed lemur are, indeed, closely related; however, they fall into two distinct clades by mitochondrial DNA sequence analyses (Pastorini 2002) and exhibit dramatic differences in the centromeric region of the X chromosome (Ventura 2001). These differences thus present a unique opportunity to study the emergence or establishment of centromere domains.

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