

## Review Article

# Sex Chromosome Evolution in Amniotes: Applications for Bacterial Artificial Chromosome Libraries

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Received 29 July 2010; Accepted 27 September 2010

Academic Editor: Hans H. Cheng

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Variability among sex chromosome pairs in amniotes denotes a dynamic history. Since amniotes diverged from a common ancestor, their sex chromosome pairs and, more broadly, sex-determining mechanisms have changed reversibly and frequently. These changes have been studied and characterized through the use of many tools and experimental approaches but perhaps most effectively through applications for bacterial artificial chromosome (BAC) libraries. Individual BAC clones carry 100–200 kb of sequence from one individual of a target species that can be isolated by screening, mapped onto karyotypes, and sequenced. With these techniques, researchers have identified differences and similarities in sex chromosome content and organization across amniotes and have addressed hypotheses regarding the frequency and direction of past changes. Here, we review studies of sex chromosome evolution in amniotes and the ways in which the field of research has been affected by the advent of BAC libraries.

## 1. Introduction

Studies of the evolutionary history and functional dynamics of amniote sex chromosomes have been enabled by bacterial artificial chromosome (BAC) libraries. Here, we review several aspects of amniote sex chromosome evolution as characterized by experimentation before and after the construction of amniote BAC libraries and we suggest that the rate of discovery and reach of comparisons have been increased by BAC resources, especially for birds and reptiles in which variability of sex chromosome organization offers great opportunities for evolutionary research [1]. In addition to describing BAC-enabled research published within the last decade, we also describe experiments that will allow researchers to use BAC libraries to describe rates of evolution, dynamics of intrachromosomal variations, and frequency of independent origins of sex chromosomes.

A genomic BAC library consists of many DNA fragments representing the whole genome of an individual. Each BAC clone contains 100–200 kb of contiguous genomic sequences, providing probes for *in situ* hybridizations on chromosomes

and for determining long-range organization of genes. The number of BAC clones in a library varies depending on the genome size of the species as well as the depth of the library representing the whole genome. For the construction of a BAC library, DNA is isolated and fragmented by hydroshearing, nebulization, or partial restriction digestion followed by preparative pulsed field gel fractionation. Fragments are size selected, ligated into a specialized vector, and maintained in *Escherichia coli* that are archived in 384-well plates at  $-80^{\circ}\text{C}$ . Colonies representing the library are gridded onto high-density nylon filters, and respective clone DNAs are anchored to filters by cross-linking [2, 3]. High-density nylon filters serve as maps to BAC libraries. Radio-labeled probes can be hybridized to filters to target clones bearing sequences of interest (Figure 1). Alternatively, “smart” pooling strategies can be employed to screen BAC libraries via polymerase chain reaction [4].

Several BAC libraries and associated resources are available through the Children’s Hospital Oakland Research Institute (CHORI), the Arizona Genomics Institute, the Clemson University Genomics Institute, Amplicon Express,

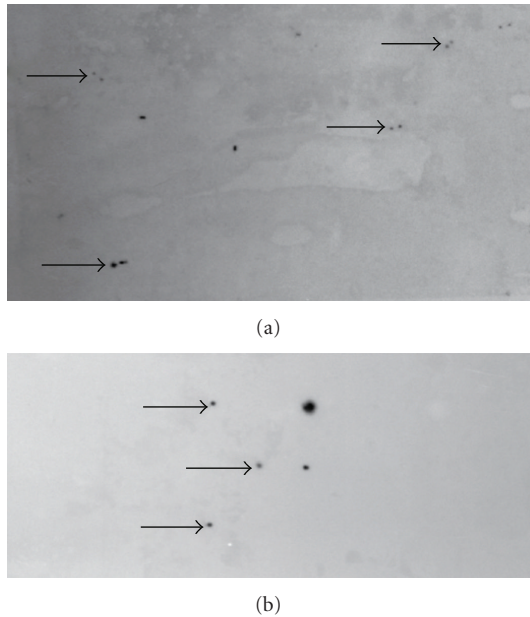


FIGURE 1: (a)  $^{32}\text{P}$ -labeled probes hybridized to sex-differentiating genes on filters representing the BAC library for painted turtle (*Chrysemys picta*). Two-dot signals permit the gridding of multiple 384-well plates into the same space on the filters. The two-dot pattern permits the gridding of up to 72 384-well plates on one nylon filter. Arrows indicate successful hybridization of one of several probes applied concurrently to filters. (b) Clones identified as putative positives from hybridization with a probe for *Dmrt1* regridded in duplicate onto a separate filter to be hybridized with a single probe as opposed to the multiprobe approach taken to initially identify positives. The secondary hybridization permits the identification of the probe that hybridizes to each positive. Arrows indicate three hybridizations that occurred on each of two identical arrays representing only positives collected from the initial large-scale hybridization. These arrays are printed side by side on the filter. Other hybridizations that were successful in only one array were rescreened to insure replicability.

