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Conserved architecture of *RAD51* recombinase in ruminants revealed through molecular cloning and characterization

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ABSTRACT

Meiotic recombination is a highly conserved process that ensures accurate segregation of homologous chromosomes and contributes to genetic variability of a species to withstand the pressure of natural selection. *RAD51* recombinase plays a pivotal role in double strand break repair during homologous recombination and also ensures that chromosomal integrity is maintained during meiotic cell cycle. The present study was aimed to clone and sequence characterize *RAD51* gene from cDNA isolated from testicular tissue of four ruminant species (buffalo, cattle, sheep and goat). The complete open reading frame of *RAD51* gene was observed to be 1020 nucleotides in length encoding a putative protein of 339 amino acids. Functional feature prediction by SMART and Pfam revealed highly conserved DNA binding h4elix-hairpin-helix motif in the N terminal domain and Walker A and Walker B motifs in the catalytic domain which confer ATP binding and hydrolysis activity, respectively. All *RAD51* orthologs and paralogs (*RAD51B, RAD51C, RAD51D, XRCC2* and *XRCC3*) were subjected to phylogenetic analysis. Evolutionary analysis between the different orthologs and paralogs suggested that these proteins are conserved from yeast to humans since all the orthologs from different species clustered in their respective clade, clearly demarcating the paralogs. Gene duplication events have expanded the *RAD51* family in eukaryotes offering them increased capacity to repair DNA and promote homologous recombination.

Key words: Characterization, Homologous recombination, *RAD51*, Ruminants

Meiosis is an evolutionarily conserved process in all sexually reproducing living organisms which contributes to genetic diversity through crossing-over. Meiotic recombination is an essential component for successful completion of meiosis which ensures accurate segregation of homologous chromosomes and generates haploid gametes from diploid precursor cells (Keeney 2008). Recombinational interactions promote the pairing of homologs and their end-to-end connection by zipper-like structures called synaptonemal complexes (Lui *et al.* 2006, Tsubouchi and Roeder 2003). In the recombination event, nuclease processing of double strand break ends generates single-stranded tails, which then assemble into nucleoprotein filaments comprising recA-family proteins and their accessory factors (Zakharyevich *et al.* 2010, Shinohara and Shinohara 2004). These filaments mediate DNA homology search and strand invasion of a homologous template chromosome to form joint molecule intermediates (Shinohara *et al.* 1992, Hong *et al*. 2001).

Bacterial *recA* was the first recombination gene to be cloned from *Escherichia coli* (Lusetti and Cox 2002). Meiotic recombination in eukaryotic cells generally requires two homologues of the *E. coli* recA protein - *RAD51* (*RAD51* recombinase) and DMC1 (disrupted meiotic cDNA) which were first identified in *Saccharomyces cerevisiae* (Bishop *et al*. 1992, Shinohara *et al*. 1992). *RAD51* is required for homologous recombination (HR) pathways in both mitotic and meiotic cells (Krogh and Symington 2004) whereas DMC1 is a meiosis-specific protein (Bishop *et al*. 1992). Cooperative action of *RAD51* and *DMC1* during meiotic recombination has been established because of their co-localization in side-by-side foci on meiotic chromatin (Bishop 1994). Although both proteins are essential for meiosis, a recent report on budding yeast, however, suggests that *RAD51* does not seem to participate directly in strand exchange, but facilitates DMC1-mediated DNA strand-exchange (Cloud *et al*. 2012). On the contrary, in the fission yeast, deletion of DMC1 causes only a moderate reduction in crossing over and a slight reduction in fertility. This suggests that *RAD51* can partially substitute for DMC1 in inter-homolog recombination (Grishchuk and Kohli 2003). In addition to playing important role in homologous recombination,

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RAD51 also ensures that chromosomal integrity is maintained in meiotic cell cycle (Li *et al*. 2006). The disruption of *RAD51* gene can lead to cell death, embryo inviability and chromosomal aberrations in mouse (Tsuzuki *et al*. 1996).

