Construction of a river buffalo *(Bubalus bubalis)* whole-genome radiation hybrid panel and preliminary RH mapping of chromosomes 3 and 10

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ABSTRACT: The buffalo (*Bubalus bubalis*) not only is a useful source of milk, it also provides meat and works as a natural source of labor and biogas. To establish a project for buffalo genome mapping a 5,000-rad whole genome radiation hybrid panel was constructed for river buffalo and used to build preliminary RH maps from two chromosomes (BBU 3 and BBU10). The preliminary maps contain 66 markers, including coding genes, cattle ESTs and microsatellite loci. The RH maps presented here are the starting point for mapping additional loci, in particular, genes and expressed sequence tags that will allow detailed comparative maps between buffalo, cattle and other species to be constructed. A large quantity of DNA has been prepared from the cell lines forming the RH panel reported here and will be made publicly available to the international community both for the study of chromosome evolution and for the improvement of traits important to the role of buffalo in animal agriculture.

Keywords: River buffalo, Whole genome, Radiation hybrid panel, Gene mapping.

INTRODUCTION - Among all the domestic animals, the buffalo holds the greatest promise and potential for production. The extensive use of buffalo in agriculture worldwide, and especially in developing countries, begs for genetic resources to evaluate and improve traits important to local and regional economies. Very few resources exist to study genomics of buffalo, besides rudimentary somatic cell maps (De Hondt *et al*.1997; El Nahas *et al*. 1999) and cytogenetic maps (Iannuzzi *et al*. 2003). Beyond its application to agriculture, a high resolution genome map of buffalo will be an important tool for evaluating chromosomal evolution among species of Bovidae which are separated by only a few million years (Ritz *et al*. 2000). Buffalo are classified as river type and swamp type. The latter are more suited to muddling terrains and are predominant in South-East Asia. River buffalo have high lactation yields

and are more suited to plowing and drafting on dry plane land, commonly found in India, Mediterranean region of Europe, South America and the Caribbean, mainly for milk and meat purposes. Two reports regarding river buffalo genome mapping (Iannuzzi *et al*. 2003; Di Meo *et al*. 2006) describe a total of 302 loci (180 of type I and 122 of type II) physically assigned to its genome. Of the 302 loci, 256 were mapped by in situ hybridization (254 by FISH), 15 by both FISH and somatic cell hybrid analysis and 33 by using only somatic cell hybrid analysis.

Currently, radiation hybrid (RH) mapping is the method of choice for producing high resolution maps (zebrafish, Geisler *et al*. 1999; mouse, Van Etten *et al*. 1999; dog, Vignaux *et al*. 1999; rat, McCarthy *et al*. 2000; horse, Kiguwa *et al*. 2000; Chowdhary *et al*. 2002; deer mouse, Ramsdell *et al*. 2006; human, Gyapay *et al*. 1996), which can then be used for integrating linkage and physical maps within a species (Quackenbush *et al*. 2001). RH mapping, like linkage mapping, shows an estimated distance between markers. Rather than relying on natural recombination to separate two markers, RH mapping uses breaks induced by radiation to determine the distance between markers. RH mapping provides a way to localize almost any marker, as well as other genomic fragments, to a defined map position and is extremely useful for ordering markers in regions where highly polymorphic genetic markers are rare. By mapping expressed sequence tags (ESTs) that are common across species, a radiation hybrid map can also be used to make comparative maps useful for identifying "positional candidate" genes putatively controlling traits that have been genetically mapped to particular chromosomal regions.

Four RH panels have been reported for cattle: two panels (5,000 and 12,000-rad) have been constructed by Womack and colleagues (Womack *et al*. 1997; Rexroad III *et al*. 2000, respectively), a 3000-rad panel (Williams *et al*. 2002), which is publicly available for purchase, and a 7000-rad radiation hybrid panel constructed by Itoh and colleagues (Itoh *et al*. 2005). The bovine 5,000-rad panel has been used in conjunction with COMPASS (Comparative Mapping by Annotation and Sequence Similarity) to develop three increasingly dense cattle-human whole genome comparative maps (Band *et al*. 2000; Everts-van der Wind *et al*. 2004, 2005).

