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Sequence analysis of the DNA-repair gene *rad51* in the tardigrades *Milnesium* cf. *tardigradum, Hypsibius dujardini* and *Macrobiotus* cf. *harmsworthi*

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ABSTRACT

Tardigrades are known for being resistant to extreme conditions, including tolerance to ionising and UV radiation in both the hydrated and the dehydrated state. It is known that these factors may cause damage to DNA. It has recently been shown that single and double DNA strand breaks occur when tardigrades are maintained for a long time in the anhydrobiotic state. This may suggest that perhaps tardigrades rely on efficient DNA repair mechanisms. Among all proteins that comprise the DNA repair system, recombinases such as RecA or Rad51 have a very important function: DNA exchange activity. This enzyme is used in the homologous recombination and allows repair of the damaged strand using homologous non-damaged strands as a template. In this study, Rad51 induction was evaluated by western blot in Milnesium *cf.* tardigradum*, after exposure to gamma radiation. The Rad51 protein was highly induced by radiation, when compared to the control. The* rad51 *genes were searched in three tardigrades*: Milnesium *cf.* tardigradum*,* Hypsibius dujardini *and* Macrobiotus *cf.* harmsworthi. *The gene sequences were obtained by preparing and sequencing transcriptome libraries for* H. dujardini *and* M. *cf.* harmsworthi *and designing* rad51 *degenerate primers specific for* M. *cf. t*ardigradum*. Comparison of Rad51 putative proteins from tardigrades with other organisms showed that they are highly similar to the corresponding sequence from the nematode* Trichinella spiralis*. A structure-based sequence alignment from tardigrades and other organisms revealed that putative Rad51 predicted proteins from tardigrades contain the expected motifs for these important recombinases. In a cladogram tree based on this alignment, tardigrades tend to cluster together suggesting that they have selective differences in these genes that make them diverge between species. Predicted Rad51 structures from tardigrades were also compared with crystalline structure of Rad51 in* Saccharomyces cerevisiae*. These results reveal that* S. cerevisiae *Rad51 structure is very similar to that of the three analysed tardigrades. On the other hand the predicted structure of Rad51 from* M. *cf.* harmsworthi *and* H. dujardini *are closer related to each other, than each of them to that of* M. *cf.* tardigradum.

Key words: Rad51, phylogeny, Tardigrada, recombinase, DNA repair.

INTRODUCTION

Among animals, resistance to gamma radiation has been reported to differ widely. Humans belong to the animals with lowest resistance, with an estimate for the LD_{50} range of 3.5 to 4.5 Gy (Gray) (Cui *et al*., 2011). For some other animals the corresponding values are: monkey 4.5 Gy, mouse 4.5 Gy, rat 6 Gy, goldfish 7.5 Gy, rabbit 8 Gy, and cockroach 50 Gy (Bolus, 2001). On the other hand, an increased resistance has been found in animals such as *Trypanosoma* cruzi Chagas, 1909, which can recover from doses of 500 Gy (Grynberg *et al.*, 2012). The LD_{50} for cysts of the crustacean *Artemia franciscana* Kellogg, 1906 after 96 h is 600-700 Gy (Dvořák and Beňová, 2002; Dvořák *et al*., 2009). In *Caenorhabditis elegans* (Maupas, 1900), doses greater than 1000 Gy (^{137}Cs) were required to reduce mean life span (Johnson and Hatman, 1988), while in *Drosophila melanogaster* Meigen, 1830 the LD_{50} is between 1238 Gy and 1339 Gy (Parashar, 2008). Insects from the family Chironomidae are even more resistant; the midge *Chironomus ramosus* Chaudhuri, Das and Sublette, 1992 has shown an LD_{50} of 2000-2300 Gy (Datkhile *et al*., 2009) and the African chironomid *Polypedilum vanderplanki* Hinton, 1951 can survive after 2000 Gy (Watanabe *et al.*, 2006). However among animals, tardigrades seem to resist the highest radiation doses, with a LD_{50} range of 3000-6200 Gy (Nilsson *et al*., 2010). Specifically, *Milnesium* cf. *tardigradum* showed a LD_{50} after gamma irradiation of 5000 Gy in hydrated animals, and 4400 Gy in desiccated animals (Horikawa *et al*., 2006).

The tardigrades are bilaterally symmetric micrometazoans with four pairs of legs (Nelson, 2002), and are able to survive not only radiation, but also a wide range of temperatures, desiccation, pesticides, vacuum, and hydrostatic pressure (Jönsson *et al*., 2005). This led to the question which mechanisms are used by these organisms to survive to extreme conditions? Among the possible answers to this question, many authors have suggested the existence of an efficient DNA repair system in these ani-

mals (Jönsson, 2003; Schill *et al.,* 2004; Jönsson *et al*., 2005; Horikawa *et al*., 2006; Wełnicz *et al*., 2011), which could repair damaged DNA after exposure to desiccation (Neumann *et al*., 2009) and radiation.