RAD51 orthologs have been well characterized in mice and humans based upon their identity to the yeast *RAD51* gene (Morita *et al*. 1993, Shinohara *et al*. 1993). Mouse *RAD51* cDNA was found to complement *S. cerevisiae RAD51* mutation (Morita *et al*. 1993) and the human homolog was shown to have ATP-dependent strand transfer activity (Baumann *et al*. 1996). Mammals possess five *RAD51* paralogs: *RAD51B, RAD51C, RAD51D, XRCC2* and *XRCC3* (Suwaki *et al*. 2011). These paralogs share 20–30% identity at the amino acid level with *RAD51* and with each other. All RAD51-like proteins share a highly conserved 230 amino acid long central domain (RecA_RAD51domain) which has two highly conserved consensus motifs, Walker A and Walker B, which are characteristic of ATPases and confer ATP binding and hydrolysis activities, respectively. *RAD51* also has a non-specific DNA-binding domain called helix-hairpin-helix (HhH) motif which is absent in other paralogs (Lin *et al*. 2006).

Although *RAD51* has been shown to have pivotal roles in meiosis, homologous recombination and double strand break (DSB) repair but it has not yet been characterized in mammals other than humans and mice. There are no reports available on characterization of *RAD51* in ruminants. Keeping in view the evolutionary divergence among mammalian species, the present study was aimed to clone and sequence characterize *RAD51* gene in four ruminant species (buffalo, cattle, sheep and goat) and to determine the phylogenetic distribution of *RAD51* and its paralogs across a diverse range of eukaryotic species.

MATERIALS AND METHODS

Sample collection: Testis tissue samples of *Bubalus bubalis* (buffalo), *Bos indicus* (cattle), *Ovis aries* (sheep), *Capra hircus* (goat) were collected from slaughter house (New Delhi and Kolkata, India) with the help of an on-site veterinary officer. The fresh tissue samples were dispensed in RNA later and stored in deep freeze (–80 °C) until further use.

RNA isolation and cDNA preparation: Total RNA was isolated from tissue samples using Trizol reagent and treated with DNAse I following manufacturer's instructions. RNA integrity was assessed by loading 2 µl RNA sample on 1.5% agarose gel. The concentration of isolated RNA was determined by measuring optical density at 260 nm using NanoDrop 1000 Spectrophotometer. About 1 µg of total mRNA was reverse transcribed to cDNA with Superscript III cDNA synthesis kit using hexamer primers according to manufacturer's instructions and stored at –80°C till further use.

RT-PCR and cloning: The full length coding sequence of *RAD15* gene was amplified from cDNA of testicular tissue. The primers for this study were designed manually using the conserved sequences from *Bos taurus* mRNA sequence (NM_001046179). Primer sequences used for amplification are as follows: F CTAAGTAATGGCTAT GCAAATGCAGCTTGAA and R TCAGTCTTTGGCA TCTCCCACTCCA. PCR was carried out on a Veriti 96 well thermal cycler in a 25 µl reaction mixture containing 2.5 µl of 10'x' *Taq* reaction buffer, 0.5 µl of 10 mM dNTP mix, 0.5 µl of 10 pM for each primer and 0.25 units of *Taq* DNA polymerase. The PCR reaction cycle was accomplished by denaturation for 3 min at 94°C; 30 cycles of denaturation at 94°C for 30 sec, annealing at 56°C for 30 sec, extension step at 72°C for 30 sec, with a final extension at 72°C for 5 min. PCR products were electrophoresed alongside DNA molecular weight marker in 1.5% agarose gel and then stained with ethidium bromide. The amplified fragments were gel purified using gel extraction kit and subcloned into pTZ57R/T vector. Recombinant clones were selected and plasmid DNA was extracted. Multiple clones were sequenced using the Sanger sequencing to obtain the coding DNA sequence of *RAD51* in four ruminant species.