Cattle and buffalo have large regions of chromosomes conserved with extended chromosome segments containing the same complement of genes (Hayes 1995; Solinas-Toldo *et al*.1995; Chowdary *et al*. 1996, Iannuzzi *et al*. 2003). The karyotypes of buffalo and domestic cattle appear very similar at the level of chromosome arms. Buffalo, *Bubalus bubalis* (BBU) chromosome 1 appears to be a fusion of *Bos Taurus* (BTA) chromosome 1 and 27, BBU 2 equals BTA2 and 23, BBU3 equals BTA8 and 19, BBU4 equals BTA5 and 28, and BBU5 equals BTA16 and 29 at the cytogenetic level with state of the art banding (El Nahas, *et al*. 2001; Iannuzzi *et al*. 2003). All the other chromosomes have a one-to-one correspondence between the two species. Assignment of genes to these buffalo chromosomes to date is consistent with cytogenetics predictions. Little is known, however, about evolutionary rearrangements within conserved segments.

The development of a comprehensive buffalo framework RH map will allow rapid and efficient transfer of buffalo linkage experiments to map-rich species, thereby enhancing positional candidate cloning in this species. Finally, comparative mapping between buffalo and other species (cattle, human and mice, for instance) can then be done. Of particular importance is the comparison of gene order between the two ruminant species, buffalo and cattle, and an assessment of rearrangements independent of the limited comparisons done with

punctual markers of the two species. The RH panel will present an opportunity to study micro-rearrangements of chromosomes in close relatives in a different order of mammals.
As a first step to creating a comprehensive buffalo radiation hybrid map, we report here the construction and initial characterization of a whole genome 5000-rad radiation hybrid panel (WGRH). The 5000-rad dose was selected from experience with other WGRH panels constructed by Womack and colleagues (bovine, horse, rhesus monkey and sheep). A preliminary RH map for BBU3 and 10 chromosomes was constructed by using the buffalo 5000-rad panel incorporating markers available on published bovine linkage and RH maps. In order to facilitate the continued use of the RH panel and the accumulation of mapping data, the whole genome-radiation hybrid buffalo panel and data reported here are publicly available upon request.

MATERIAL AND METHODS - Construction and preliminary characterization of a whole genome buffalo radiation hybrid panel ($BBURH_{5000}$): Procedures developed at Texas A&M University during the construction of 5,000 and 12,000-rad RH panels in cattle, as described by Womack *et al*. (1997) and Rexroad III *et al*. (2000), were used. A normal male fibroblast cell line was established for the panel construction. Approximately 10^7 cells were irradiated with gamma rays from a CO-60 radiation source at 185 rad/min for a total of 5,000-rad and fused with thimidine kinase deficient TK^- A23 hamster fibroblast cell line. One hundred and seventy five hybrid cell lines were established from one fusion. All 175 hybrid cell lines were tested for the presence of buffalo DNA using BOV-A SINE-PCR amplification (Lenstra *et al*. 1993). A subset of 103 cell lines with the most intense PCR products was selected for the marker screen. The remaining clones were reserved to accommodate any future needs for inclusion in the panel.
A total of fifty-four markers derived from all bovine syntenic groups, including coding genes, ESTs and microsatellite loci were selected to represent each corresponding buffalo chromosome or chromosome arms. It was anticipated that PCR primers for most of the bovine markers from corresponding cattle chromosomes would amplify from buffalo DNA. From the selected markers, nine were discarded because did not amplify with buffalo DNA or generated unspecific amplification. The remaining forty-five markers amplified PCR products suitable for the genotyping. In most cases, the PCR was specific for buffalo DNA; however, in some cases a PCR product was also observed from the hamster control. A subset of 90 hybrids was selected, excluding those with very low retention of the 45 markers. Three 900-cm^2 roller bottle cultures from each clone were grown to produce the final harvest for DNA extraction.
DNA was isolated from cell pellets by phenol/chloroform/isoamyl alcohol extraction method. Briefly, cell pellets were stored frozen at -80°C between harvest and DNA extraction in 15-ml conical centrifuge tubes. After removing the tube from -80°C, the following reagents were added to the tube: 7 ml saline-EDTA, 360-µl 20% SDS and 180-µl Proteinase K (20 mg/ml). Samples were incubated with continuous rotation for 12-15 hours at 55°C, then washed once with phenol/chloroform/isoamyl alcohol (25:24:1, v/v) and washed twice with chloroform/isoamyl alcohol (24:1, v/v). The tubes were centrifuged at 900 x g for 10 minutes during each wash. After the second chloroform/isoamyl alcohol wash, 0.450-ml of sodium acetate, pH 5.2, and 14-ml 95% ethanol were added. The samples were kept at room temperature for at least 1 hr, and then centrifuged at 900 x g for 7 min. The resulting DNA pellet