In the living cell, ionizing radiation exposure causes lesions in DNA inducing genetic instability, related to cancer generation or apoptosis of the cell (Jeggo and Lavin, 2009). The damage to the DNA from radiation can arise by direct (ionization of DNA) and indirect effects (reaction of radicals like OH^-, formed as a by-product during radiolysis of H_2O) (Sonntag, 1991). The most dangerous effect on DNA is the formation of double strand breaks (DSBs), and in many cases failure to recognise and/or repair these lesions determines the radio-sensitivity status of the cell (Jeggo and Lavin, 2009). Homologous recombination (HR) and non-homologous end joining (NHEJ) are the main pathways for repairing DSBs in eukaryotic cells, but there are distinct sub-pathways depending on the characteristics of the break site (one or two ends, or flanking homologies) (Symington, 2005). HR is present in the mammalian cell cycle during late S phase to G2 phase. Its main function is the accurate repair of damaged DNA using the sister chromatid as reference for repairing. When there is a deficiency in homologous repair, more error prone repair pathways are activated, including non-homologous end joining and single-strand annealing (SSA). Error-prone repair contributes to genome instability through the accumulation of mutations (Insertion/Deletion) and bigger changes such as chromosomal aberrations (Moynahan and Jasin, 2010). The core reactions of homologous recombination, homology recognition and strand exchange, are mediated by recombinase proteins such as RecA in prokaryotes and Rad51 in eukaryotes, including yeasts and mammals (West, 2003; Holthausen *et al*., 2010).

Rad51 is a very important protein in the DNA repair system and also is highly conserved. The aim of the present study was to identify *rad51* genes from 3 different tardigrades species in order to perform *in silico* analysis of the predicted proteins, comparing these among the tardigrade species and with other phyla. Initially, we observed Rad51 induction in *M.* cf. *tardigradum* after radiation exposure. We also found a single gene corresponding to a putative Rad51 protein in three different species of tardigrades: *Milnesium* cf. *tardigradum*, *Hypsibius dujardini* (Doyère, 1840) and *Macrobiotus* cf. *harmsworthi*. These protein sequences were compared with *rad51* genes of other organisms. This comparison revealed conserved motifs which are important for the protein function. The predicted crystalline structures of the proteins showed interesting differences and similarities between the putative Rad51 proteins. Finally we performed comparative analysis between the putative Rad51 proteins sequences and with respect to homologous proteins from other species. We found interesting similarities and differences among them.

METHODS

Specimens

The *Milnesium* population used in this study was qualitatively identified initially as *Milnesium tardigradum* Doyère, 1840 (Eutardigrada, Apochela, Milnesiidae) from the diagnostic key by Michalczyk *et al.* (2012a, b), but due to lack of morphometric data it was subsequently called *Milnesium* cf. *tardigradum*. Specimens were extracted from moss collected at Svinninge (Åkersberga, north of Stockholm, Sweden; N 59° 26', and E 18 17'). They were reared on solid agar plates with a thin layer of distilled water (Suzuki, 2003). The culture was maintained at room temperature and rotifers were used as food source. Rotifers of the species *Adineta ricciae* Segers and Shiel, 2005 were acquired from Institute of Biotechnology, Cambridge University, and cultured with fish food as the energy source. Specimens of *Macrobiotus* cf. *harmsworthi* (Eutardigrada, Parachela, Macrobiotidae) were obtained from moss samples collected in the Volkspark Großdeuben near Leipzig (Saxony, Germany; N 51°14', E 12°23'). Specimens of *H. dujardini* (Eutardigrada, Parachela, Hypsibiidae) were obtained commercially from Sciento (Manchester, UK) and cultured at room temperature in the commercial bottled spring water *Volvic* (Danone Waters Deutschland GmbH, Frankfurt, Germany) in 100 mm Petri dishes.

Tardigrade irradiation, protein extraction and Western blot analysis

A sample of 300 individuals of *M.* cf. *tardigradum* was selected and starved for 48 h in agar plates with a thin layer of sterile distilled water. Then the sample was exposed during 10.5 min to gamma irradiation (137Cs, Gammacell 1000; Isomedix Inc., Kanata, Ontario, Canada) at a dose rate of 6.74 Gy/min at room temperature. During the time of recovery, water changes were done every 15 minutes in order to provide oxygen. The control sample (300 tardigrades) was kept at the same conditions as the irradiated sample. After 3 h, the proteins were extracted with Laemmli buffer (Laemmli, 1970) supplemented with 25x proteinase cocktail (Complete Roche) and 50 mM PMSF (Phenylmethylsulfonyl fluoride P7626 Sigma). Afterwards, the samples were immediately lysed with a rotor homogenizer during 10 seconds. After this, 2 µL of chitinase 2 µg μL^{-1} (Sigma C6137) was added and proteins were incubated 37°C during 30 minutes. Finally, they were sonicated on ice for 4 seconds with 45% amplitude, boiled and spun 11000 rpm/10 min. Then, samples (60 µg μL^{-1} in all) were loaded in a SDS-PAGE precast gel NuPAGE 4-12% Bis tris 1.5 mm (NPO

335 Invitrogen; Invitrogen, Carlsbad, CA, USA). The molecular weight markers were also loaded (Fermentas PAGE Ruler Plus Prestained Protein). Once proteins were resolved, they were transferred to an activated PVDF membrane (RPN 303F 45 µm Amersham Biosciences). The membrane was washed twice with TBST 1x (12.11 g Tris base, 87.66 g NaCL, 5 mL Tween 20 Promega H5152 and H_2O to 1 L, pH approx. 7.4), and the blocking was carried out by incubation for 2 h shaking with non-fat milk 5% at 37°C. Following TBST washes, the commercial antibody (H-92 sc-8349 Santa Cruz) was added in a 5:5000 dilution and incubated at ambient temperature (20°C) for 1 h. After washing with TBST, the secondary antibody (Donkey anti-rabbit IgG-HRP) was added 1:2000. The membrane was rinsed with TBS (Tris base 11 g, and NaCL 87.66 g to 1 L H_2O) and developed with Amersham ECL Plus™ Western Blotting Reagent (GE Gealth Care sciences RPN2124) in a Fuji CCD camera (Run on LAR-1000 program). Actin was used for normalizing protein levels (Mouse monoclonal antibody Actin, pan Ab-5 MS 1295 P0 Thermo scientific). Tk6 Human Lymphoblast cell line was used as positive irradiated control following the same procedure as described previously, except for the protein extraction method that did not include the chitinase treatment and sonication. The experiment was repeated twice.