Data extraction and sequence analysis: RAD51 nucleotide sequences were used as query in BLAST search against NCBI (http://www.ncbi.nlm.nig.gov/sites/entrez) database to collect all possible orthologous and paralogous sequences in different species of eukaryotes (yeast, plants and animals). BLASTp (Altschul *et al*. 1990) search was performed using protein sequences corresponding to *RAD51* gene to retrieve similar sequences from other species such as *Bos taurus, Bos mutus, Bubalus bubalis, Capra hircus, Ovis aries, Gallus gallus, Pan troglodytes, Homo sapiens, Mus musculus etc*. Percentage identity matrix was obtained by pair wise alignment of all the sequences with each other using clustal omega (Sievers *et al.* 2011). SMART (http://smart.emblheidelberg.de) and Pfam (pfam.sanger.ac.uk/search) were used to predict the functional domains of the *RAD51* protein.

Evolutionary analysis: Multiple protein sequence alignment was performed using MAFFT (http://mafft.cbrc.jp/alignment/server/) and Bioedit. Phylogenetic analysis was performed using Neighbour Joining and Maximum Likelihood algorithm with MEGA 6.0 package (http://www.megasoftware.net) and in each case, branch

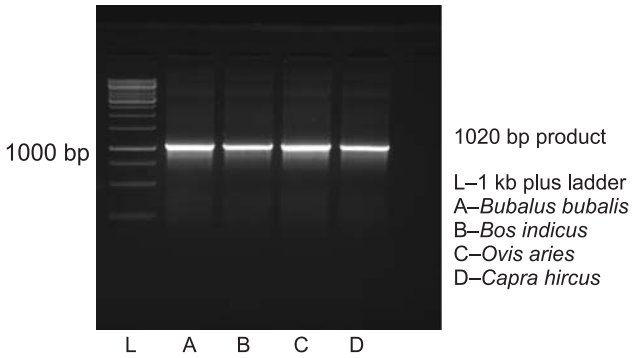

Fig. 1. Gel image of RT-PCR product of *RAD51* gene in ruminants.