were washed once in 20-ml ice cold 70% ethanol centrifuged again at 900 x g for 7 min, then transferred to a 1.5-ml micro centrifuge tube. Excess ethanol was aspirated and the sample was dried using a speed-vac. One ml 1X TE, pH 8.0, was added to each tube, and the tubes were placed in a 37°C water bath overnight. The following day, DNA samples were gently mixed on a rotator for 1 hr. Concentrations was determined by spectrometry.

Development of a preliminary map of BBU3 and BBU10 containing cattle microsatellites, cattle ESTs and coding genes

Selection of the markers:

A total of 75 cattle markers were selected for the development of the preliminary RH map of BBU3 and BBU10, including microsatellites, coding genes and ESTs. Primer pairs sequences from microsatellite markers were selected from published cattle linkage maps and those from coding genes and ESTs from published RH maps based on their location on cattle chromosomes homologous to buffalo chromosomes 3 and 10, as follows: BBU3p = BTA 19; BBU3q = BT8 and BBU10 = BTA 9. From the selected markers, 72 generated PCR products suitable for the genotyping with the panel cell lines.

RH panel genotyping:

DNA obtained from each RH cell line was diluted to a concentration of 25ng/ul. The markers were typed on DNA from the 90 radiation hybrid lines together with control bovine and hamster DNA by PCR in 96-well micro titer plates. Each PCR reaction was performed in 10-µl reaction mixtures containing 50ng of DNA; 1.5mM $MgCl_2$; 10mM Tris-HCl; 50mM KCl; 0.2mM dGTP, dTTP, dATP and dCTP; 10pmol each forward and reverse primer and 0.5U of Taq DNA polymerase (AmpliTaq Gold; PE Applied Biosystems, Foster City, CA, USA). The reactions were performed in 96-well PCR plates on a Techne thermal cycler with thermal gradient software. PCR conditions included 94°C for 10 min; 35 cycles of 94°C for 30 sec, 65°C for 30 sec and 72°C for 30 sec; with a final extension cycle of 72°C for 5 min. PCR products were visualized on 2% agarose gels in 1.0X TBE buffer and stained with ethidium bromide. Each marker was typed twice on the RH panel to ensure reproducibility. Strong amplification products were scored as (1), weak products as (2), and absence of amplification products was assigned as (0). Markers with discrepancies between the results from the first two runs were retyped a third time. Scores from each RH clone for each primer was entered into a Microsoft Excel spread sheet.

Computation of RH maps:

Radiation hybrid maps were computed using the software rh_tsp_map (Agarwala *et al*. 2000) and CONCORDE (Applegate *et al*. 1998) linked to QSopt. We used the maximum likelihood criterion and our framework maps are called "MLE-consensus" maps because they are the optimal order for all three formulations of maximum likelihood given in (Agarwala *et al*. 2000). To compute the MLE-consensus maps, we used the same general procedure as is described in detail in (Brinkmeyer-Langford *et al*. 2005), but some thresholds were set differently. Linkage groups were computed at a threshold of 5.0. This gave 1 group each on 3p, 3q and 10. We verified that the MLE-consensus maps are at least 0.25 LOD units better than any alternative. Markers were assigned to their best MLE-consensus interval (sometimes called a "bin") if that placement was at least 0.50 LOD units better than the second best placement.