rad51 identification from *Macrobiotus* cf. *harmsworthi*, *Hypsibius dujardini* and *Milnesium* cf. *tardigradum*

The identification of *rad51* genes was performed using the same protocol (transcriptomic library) for *M.* cf. *harmsworthi* and *H. dujardini* (Mayer and Hering 2012, personal communication). A different protocol was designed for *Milnesium* cf. *tardigradum* based on alignments of the previous sequences, and the walking genomes and rapid amplification of cDNA ends (RACE) techniques.

A brief description of the transcriptomic library protocol is described below. Total RNA was extracted from specimens of each of the two species, *M.* cf. *harmsworthi* (50-100 animals) and *H. dujardini* (500-1000 animals), using the TRIzol® Reagent (Invitrogen) and the RNeasy MinElute Cleanup Kit (Qiagen, Hilden, Germany) according to the manufacturers' protocols. Transcriptome library preparation and sequencing were performed following Illumina's mRNA-seq sample preparation guide (Part #1004898 Rev. D) with modifications as described in Hering *et al*. (2012). Sequencing was done with 76 cycles paired-end on the Genome Analyser IIx platform (v4 sequencing chemistry and v4 cluster generation kit; Illumina, San Diego, CA, USA). Raw sequences were analysed with IBIS 1.1.2 (Kircher *et al*., 2009). For highly accurate sample identification, sequences with falsely paired indexes were discarded. Paired-end reads from a single cluster were merged if at least 11 bp were overlapping (Kircher *et al.,* 2011). From these data, reads with more than five bases below a quality score of 15 and reads with low complexity were removed. For *H. dujardini* a total of 68,214,238 filtered reads (*M.* cf. *harmsworthi*: 14,533,817) were assembled *de novo* using the CLC Genomics Workbench 5.1 (CLC bio, Aarhus, Denmark) with the following parameters: mismatch cost 3; insertion cost 3; deletion cost 3; length fraction 0.5; similarity fraction 0.8; minimum contig length 200; automatic word size; automatic bubble size; contig adjustment by mapped reads. To obtain *rad51* orthologs from both species studied, tBLASTx searches (Altschul *et al.,* 1997) were performed with known *rad51* sequences from other organisms obtained from GenBank as queries [*D. melanogaster* (accession number D17726.1), *Bombyx mori* Linnaeus, 1758 (U94993.1), *C. elegans* (NM_001028294.2), *Saccharomyces cerevisiae* Hansen, 1883 (NM_001178986.3), *Arabidopsis thaliana* (L.) Heynh (NM_122092.2)]. For both tardigrade species (*M.* cf. *harmsworthi* and *H. dujardini)*, a single sequence was obtained (Mayer and Hering 2012, personal communication).

Identification of *rad51* sequence from *Milnesium* cf. *tardigradum* was performed according to the following specifications. DNA and RNA were extracted from 100 and 300 animals, respectively. For DNA and RNA, *M.* cf. *tardigradum* animals were isolated from culture in a Petri dish with 1 mL of sterile distilled water. DNA was extracted after two days of starvation to avoid extraction of contaminant RNA/DNA from food in the gut (Reuner *et al*., 2010). Animals were processed following the instructions from the GenElute™ Mammalian Total DNA Miniprep Kit (G1N70 Sigma). In order to enrich *rad51* gene transcripts the animals were irradiated with 70 Gy of gamma radiation (^{137}Cs). Two h later, the RNA was extracted using the GenElute™ Mammalian Total RNA Miniprep Kit (RTN70 Sigma). Complementary DNA was generated using Superscript II retrotranscriptase (Invitrogen) and instructions provided by supplier were followed.

Primer design and rapid amplification of cDNA ends

In order to amplify fragments of the *rad51* gene from *M.* cf. *tardigradum*, six primers were designed (Tab. 1). First, we designed the primers 366F and 518R based on

Tab. 1. Primer sequences.

Name	Sequence 5'-3'
366F	GGATCCAAGATGGTTCCGATGGGCTTC
518R	CCACACACTCGCAGTCACATGAAGCTT
GeneRacer 5'1 Primer	GCACGAGGACACUGACAUGGACUGA
Race Rev 653	GTGCCTTCGGTGTCGATATACAG
Rad2350	CGATGATGTTACCGCCGATCG
Rad-Ext- R	CCTCATTAGCGATCGCGAACA

alignments of *rad51* from *M.* cf. *harmsworthi*, *H. dujardini* and other organisms. After amplification with Taq polymerase Platinum from Invitrogen a 200 bp sequence was obtained from both cDNA and DNA. This amplicon was cloned using the TOPO cloning kit (Invitrogen) and then sequenced with ABI 3730xl 96-capillary DNA Analysers (Eurofins). Once the identity of the sequence with *rad51* from other species was confirmed by BLAST searches against the GenBank database, additional primers (Rad2350 and Rad-Ext-R) were designed in order to determine the 3' end. Finally, the RACE technique was used to determine the sequence on the 5' end of the gene. For this purpose, 2000 specimens of *M.* cf. *tardigradum* were used as described before for RNA extraction (GenElute™ Mammalian Total RNA Miniprep Kit). The isolated RNA was processed according to manufacturer instructions of the GeneRacer RACE Ready cDNA Kit (Invitrogen). The designed primer Race_Rev_653 and GeneRacer_5'1-Primer (Invitrogen) were used for amplification of the 5' end of the gene.