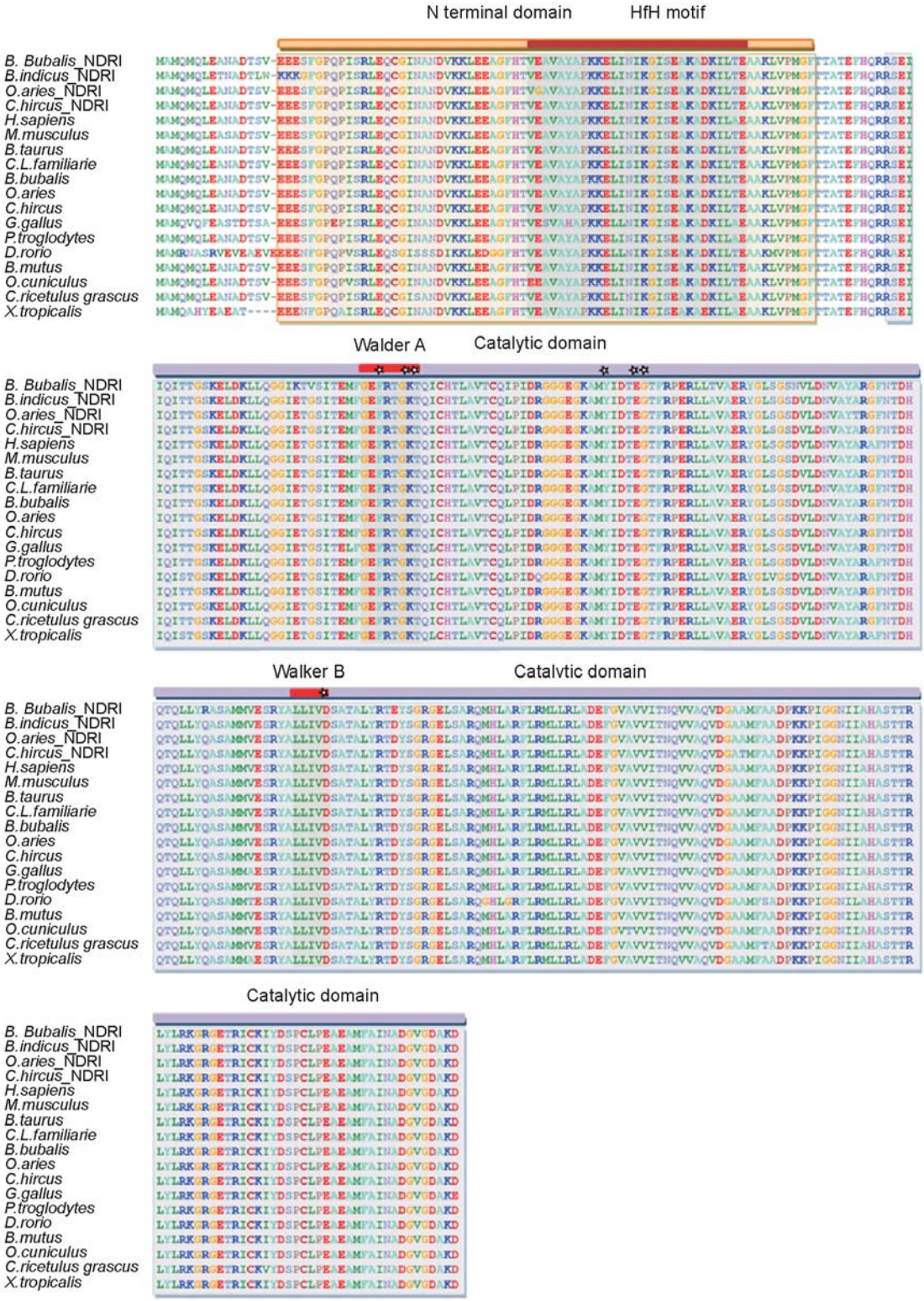

Fig. 2. Multiple sequence alignment of *RAD51* gene in different eukaryotes. All the ruminants sequences characterized in the present study (suffixed with NDRI) as well as sequences retrieved from NCBI are included in the alignment. The N terminal domain and catalytic domain are shown by bars above the sequence alignment. HhH motif, Walker A and Walker B motifs have been highlighted and ATP binding sites are marked by asterisks.

confidence value was calculated with bootstrapping with 1000 iterations using pairwise deletion option of amino acid sequences with gamma parameters. Here, the Jones, Taylor and Thorton (JTT) model for amino acid sequences and gamma parameters was used (Jones *et al.* 1992).

RESULTS AND DISCUSSION

Homologous recombination is an enigmatic process

which not only creates genetic diversity but also provides an important way of repairing DNA damage without errors. Meiotic recombination is initiated by the introduction of programmed double-strand breaks (DSBs) catalyzed by an evolutionarily conserved, meiosis-specific, topoisomerase-like protein, SPO11 (Keeney 2008). Resection of the 5' ends of the breaks results in 3' single stranded tails, which are bound by 2 conserved recA-like proteins, *RAD51* and DMC1 in many organisms, including yeast and mammals which are involved in DNA repair (Bishop *et al*. 1992, Shinohara *et al*. 1992). *Escherichia coli recA* gene is among the most studied recombination gene which encodes a DNA-dependent ATPase that binds to single-stranded DNA and promotes strand invasion and exchange between homologous DNA molecules. Eukaryotic *recA* homologs are *RAD51* and *DMC1*, which were first discovered in the budding yeast *Saccharomyces cerevisiae* (Shinohara *et al*. 1992).