Unlike the dense maps in (Brinkmeyer-Langford *et al*. 2005), centiRay positions were not assigned to the binned markers because these are intended to be a coarse maps. At the end two markers with identical vectors to MLE-consensus markers were added to the map at the same position as their "twin".

RESULTS AND DISCUSSION - Initial characterization: Our initial efforts indicated that the $BBURH_{5000}$ panel contained chromosomal regions from each river buffalo chromosome. Initially, all 175 hybrid clones were tested for the presence of river buffalo DNA in the hybrid cell lines using BOV-A SINE-PCR amplification (Lenstra *et al*. 1993). A subset of 103 cell lines with the strongest SINE amplification was selected for analysis of retention frequency.

Fifty-four markers derived from all bovine syntenic groups, including coding genes, ESTs and microsatellite loci were selected to represent each corresponding buffalo chromosome or chromosome arms. A set of forty-five markers amplified PCR products suitable for the genotyping. The markers discarded, did not amplify a single PCR product with buffalo DNA or amplified unspecific bands. All buffalo chromosomes or chromosome arms were represented with at least one marker in the remaining set genotyped with the panel cell lines. The average retention frequency for the 45 markers was 32.75%. Retention frequency for individual chromosomes varied from 20% on XBM111 (BBUX) to 50% on HUJ614 (BBU5q). The range in retention frequency for each marker across the autosomal chromosomes varied from 21% for LGB (BBU12), F10 (BBU13), OXT (BBU14), RM074 (BBU24) to 50% for HUJ614 on BBU5q (Fig. 1). Ten clones with a retention frequency of < 5% and three clones with retention frequency > 80% were eliminated from the final panel after amplifying a total of 45 markers. The selected 90 hybrid clones were grown to produce the final harvest for DNA extraction. DNA extractions from the hybrid lines produced an average of 3.5 mg of DNA for each clone, sufficient for an estimated 70,000 PCR reactions, assuming 50ng required per reaction.

The preliminary RH maps from BBU3 and BBU10: A total of 72 markers were scored on the $BBURH_{5000}$ panel. Of these, 66 markers were placed in RH maps as follows, twelve markers (eleven microsatellites and one coding gene) on the BBU10 RH map and 54 markers (11 coding genes, 15 cattle ESTs and 28 microsatellites) were placed on BBU3. Out of the 72 markers, 56 were on MLE-consensus map of which 54 were on the computed map and 2 were added manually as they had identical RH vectors (BMS836 same as BMS2847 on BBU3; MB009 same as D9S1 on BBU10); 10 markers were placed in bins, and 6 were not included on the maps as they were placed with LOD < 0.5 (BMS1290, BMC5012, BMS501, BMS1234, FLJ10853, BMS2377).

Retention frequencies of individual markers ranged from 18.8% for BM4208 (BBU10) to 91.1% for BMS1069 (BBU3p). A higher retention frequency from markers on BBU 3 was expected, since this chromosome contains the selectable marker, TK. BBU3p RH map size spans 472.7 cR_{5000} from marker PSMC5 to BM6000, with the markers distributed in a single linkage group. The markers on BBU3q also generate a single linkage group spanning 795.2 cR_{5000} from marker BM1864 to CSSM047. The total length of BBU10 RH map is about 291.2 cR_{5000} from marker MB009 to BM4208. The marker order within the linkage groups for both chromosomes is consistent with the cattle linkage and RH maps (Ihara *et*

al. 2004; Everts-van der Wind *et al*. 2004 and 2005) and, where information is available, also in agreement with the corresponding position with markers cytogenetically assigned on BBU3 (GAS, TAU, CSSM47) and BBU10 (CGA).