Sequence analysis

The putative tardigrade *rad51* sequences translated to amino acid sequences were initially compared with the GenBank database using BLASTP searches (Altschul *et al.*, 1990) and afterwards aligned with other Rad51 orthologs from several different species; yeast: *S. cerevisiae* (CAA45563.1), silkworm: *B. mori* (AAB53330.1), plant: *A. thaliana* (NP_568402.1), pig: *Sus scrofa* Linnaeus, 1758 (NP_001116653.1), frog: *Xenopus laevis* (Daudin, 1802) (NP_001081236.1) using EMBI clustalW (Larkin *et al*., 2007) The alignment was then used to obtain a structure-based sequence alignment using ESPript 2.2 (Gouet *et al.*, 1999) by using the Rad51 crystal structure of *S. cerevisiae* as reference (1szp PDB Accession number). Finally the alignments were analysed and percentage of identity and similarity were determined.

Cladogram analysis was performed with the programme Phylogeny.fr (http://www.phylogeny.fr/version2_cgi/simple_phylogeny.cgi) (Wheeler *et al*., 2007; Dereeper *et al*., 2008). The program processed the alignment with MUSCLE (version 3.7), then it was refined with GBlocks (0.9b), analysed with phyML (3.0) and the tree was generated with TreeDyn (198.3). We used additional organisms for this analysis: the Chinese liver fluke *Clonorchis* Coddald, 1875*,* (GAA50958.1), the tick *Amblyomma maculatum* Koch, 1844 (AEO35092.1), the protozoan *T. cruzi* (AAZ94621.1), the nematodes *Trichinella spiralis* (Owen, 1835) (XP_003378865.1), *C. elegans* (NP_001023465.1), and *Caenorhabditis briggsae* (Dougherty and Nigon, 1949) (XP_002634402.1), the dipterans *Drosophila pseudoobscura* Frolova and Astaurov, 1929 (EAL26777.1) and *D. melanogaster* (BAA04580.1) and sleeping chironomid *P. vanderplanki* (ADM26629.1), the plants *A. thaliana* (NP_568402.1) and *Oryza sativa* L. Japonica group (BAB85491.1), the archaeans *Pyrococcus furiosus* Erauso *et al.,* 1993 (AAC34998.1), and *Pyrococcus abyssi* Erauso *et al.,* 1993 (CAB49165.1)*,* the fungi *Schizosaccharomyces pombe* Lindner, 1893 (BAA02963.1), *Aspergillus fumigatus* Fresenius, 1863 (EAL90369.1), and *Candida albicans* (Robin, 1853) (EAK94324.1).

Finally, predicted Rad51 structures were obtained for *M.* cf. *tardigradum, M.* cf. *harmsworthi* and *H. dujardini.* They were compared with each other and with Rad51 proteins from other species that showed close similarity using Itasser software http://zhanglab.ccmb.med.umich.edu/I-TASSER (Roy *et al*., 2010). Also the program PDBe Fold v2.51 28 Mar 2012 http://www.ebi.ac.uk/msd-srv/ssm/cgi-bin/ssmserver was used for multiple comparison and 3D alignment of protein structures. The analysis of conserved domains was carried out with the programme SMART (http://smart.embl-heidelberg.de/) (Letunic *et al.,* 2012) and DELTA-BLAST Domain Enhanced Lookup Time Accelerated BLAST (Boratyn *et al.*, 2012).

RESULTS

Rad51 radiation induction

The western blot assay revealed a high induction of the *M.* cf. *tardigradum* MtRad51 after 3 h recovery from gamma radiation exposure when compared with the control. The relative units of Rad51 protein normalised with actin are shown in Fig. 1. For comparison, Tk6 cells from humans were used as a positive control of irradiation. The

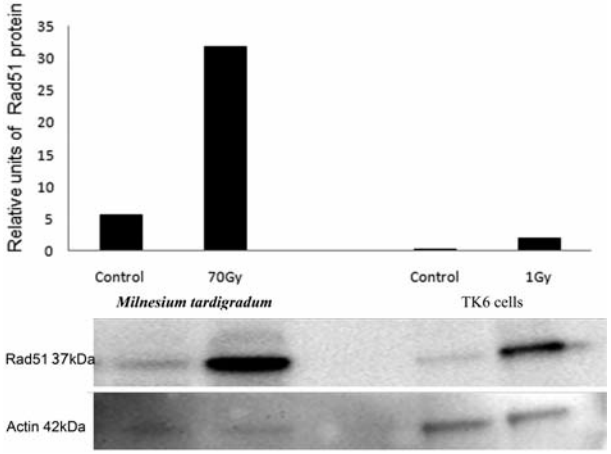

Fig. 1. Rad51 induction in *Milnesium* cf. *tardigradum* and Tk6 *Homo sapiens* cells exposed to gamma radiation. Rad51 levels (37kDa) were normalized with actin levels (42kDa) as shown in this western blot (lower panel) and relative values were plotted (upper panel). This assay was carried out twice and the image presented is representative of these assays.

human cells also showed an increase in the protein levels when exposed to 1 Gy.