In the present study, the complete open reading frame of *RAD51* gene was amplified by RT-PCR from the mRNA isolated from testis tissue of buffalo, cattle, sheep and goat. The amplified products were sequenced in both directions after cloning in pTZ57R/T vector and the DNA sequences from these 4 species were submitted to NCBI and are available under accession numbers: KR935222, KR935223, KR935224 and KR935225 for buffalo, cattle, sheep and goat, respectively. The complete ORF of *RAD51* gene in all the four species was observed to be 1020 nucleotides in length (Fig. 1) encoding a putative protein of 339 amino acids. Comparative analysis of cDNA sequence of 4 ruminant orthologs revealed limited sequence divergence at both the nucleotide and the amino acid level indicating a high degree of evolutionary conservation. The sequence identity at nucleotide level between cattle and buffalo was 97.5% whereas between sheep and goat, it was 99.6%, as determined by percentage identity matrix in Clustal Omega. The homology of sheep and goat *RAD51* nucleotide sequence was more with that of cattle (98.1 and 98.3%, respectively) as compared to buffalo (97.7 and 97.9% respectively).

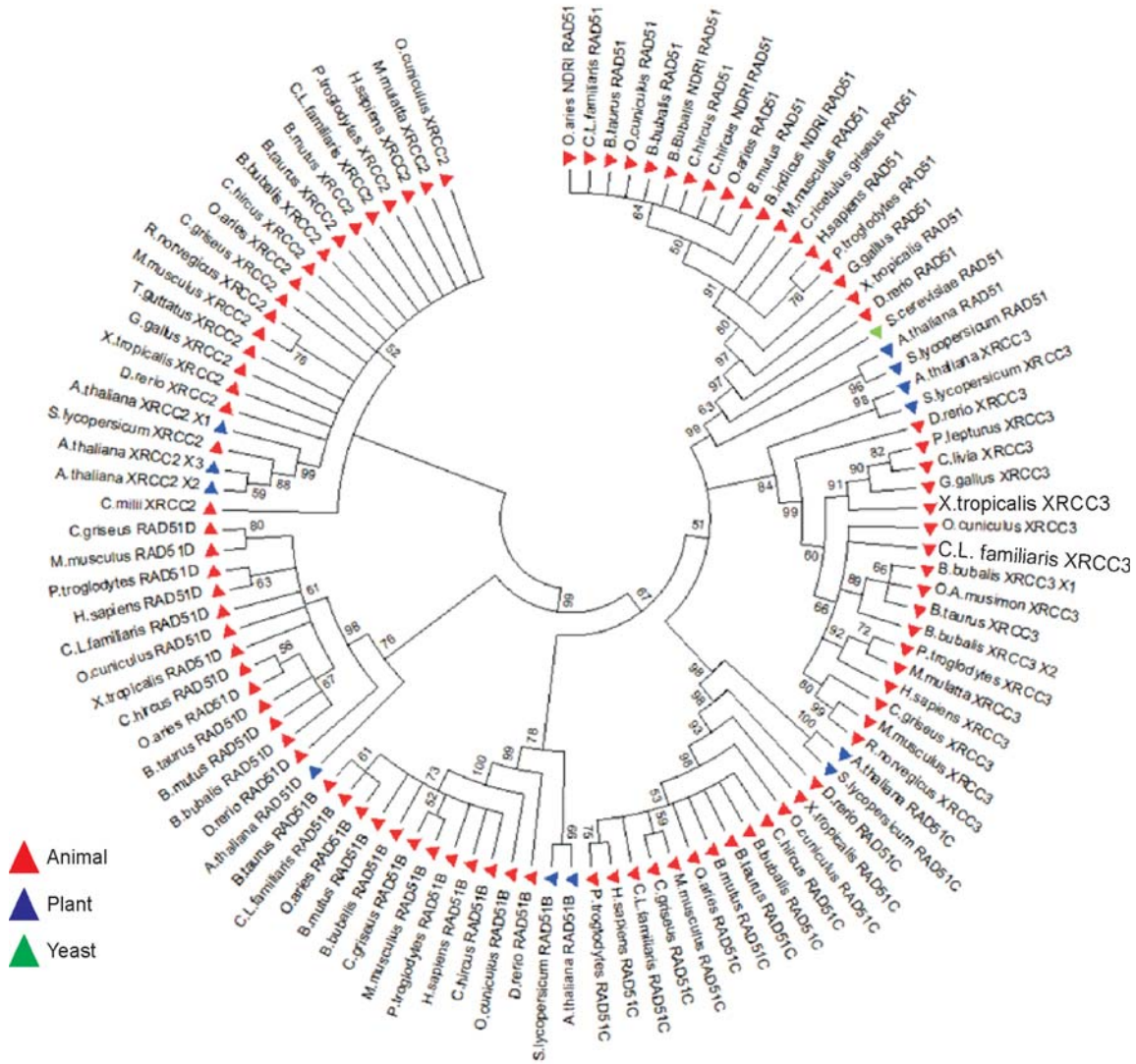

Fig. 3. Phylogenetic tree of *RAD51* and its paralogs in eukaryotes constructed by neighbour method (Poission correction with gamma parameters).

Multiple sequence alignment of the ruminant *RAD51* protein with other orthologs revealed highly conserved structure of this protein in different species (Fig. 2). Functional feature prediction by SMART and PDBsum also revealed significant identity of cattle, buffalo, sheep and goat *RAD51* protein with humans as well as other animal species. *RAD51* protein in humans and mice typically has two major domains: N terminal domain (~70 AAs) and Catalytic domain (~240 AAs). We also observed these domains to be present in the RAD51 protein of ruminants. The N terminal domain revealed the putative double stranded DNA binding helix-hairpin-helix (HhH) motif which is characteristic of the *RAD51* protein (Fig. 2). The catalytic domain has Walker A and Walker B motifs which are present in ATPases and confer ATP binding and hydrolysis activity respectively (Walker *et al*. 