Empire Genomics, and other vendors. Typically, whole plates, individual clones, or high-density nylon filters are available for purchase. The past decade has been a period of great discovery regarding amniote sex chromosomes [1, 6–9], and the next ten years should be even more productive as more laboratories incorporate BAC technology.

## 2. A Primer on 20th Century Sex Chromosome Research

The study of sex chromosome evolution within and beyond Amniota has a rich history that extends beyond the advent of BAC technology. Sex-specific functions of a chromosome pair in sex determination were first reported by McClung more than a century ago [10]. Since then, a series of quantitative and cytogenetic studies have generated hypotheses that still serve as pillars of contemporary experimentation [11–13]. Muller expanded on McClung's observation by suggesting that sex chromosomes evolved from an ancestral

autosome pair, leading to the degeneration of the Y chromosome but not the X [14]. Haldane posited that inheritance of the degenerated sex chromosome could explain sex-specific inviability in hybrid offspring. In male heterogametic species, in which males carry X and Y sex chromosomes and females carry two X sex chromosomes, Haldane predicted male inviability whereas in female heterogametic species, in which females carry Z and W sex chromosomes and males carry two Z sex chromosomes, sex-specific inviability should affect female hybrid offspring [13]. Dobzhansky reported patterns of inheritance for sterility factors found on the X chromosome in a fly (*Drosophila pseudoobscura*), further suggesting that sex chromosomes are key to offspring viability but noted that such factors were also found on other chromosomes and the greater number of factors on the X could be attributed to differences in chromosome length rather than sex-specificity of chromosomes [15]. Three decades later, Ohno argued for a rate of sex chromosome degeneration that matches divergence times, presenting karyotypic data from snakes as evidence. W chromosomes appear to be less degenerated in characteristically ancient snakes such as colubrids that diverged from a common ancestor earlier than characteristically derived snakes like viperids that exhibit extremely degenerated W chromosomes [12]. Contemporary to Ohno's hypothesis, cytogenetic surveys rapidly improved in reporting karyotypes of many species, including amniotes [16, 17]. In 1990, *Sry*, the master gene for human sex determination was identified and localized to the short (p) arm of the Y chromosome [18]. These and many other characterizations of the evolution and functional dynamics of sex chromosomes were enabled throughout the 20th century by integrating mating arrays, tissue culture, and chromosome staining, but molecular tools including fosmids, cosmids, and BAC libraries increased the pace and breadth of discovery.

## 3. BAC-Enabled Contemporary Research on Amniote Sex Chromosomes

Sex chromosomes house the sex-determining genes responsible for activating the developmental cascade that directs embryonic development to a male or a female fate [19]. The techniques needed to detect sex chromosomes depend in part on the evolutionary history of the sex chromosome pair. Sex chromosomes originate when a sex-determining mutation arises in a pair of autosomes [12, 20]. This initial step is followed by the accumulation of additional mutations conferring some sex-specific advantage and by decreased recombination, sometimes involving chromosomal inversions or rearrangements [11, 12, 21]. This process may lead to the formation of two morphologically distinct sex chromosomes, exhibiting different patterns of heterochromatin accumulation and deletions, and to the degeneration of the nonrecombining heterogametic sex chromosome (Y or W) due to its higher mutation accumulation rate and ineffective selection [12, 22, 23]. In such cases, the detection of this heteromorphic pair of sex chromosomes can be carried out using classical cytogenetic techniques. For instance, a simple

Giemsa-stained karyotype will reveal sex chromosome heteromorphisms due to differences in size while banding techniques (e.g., G-, C-, replication banding, DAPI banding, etc.) will reveal heteromorphic heterochromatin patterns.