Identification of Rad51 protein homologs from tardigrades

After obtaining an induction of Rad51 we decided to analyse its gene in tardigrades. For this, three putative *rad51* sequences were obtained from different tardigrades: *M.* cf. *tardigradum* (MtRad51), *H. dujardini* (HdRad51) and *M.* cf. *harmsworthi* (MhRad51). The MtRad51 sequence is 1072 bp long and the predicted protein is 358 amino acids long (GenBank accession number=HF544509). The result of a comparison by BLASTP against the GenBank database showed a very high similarity to the Rad51 of the nematode *T. spiralis*, with an identity of 71% and E value of 0.0. The HdRad51 putative amino acid and nucleotide sequence is 403 amino acids and 1209-bp long (Accession number=KC152448) and the MhRad51 contains 375 amino acids and 1125-pb (GenBank Accession number=KC152449). When HdRad51 and MhRad51 were compared individually with the GenBank database, they both showed a higher similarity with the Japanese firebelly newt *Cyanops pyrrhogaster* Boie, 1826 Rad51 with a 75% of identity and an E value of 5e-179.

A structure-based sequence alignment of Rad51 from tardigrades and other organisms (Fig. 2) was also performed. It revealed the presence of conserved motifs such as Gly103, HhH, Walker A, Walker B, L1, L2 and ATP cap suggesting that Rad51 proteins from tardigrades possess the characteristic properties of recombinase, mainly DNA binding and ATPase activity.

Important residues in *S. cerevisiae* Rad51 for Rad52 interaction have been identified by specific mutagenesis: G210C, G211S, A248T, and A320V (Krejci, 2001). These residues are conserved in the alignment of Fig. 2 for all the organisms, including tardigrades. However, other identified residues for the single interaction Rad52-Rad51 located in the C terminal part of ScRad51 such as Y388H, G693D, G393S (Krejci, 2001) are not conserved, as shown in the alignment (Fig. 2). Also, the N terminal region of ScRAd51 (1-152 amino acid residues) was suggested to bind ScRad52, but this region does not correspond to Human Rad51 interaction site with Rad52 (Kurumizaka *et al*., 1999). As shown in Fig. 2, almost half of it is not conserved, which could indicate that its interaction with Rad52 is species-specific. Besides, the RPA interaction region is located in the N terminal part of the protein (Residues 16 to 20) (Stauffer and Chazin, 2004), indicating possible species-specificity of this interaction region as well. The DNA binding domain is also located in the N terminal region; despite its variability, it is characterized by a G 103 (Galkin, 2006) that is conserved, as shown in the alignment (Fig. 2).

Conserved domains among the putative Rad51 from tardigrades

The analysis of conserved domains was carried out with the programme SMART (http://smart.embl-heidelberg.de/) (Letunic *et al*., 2012). The same domains were identified in all three sequences from tardigrades: HhH1 and AAA. The HhH motif is a 20 amino acids region present in prokaryotic and eukaryotic non-sequence-specific DNA binding proteins (Thayer, 1995; Aravind and Koonin, 1999; Provvedi and Dubnau, 1999). AAA ATPases have been associated with diverse cellular activities. They have in common the presence of a highly conserved AAA domain (the AAA module). This domain of 200-250 residues is responsible for ATP binding and hydrolysis, among them there are two important motifs, Walker A (GX4GKT) and Walker B (HyDE) (Kedzierska, 2006).

A delta blast analysis was also performed in order to have more information about domains. The three putative proteins MtRad51, MhRad51 and HdRad51 showed the presence of both domains for recombinases: Rad51_DMC1_radA [cd01123], radA,B (E-value 1.38e-139) and PTZ00035, Rad51 protein. However *Hypsibius dujardini* HdRad51 presented an additional domain ENDO3c super family [cl14786], endonuclease III, which includes endonuclease III (DNA-(apurinic or apyrimidinic site) lyase, alkylbase DNA glycosidases (Alka-family) and other DNA glycosidases (Marchler-Bauer, 2004, 2009, 2011).

Moreover, significant alignments from the delta blast analysis showed that MtRad51 has a higher domain similarity with the firebelly newt *C. pyrrhogaster* with a maximum identity of 75% and an E-value of 5e-143. MhRad51 showed higher similarity with the Chinese liver fluke *C. sinensis,* with a maximum identity of 74% and an E-value 3e-141, although it presents similarity as well with *C. pyrrhogaste*r with a lower coverage of 84% and maximum identity of 77% (E-value 5e-139). On the other hand, HdRad51 showed a maximum identity of 74% with a hypothetical protein from the tick *A. maculatum* with an E value of 5e-143 and coverage of 80%. See Tab. 2 for comparison.

Alignments and cladogram

The alignments with ClustalW generated among Rad51 (amino acid sequences) from different species were used to obtain phylogenetic trees. When compared with other organisms, tardigrade Rad51 has a 36.9% identity and 78.4% similarity, while a comparison among tardigrades revealed a higher conservation level with an identity of 57.7% and similarity of 83.4%. Thus, in the cladogram, tardigrade Rad51 showed a closer relationship. Moreover, MhRad51 and HdRad51 had higher identity (68.9%) and similarity (77.3%) with each other than