1982). These motifs were also found to be highly conserved. Put together, a high degree of evolutionary conservation was evident between ruminant sequences generated by us as well as those retrieved from NCBI.

Numerous gene duplication events have expanded the *RAD51* family in vertebrate animals producing 5 *RAD51* paralogs (*RAD51B, RAD51C, RAD51D, XRCC2* and *XRCC3*) and all the corresponding proteins have acquired new functionalities (Suwaki *et al*. 2011). All the *RAD51* paralogs have DNA stimulated ATPase activity and are important for homologous recombination and DNA repair. Animal cells defective in any of the *RAD51* paralogs are hypersensitive to DNA cross-linking agents, such as Cisplatin and Mitomycin C, and show spontaneous chromosomal aberrations (Takata *et al*. 2001). Embryonic lethality is observed in mouse if any of the *RAD51* paralog is individually knocked out suggesting that these paralogs are imperative for animals' viability (Deans *et al*. 2000, Tsuzuki *et al*. 1996). For instance, disruption of *XRCC2* in mice resulted in embryonic lethality occurring from mid-gestation but the embryos that survived until later stages of embryogenesis showed developmental abnormalities and died at birth. Genetic instability was evident in embryonic cells with a high degree of chromosomal aberrations (Deans *et al*. 2000). Similarly, haploinsufficiency of *RAD51B* has been reported to cause centrosome fragmentation and aneuploidy in human colon cancer cell line (Date *et al.* 2006). This suggests that all *RAD51* paralogs play a key role in preserving genomic integrity during the early stages of development, as well as during physiologically normal cell proliferation.