However, sex chromosomes need not be grossly heteromorphic in size or banding pattern as the degeneration of the Y or W sex chromosome is not ubiquitous (e.g., [24] reviewed in [19]). This may be the case for sex chromosome systems at their early stages of evolution [11, 23, 25–28] or at an evolutionary stable state in some ancient sex chromosome systems (e.g., [29–31]; reviewed in [19]) Homomorphic sex chromosomes may therefore be cryptic and their detection may require molecular cytogenetic techniques such as comparative genome hybridization methods (CGH; [32]) that can reveal more subtle differences in DNA content. Recent examples of cryptic sex chromosomes in amniotes revealed by CGH include microchromosome systems in the dragon lizard (*Pogona vitticeps*) [33], the Chinese soft-shelled turtle (*Pelodiscus sinensis*) [34], and the Australian snake-necked turtle (*Chelodina longicollis*) [35], as well as a macrochromosome system in the Macquarie turtle (*Emydura macquarii*) [36]. Using CGH, differences in DNA content between the sexes are revealed by the differential hybridization pattern on chromosomal spreads of male and female genomic DNA previously labeled with two different fluorochromes.

In the past ten years, mapping of sex chromosomes has continued apace of overall genome research, enabled, in part, by fluorescent *in situ* hybridization (FISH) of cDNAs, cosmids, and BACs. BAC-FISH uses DNA probes derived from BAC libraries for the detection of sequences in target chromosomes [37, 38]. This technique can be used to determine the chromosomal location of specific genes (gene mapping) and to detect rearranged regions in chromosomes (breakpoints) [39, 40] where genes have changed order or location. Thus, when applied in a phylogenetic and comparative framework, BAC-FISH can be used to test hypotheses about the evolutionary history of chromosomes [39]. Recent studies in this area exemplify the utility of applying this technique to our understanding of sex chromosome evolution in amniotes [5, 41–45]. For example, gDNA-FISH permitted the localization of *Dmrt1* to the Z but not the W chromosome in emu (*Dromaius novaehollandiae*) [46]. In emu, *Dmrt1* is the only known marker identified to date that is solely Z linked. Beyond *Dmrt1*, emu sex chromosomes appear to consist entirely of the shared pseudoautosomal region (PAR) [47]. The singular Z-linkage of *Dmrt1* in emu lends additional support to the report that *Dmrt1* is the sex-determining gene in birds, akin to *Sry* in most mammals [48]. Janes et al. [47] mapped random and targeted BACs to either autosomes or the PAR of sex chromosomes in emu. End-sequences of mapped BACs demonstrated higher rates of recombination in the PAR than on autosomes, despite equivalent population sizes of pseudoautosomal and autosomal loci in wild emu populations, assuming balanced sex ratios. Also, cDNA clones have been mapped to sex chromosomes of the Japanese four-striped rat snake (*Elaphe quadrivirgata*), Burmese python (*Python molurus bivittatus*), the habu (*Trimeresurus flavoviridis*), a species of gecko

(*Gekko hokouensis*) [49], and the Chinese soft-shelled turtle (*Pelodiscus sinensis*) [50], demonstrating a lack of homology of Z and W chromosomes among snakes, birds, and turtles, indicating independent origins of sex chromosomes in these clades [51]. However, homology between chicken and a gecko over six markers suggests the possibility of shared ancestry.

Bacterial artificial chromosomes are also frequently used as fingerprint maps to assign sequences to chromosomes and organize linkage groups for the improvement of genome assemblies [52]. BACs are particularly useful for sequencing fractions of genomes, as in the assembly of the male-specific region of the Y chromosomes in human (*Homo sapiens*) and chimpanzee (*Pan troglodytes*) [53]. Comparative physical mapping of sex chromosomes has employed BACs for species including but not limited to cow (*Bos taurus*) [54], domestic cat (*Felis catus*) [55], black muntjac (*Muntiacus crinifrons*) [56, 57], elephant (*Loxodonta africana*) [58], horse (*Equus caballus*) [59], South American opossum (*Monodelphis domestica*) [60, 61], tammar wallaby (*Macropus eugenii*) [60, 62, 63], short-beaked echidna (*Tachyglossus aculeatus*) [64, 65], platypus (*Ornithorhynchus anatinus*) [63–68], and chicken (*Gallus gallus*) [69–71]. Mapping of degenerated sex chromosomes, in particular, is aided by BACs because of the low gene and high repeat content that are typical of degenerated sex chromosomes. These characteristics can complicate shotgun sequencing and linkage map analyses.