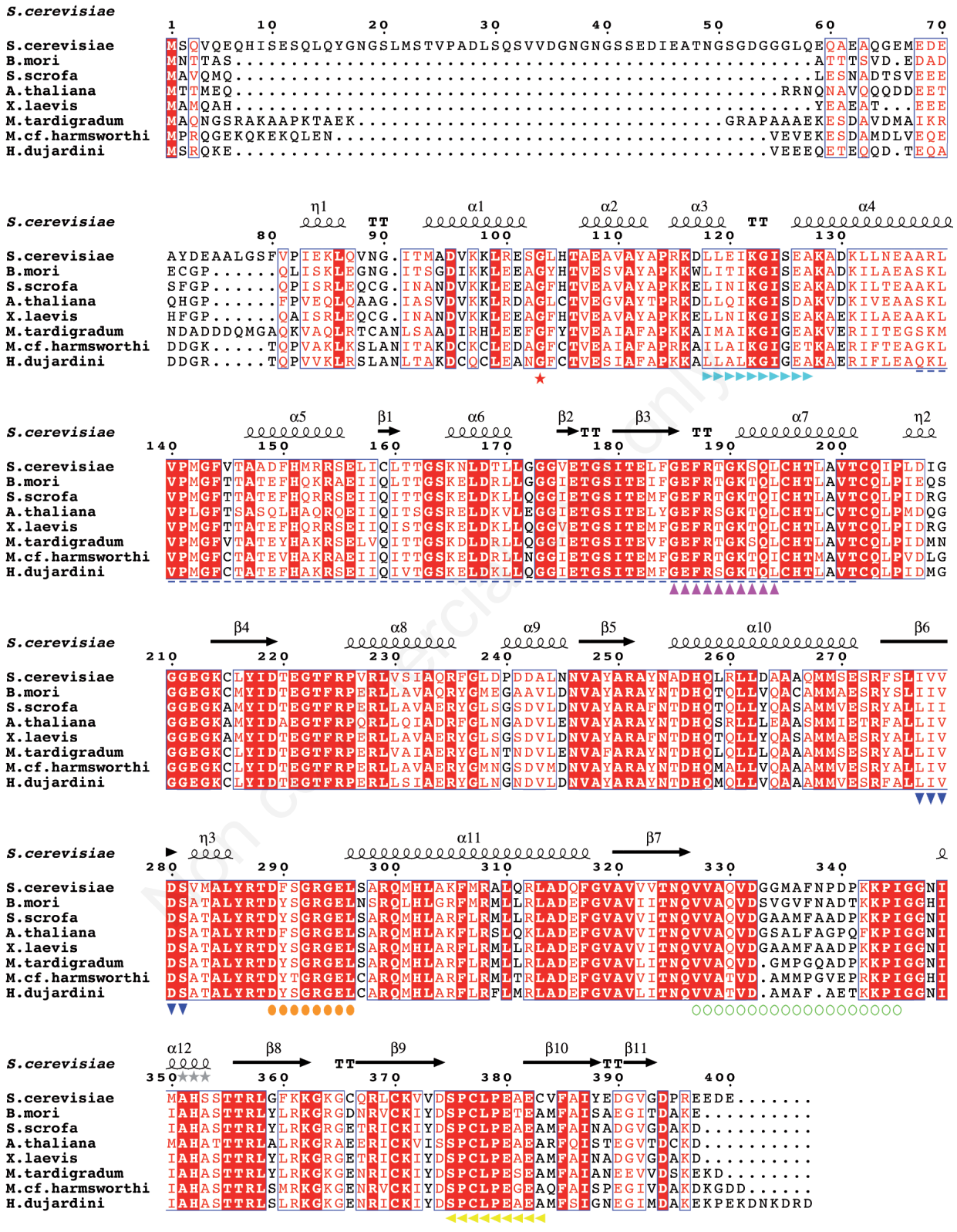

Fig. 2. Homology within a subgroup is indicated with boxes around amino acids showing similarity and white letters over a dark background that highlights identity, across the whole alignment. η indicates 3_{10} helices and TT β turns. The functional motifs are shown with different geometrical figures, from top to bottom: Gly103 (star), HhH (triangles towards right), walker A and B (triangles towards up and down respectively), L1 and L2 (full and empty circles, respectively) and ATP cap (triangles towards left).

MtRad51 had with these species (67.6 - 79.5% and 64.7 - 73% of identity and similarity, respectively). This is consistent with the phylogenetic position of the species previously obtained with 18S RNA (Mali *et al.,* 2010). This is also evident in the cladogram, where *M.* cf. *tardigradum* Rad51 is in basal position with respect to the other two species (Fig. 3). Interestingly, tardigrade proteins are in the same clade as the nematode (*T. spiralis*) and trematode (*C. sinensis*), while *D. melanogaster* and *C. elegans* are in another branch (Fig. 3).

Structural analysis of Rad51 from tardigrades

Using the program I-TASSER, modelled structures of Rad51 were generated (Fig. 4, upper panel). In accordance with the individual analysis in the top 10 identified structural analogs in PDB, all MtRad51, HdRad51, and MhRad51 showed the highest structural similarity with Rad51 H352Y from *S. cerevisiae* ScRad51 (Filament Interface Mutant 3ldA) and the protein 1xu4A a Rad51 homolog from the archaean *Methanococcus voltae* (Balch *et al*., 1981 emend. Ward *et al.*, 1989). Modelled putative Rad51 structures from the three tardigrades species were compared among each other, and with that from *S. cerevisiae* (Fig. 4, lower panel). These results revealed that while *M.* cf. *tardigradum*, *M.* cf. *harmsworthi* and *H. dujardini* Rad51 have a high similarity, as expected, with the protein reference ScRad51, there are some interesting differences among them when the tardigrades Rad51 structures are superimposed.