It was validated by 2 hybrid and immunoprecipitation experiments that the 5 *RAD51* paralogs form 2 complexes: a heterotetramer composed of *RAD51B*, *RAD51C*, *RAD51D* and *XRCC2* referred to as the *BCDX2* complexes and a heterodimer called CX3 composed of *RAD51C* and *XRCC3* (CX3). These complexes facilitate the binding of *RAD51* with single stranded DNA and hence promote homologous recombination (Masson *et al*. 2001, Schild *et al*. 2000). Genetic complexity of the eukaryotic organisms dictates the need for functional innovations for each member of this family. *RAD51* paralogs, when present in a different complex, function at least in part independently in assisting *RAD51* mediated recombination. They have evolved to perform specific functions during meiotic and mitotic recombination but all are integral components of DNA damage signaling pathways which are critical for efficient DNA damage repair, thus conforming to a single *RAD51* gene family.

Amongst all the 5 paralogs, *RAD51C* is thought to play more prominent cellular functions since it is the component of both BCDX2 and CX3 complexes. In fact, *RAD51C* plays an important role in DNA damage response, acting as a transducer of the damage signal to ensure engagement of the homologous recombination pathway of repair (Badie *et al*. 2009). *RAD51C* or *RAD51C*-associated proteins were also proposed to be components of Holliday junction resolvase (Liu *et al*. 2004) and hence are involved in the processing of the Holliday junction in mammalian cells. *RAD51C* and *XRCC3* play key roles in the homology search together with *RAD51*, and were shown to act as cofactors facilitating loading of *RAD51* onto DNA (Bishop *et al*. 1998, Badie *et al*. 2009). *XRCC3* is shown to be required for stabilizing *RAD51C* possibly by forming dimers, and this function requires the ATPase activity, suggesting that *XRCC3* may regulate the dimerization through its ATP binding and hydrolysis activity (Yamada *et al*. 2004). Biochemical evidence has suggested that *RAD51B* may play a role in the late phase of homologous recombination by binding to the Holliday junction (Yokoyama *et al*. 2003). Chicken DT40 cells deficient in *RAD51B* show hypersensitivity to DNA-damaging agents, impaired damage dependent *RAD51* focus formation, increase in chromosome aberrations and decrease in sister chromatid exchange (Takata *et al*. 2001). While, *RAD51D* is involved in promoting HR reactions at telomeres to the extent that the cells deficient in it show loss of telomere capping and telomere shortening in the presence of telomerase, consistent with a role for homologous recombination (HR) in telomere protection (Tarsounas *et al*. 2004).

To analyze the level of conservation and evolutionary relationship of the *RAD51* with its paralogs, all the *RAD51* orthologs and paralogs from a diverse range of species were subjected to phylogenetic analysis. The phylogenetic tree generated by both neighbour joining and maximum likelihood methods resolved into 4 main branches. The *XRCC2*, *RAD51D*, *RAD51B* members formed 3 separate branches whereas *RAD51*, *RAD51C* and *XRCC3* clustered into a fourth branch (Fig. 3). The phylogeny separated all the 6 genes into different and distinct clades. *XRCC2* is the first to separate amongst all the paralogs followed by *RAD51D* and then by *RAD51B*. *RAD51*, *XRCC3* and *RAD51C* form well supported sister groups in the phylogenetic tree. All the orthologous sequences of other eucaryotic species for all the genes of the *RAD51* family were components of their respective clades without any admixing indicating sufficient divergence between the paralogs across all taxa. Plants form a separate cluster in

all the clades being well separated from animal species with good bootstrap support value. Humans and chimpanzees always clustered together for all the genes in their respective clades. The same picture was seen in case of rat, mouse and Chinese hamster indicating sequence similarity between the orthologs for all the paralogs.

Evolutionary analysis of ruminant *RAD51* with other animals, humans, fishes, plants and yeast clearly separated *Saccharomyces cerevisiae* (yeast) gene from all other sequences. Plants such as *Arabidopsis thaliana* and *Solanum lycopersicum* formed a separate cluster (with 100% bootstrap support) which was quite distinct from all other animal species. *Danio rerio* (Zebra fish) is the first to separate from the group followed by *Xenopus tropicalis* (frog) and *Gallus gallus* (Jungle fowl) indicating less sequence similarity as compared to homology between different mammals. As is expected, *Homo sapiens* and *Pan troglodytes* clustered together with 85% confidence value. All the ruminants (our sequences as well as sequences retrieved from NCBI) clustered together indicating high sequence similarity.