Contemporary research on the evolutionary history of amniote sex chromosomes is concerned with the causes and frequency of origination of sex chromosomes from ancestral autosomes and the implications of subsequent chromosomal degeneration [72]. Comparison of sex chromosomes within and among species has benefited enormously from the availability of BAC libraries, because they provide researchers the opportunity to find the genomic position of long DNA sequences that contain a targeted marker of phenotypic interest (such as a disease-causing locus) or represent a landmark genomic location for comparative purposes. Once a BAC clone that represents a marker or map location has been identified, then cross-species FISH allows researchers to reconstruct evolutionary history. For example, BAC clones from the library for the Australian dragon lizard that were known to contain genes that mapped to sex chromosomes of either snake or chicken were FISH mapped to dragon lizard chromosome spreads, demonstrating a lack of homology of either snake or chicken sex-linked genes to sequence on the sex chromosomes of dragon lizards [5]. Interestingly, while chicken sex chromosomes are homologous to chromosomes 2 in both snakes and dragon lizards, snake sex chromosomes were found to be homologous to chromosome 6 in dragon lizards, suggesting independent evolution of ZW sex chromosomes in squamate reptiles (Figure 2; [5, 51]).

#### 4. Future Directions: Potential Applications of BAC Libraries

Several strategies can be used to identify sex-linked markers in species with heteromorphic sex chromosomes. For

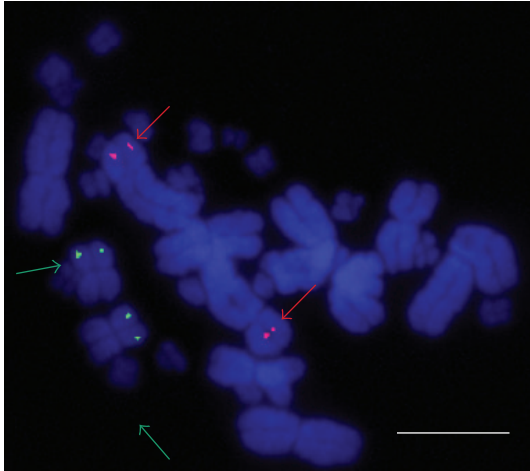


FIGURE 2: An example of two-color BAC-FISH showing hybridizations of BAC clones containing sex-linked genes from snake and chicken in a metaphase chromosome spread from an Australian bearded dragon (*Pogona vitticeps*). Red arrows indicate hybridization signals of a BAC clone containing chicken sex-linked gene CHD1 (red signals) onto the short arms of chromosomes 2 in *P. vitticeps*; green arrows indicate hybridization signals of a BAC clone containing snake sex-linked gene KLF6 onto the short arms of chromosomes 6 in *P. vitticeps*, thus showing nonhomology of ZZ/ZW sex chromosomes in reptiles [5]. Scale bar indicates 10  $\mu\text{m}$ .

organisms with a sequenced genome, targeted BAC library screening can be performed to generate sex-chromosome-specific probes [73]. For nonmodel organisms lacking a sequenced genome, several approaches are possible. First, a large number of random BAC clones could be FISH mapped onto chromosomal spreads of the target species to determine which clones map to the sex chromosomes. The total number of hybridized BAC clones depends on the coverage of the BAC library and the diploid number of target species, and it should be in large enough numbers to ensure that at least a few clones hybridize to the sex chromosome pair. While this is a time-consuming and probabilistic approach, it may be useful for some target nonmodel species. A second approach involves the capture of the target sex chromosomes by microdissection or flow sorting, which can then be amplified to generate probes for BAC library screening (e.g., [62, 74]). For nonmodel organisms that also lack a BAC library, a BAC library from another closely related species could be used for screening. An additional approach to detect sex-linked genes in a nonmodel taxon is to screen for BAC clones containing genes known to be linked to sex chromosomes in other amniotes. Sex-linked BAC-clone probes generated by any of these approaches can then be employed for physical mapping in candidate species as described above to identify their location and synteny. Such mapping data can then be used in subsequent construction of a comparative genomic map among amniotes to test evolutionary hypotheses on the origin and evolution of sex chromosomes.

## Acknowledgments

The authors thank Joel Sweetser, Samuel Pokross, Chad Kohler, and Katherine Falb for their thoughtful reviews of this manuscript. Funding for this paper and for continued experimentation to characterize sex chromosome evolution in turtles through the use of BAC libraries was provided by NSF Grant # MCB0817687 to N. Valenzuela and S. V. Edwards. D. E. Janes was also supported by NIH NRSA Grant # 1F32GM072494. The construction of BAC libraries for several amniote species was supported by the NSF Grant no. 0431717 to S. V. Edwards, C. Amemiya, and J. Robert Macey. T. Ezaz was partially supported by the Discovery Project Grant #DP0881196 awarded by the Australian Research Council to Stephen Sarre, Arthur Georges, and S. V. Edwards. Also, they thank Dr. Engy Edward for inviting this paper as a contribution to the Journal of Biomedicine and Biotechnology.

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