DISCUSSION

Rad51 is the eukaryotic homolog of RecA, and it is essential for the homologous recombination pathway. This protein is also essential for most of the vertebrates, and *S. cerevisiae* mutants lacking Rad51 show hypersensitivity to DNA damaging agents and meiotic defects (Markmann-Mulischa, 2007). This effect is seen as well in *D. melanogaster* (Rinaldo *et al*., 2002) and *C. elegans* mutants (Staeva-Vieira *et al*., 2003) when exposed to ionizing radiation. Given the important function of the protein, it is conserved from bacteria to humans (Thacker, 1999). In order to understand the DNA repair system and the homologous repair pathway in tardigrades, the Rad51 protein is of major interest.

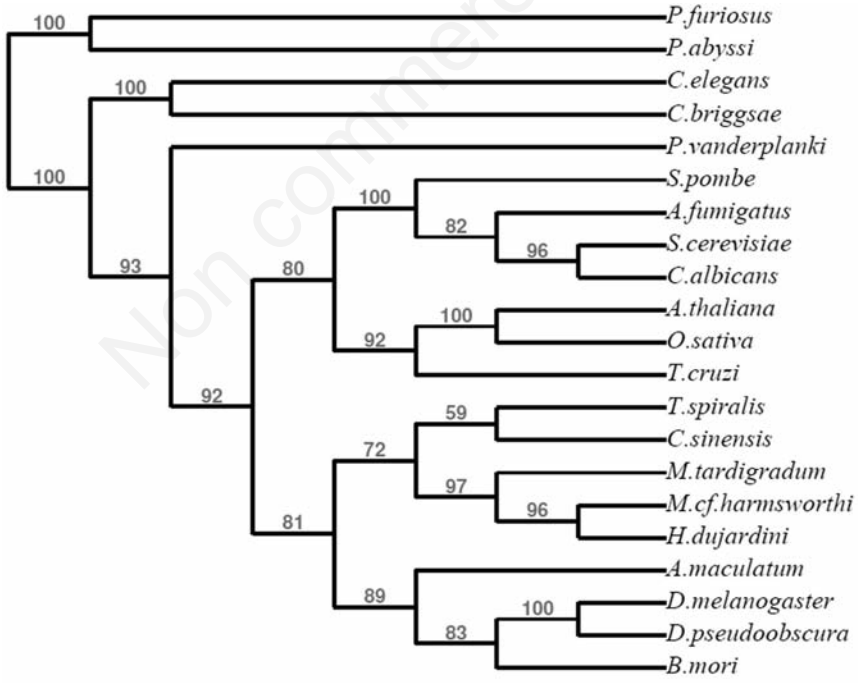

Fig. 3. Cladogram of Rad51 from different organisms: the tardigrades *Milnesium* cf. *tardigradum*, *Hypsibius dujardini* and *Macrobiotus* cf. *harmsworthi*. The silkworm *Bombyx mori,* the Chinese liver fluke *Clonorchis sinensis*, the tick *Amblyomma maculatum*, the sleeping chironomid *Polypedilum vanderplanki*, the protozoan *Trypanosoma cruzi*, the nematodes *Trichinella spiralis*, *Caenorhabditis elegans*, and *Caenorhabditis briggsae*, the plants *Arabidopsis thaliana* and *Oryza sativa*, the archeans *Pyrococcus furiosus* and *Pyrococcus abyssi,* the fungi *Saccharomyces scerevisiae, Schizosaccharomyces pombe, Aspergillus fumigatus* and *Candida albicans*, and the flies *Drosophila pseudoobscura* and *Drosophila melanogaster*. This tree was generated by using at least 1000 replicates.

Using western blot to detect whether there was some involvement of the DNA repair system in tardigrades in response to radiation, we observed a high induction of Rad51 protein in *M.* cf. *tardigradum* after 3 h of radiation exposure. This strongly suggested that the homologous repair system is activated by radiation in tardigrades and supports the hypothesis that the DNA repair system may play an important role in tardigrades when recovering after radiation exposure. Rad51 was also upregulated in the radiation resistant African chironomid, *P. vanderplanki* (Gusev *et al*., 2010). However, tardigrades seem to tolerate more radiation (5000 Gy; Horikawa *et al*., 2006) than P. *vanderplanki* (2000 Gy; Watanabe *et al.,* 2006). According to results of Gusev *et al*. (2010), there seems to be a correlation between DNA repair response after anhydrobiosis and the radiation resistance, because they observed similarities between the DNA fragmentation level and its recovery after desiccation and radiation exposure in the spleeping chironomid. As tardigrades show the ability to survive desiccation as well, this may be a logical explanation, also expressed by other authors (Jönsson and Schill, 2007). Our results complement the comet assay studies (Neumann *et al*., 2009; Gusev *et al*., 2010) that revealed the DNA damage caused by desiccation and radiation, by showing that the homologous repair system is activated when double strand breaks are present in somatic cells. An alternative mechanism is the increase of enzymes involved in the oxidative stress defense, and this has also been shown to be involved in radiation/desiccation resistance (Gusev *et al*., 2010; Rizzo *et al*., 2010). Accumulation of polyunsaturated fatty acids and thiobarbituric acids have also been indicated as part of the mechanism for anhydrobiosis (Rizzo *et al*., 2010). Other pathways that have been shown to act in tardigrades when they are exposed to desiccation and radiation are the heat shock proteins, especially the protein HSP70 (Schill *et al.,* 2004; Jönsson and Schill, 2007) and a putative protein form the LEA family (late embryogenesis abundant) (Schokraie *et al*., 2010).