Lin *et al*. (2006) conducted extensive searches for *RAD51*-like genes from public databases and performed detailed phylogenetic analyses of the genes identified. They proposed that *RAD51* family members can be categorized into three distinct groups, designated as the *recA*, *RAD*α and *RAD*β subfamilies. The *recA* subfamily includes members from eubacteria, plants, and protists, whereas the *RAD*α and *RAD*β subfamilies each constitute genes from archaea and eukaryotes. *RAD*β subfamily was proposed to contain *RAD51* and *DMC1* genes, both of which are important enzymes of recombination. *RAD*β subfamily members form separate monophyletic group which is different from the monophyletic group formed by *RAD*α subfamily members viz. *RAD51C*, *XRCC3*, *RAD51B*, *RAD51D*, and *XRCC2* genes. This research group went on to state that these five eukaryotic *RAD51*-like genes were derived from a single ancestral *RADB* gene by successive duplication events which probably occurred before the divergence of plants from fungi and animals. The phylogenetic tree constructed with *RAD*α and *RAD*β subfamilies showed that these subfamilies form two separate groups. In the *RAD*β subfamily, the *RAD51C* group first separates from the other four eukaryotic *RAD*β genes, and *XRCC3* is next to follow. *RAD51D* and *XRCC2* genes formed two well-supported sister groups indicating that they have emerged most recently among the eukaryotic *RAD*β genes.

However, our analysis indicated that *XRCC2* is the first to separate from the group suggesting most distinct structure and function. *RAD51D* and subsequently *RAD51B* separate from other members. In fact, in our study, *RAD51*, *XRCC2* and *RAD51*C seem to be members of one sub-family by virtue of sharing highest sequence similarity. This discrepancy in the evolutionary analysis could be because Lin *et al*. (2006) compared sequences of humans, chicken, zebrafish, sea urchin, *Arabidopsis* and rice which encompass members of different taxa literally. On the contrary, in our study we have analyzed different animal species and kept plants as an out-group for comparison. However, we agree with the observations of Lin *et al*. (2006) who reported that flowering plants *Arabidopsis thaliana* and rice (*Oryza sativa*) possess four conserved *recA*-like genes that have higher levels of sequence similarity with eubacterial *recA* genes than with the eukaryotic *RAD51*-likes genes. This observation was also seen in our analysis since all plant *RAD51* genes formed separate cluster that was distinct from animal *RAD51* genes sequences.

The mechanism of double-strand break repair process can be elucidated by understanding the effect of ATP hydrolysis on the assembly of DNA repair complexes. *RAD51* undergoes conformational changes during ATP binding or hydrolysis that regulates its polymerization on single stranded DNA. Similarly, other RAD51-related protein complexes are also controlled by this small effector molecule (Namsaraev and Berg 1998, Kim *et al*. 2002). ATP stimulates the binding of RAD51D-XRCC2 to single-stranded DNA (Kurumizaka *et al*. 2002) and ATP hydrolysis is necessary for normal dynamics of the RAD51C-XRCC3 complex (Yamada *et al*. 2004). Because of the important roles that Walker A and Walker B motifs play in DNA inter-strand crosslink repair, their structure and function have been conserved in all *RAD51* paralogs. Functional feature prediction by SMART and Pfam in our study in ruminants confirmed the presence of Walker A, Walker B and HhH motifs which are characteristic of the *RAD51* protein. These domains were also identified in other orthologs by *in silico* analysis which reinforces that the structure and function of this protein is conserved across different taxa such as chicken, rabbit, humans, mice and ruminants. Put together, our study first time reports the cloning and characterization of *RAD51* in four ruminant species and establishes conserved architecture of this recombinase across diverse species.

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