There are presently no genetic linkage maps for river buffalo. To compare our RH mapping results with the results of previous linkage maps, extrapolations from centirays to centimorgan was made based upon the information from cattle linkage maps. Nevertheless, the relationship between centiray and centimorgan is just tentative until linkage maps from the buffalo genome are available. The current bovine linkage map length of BTA8, homologous to BBU3 long arm, spans 128.6cM (Ihara *et al*. 2004). The BBU3q RH map shown in Fig. 2 contains 35 markers, including 5 genes, 10 ESTs and 2 microsatellites not present in the BTA8 linkage map. The marker order within the linkage group on the BBU3q RH map is consistent with the cattle linkage map (Ihara *et al*, 2004) except for two markers located on the terminal region of the BBU3q arm (BM2629 and CSSM047), that suggest an intrachromosomal segmental inversion. Not considering the terminal region of the BBU3q RH map showing the inversion, the most distant common markers in both maps are BM1864 and SRC259. Both markers are separated by 668.3 cR_{5000} on the BBU3q RH map and 97.1 cM apart on the BTA 8 linkage map (Ihara *et al*. 2004). Then on average, 1 cM corresponds to approximately 6.8 cR_{5000}.

The current linkage map of BTA19, homologous to BBU3 short arm, spans 109.6 cM (Ihara *et al*. 2004). The BBU3p RH map, also shown in Fig.2, contains 19 markers, including 6 genes, 5 ESTs and one microsatellite not present in the BTA19 linkage map. The markers BM6000 and FCB193 are the most distant common markers in both maps, separated by 338.6 cR_{5000} on the RH map and 53.8 cM apart on the linkage map, resulting to an average of 1cM to approximately 6.3 cR_{5000}. According to cytogenetic band comparison between river buffalo and cattle (El Nahas *et al*. 2001; Iannuzzi *et al*. 2003), BBU3p originated by centric fusion translocation of BTA19 with the position of its centromere flipped on the biarmed BBU3. Therefore, markers included on BBU3p RH map are in the same order on the two chromosomes, but with the gene order in opposite orientations. Since the RH map constructed does not cover the entire length of the buffalo p arm and the markers assigned cytogenetically on BBU3 short arm are located in the middle of the chromosome, there is not enough information to characterize signs of disrupted conservation.

The BBU10 RH map shown on Fig.3 contains 12 markers, including one gene and three microsatellites not present on the current BTA9 linkage map, which spans116.2 cM (Ihara *et al*. 2004). The marker order within the linkage group on the BBU10 RH map is consistent with the cattle linkage map (Ihara *et al*, 2004).The most distant common markers in both maps are MB009 and BM4208, separated by 291.2 cR_{5000} on the buffalo RH map and 77.9 cM on the cattle linkage map. Then on average, 1 cM corresponds to approximately 3.73 cR_{5000}.

The preliminary characterization and mapping of this river buffalo WGRH panel compares well with the initial characterization of other domestic animals WGRH panels (cattle, Womack *et al*. 1997 and Williams *et al*. 2002; horse, Kiguwa *et al*. 2000; Chowdhary *et al*. 2002; pig, Hawken *et al*. 1999). PCR primers for cattle derived markers, including microsatellites, coding genes and ESTs, amplify buffalo sequences in homologous regions of the respective genomes, representing a very important source of markers that can be incorporated into the buffalo RH mapping effort. Although some PCR amplification failures were observed, on average, 85% of the microsatellites and 90% of the cattle PCR primers from ESTs and co-

ding genes produced reliable genotypes with the buffalo DNA. With the current availability of the bovine genome sequence and the high number of markers, it will be possible to generate a buffalo RH map with the bovine genome and to obtain a large amount of information on the genes likely to be found at a particular chromosomal location. Mapping a large number of genes on the buffalo genome and cross-referencing these with the map locations for the genes in the other species can achieve the alignment from conserved segments between these species. Considering that linkage mapping of genes is nonexistent in river buffalo at this time, alternative mapping approaches such as RH mapping allows including genes in the buffalo map.

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