Continuing with the *in silico* characterization of the putative *rad51* sequences from tardigrades, an analysis of structure-based sequence alignment (Fig. 2) was made. This indicated the presence of conserved motifs Gly103, HhH, Walker A, Walker B, L1, L2 and ATP cap in thethree

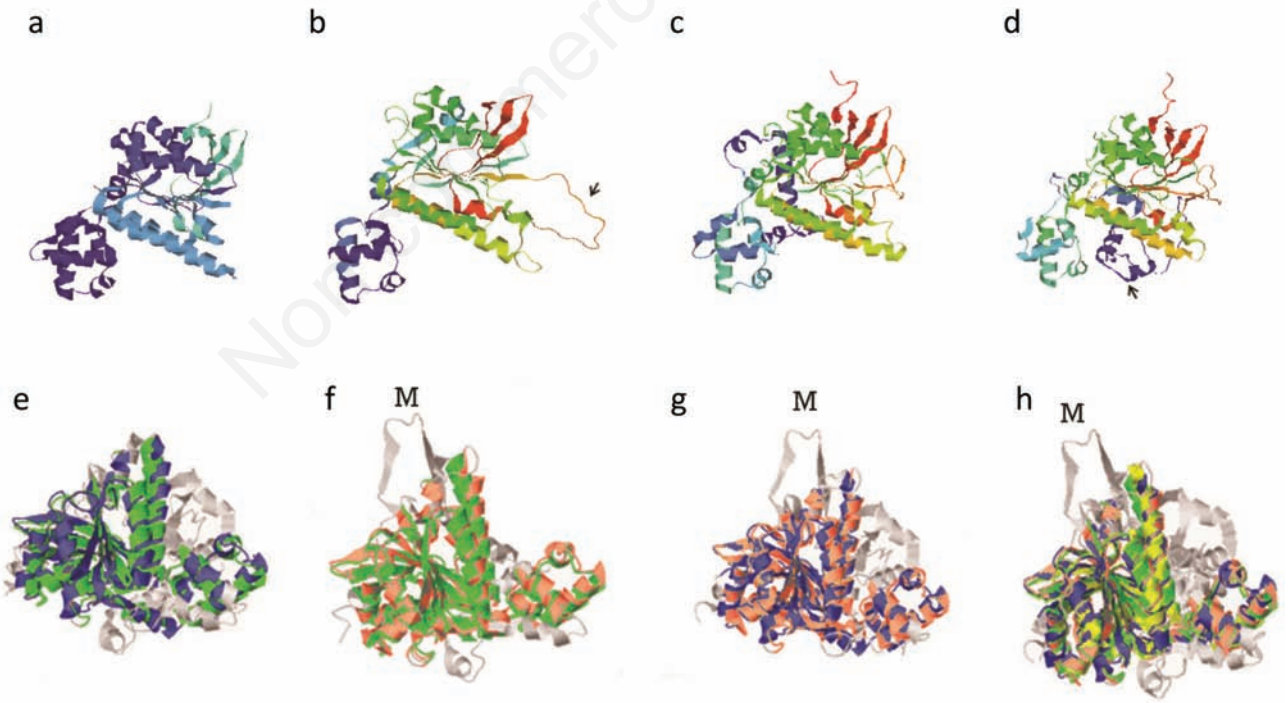

Fig. 4. Rad51 structures modelled by I-TASSER. Upper panel: a) Crystalline structure of *S. cerevisiae* Rad51, as a reference; predicted structures are presented in the same angle utilizing Rasmol for comparison; b) *Milnesium tardigradum* MtRad51; c) *Hypsibius dujardini* HdRad51; d) *Macrobiotus* cf. *harmsworthi* MhRad51. Black arrow indicates evident differences in structure. Lower panel: superimposed Rad51 structures generated with PDBe Fold v2.51 28 are shown; e) *M.* cf. *harmsworthi* - *H. dujardini*; f) *M.* cf. *tardigradum* - *M.* cf. *harmsworthi*; g) *M.* cf. *tardigradum* - *H. dujardini;* h) all Rad51 proteins from tardigrades plus ScRad51 from *Saccharomyces cerevisiae.* The different MtRad51 structure is pointed out with an M letter over a salient β sheet. Notice the difference between figure e respect the other figures, showing structural similarities between modelled *M.* cf. *harmsworthi* and *H. dujardini* Rad51.

tardigrade proteins. Furthermore, the analysis of conserved domains (SMART programme) detected the domains HhH1 and AAA as well. They contain some of the motifs mentioned previously (HhH, and Walker A, Walker B), corroborating our analysis. The HhH domain binds to DNA without sequence specificity and it is more common in proteins that carry out enzymatic activities (Dohertly *et al.*, 1996). The AAA domain is in charge of the main protein functions ATP binding and hydrolysis (Kedzierska, 2006). Glycine 103 is important for binding to both single-stranded and duplex DNA, while L1 and L2 correspond to disordered loops that bind to single strand DNA only. Finally, the ATP cap mediates the stability of the nucleoprotein (Amunugama *et al.*, 2012). The alignment (Fig. 2) also showed the high variability of the amino terminus region of the Rad51 protein among species. This characteristic is significant because Rad52 and RPA proteins interact with Rad51 within this region, and the HhH domain is located here as well (Thayer *et al.*, 1995), thus this region provides specificity for interacting proteins (Shen *et al*., 1996). These results could indicate possible species-specificity in tardigrades for interactions between Rad51 and Rad52. Moreover, Gly103, is also present in this region, but it is extremely conserved in all Rad51 proteins, except for *Drosophila* Rad51 (Zhang *et al.*, 2005).

The putative single Rad51 sequences from three different species of tardigrades were compared with each other and also with the available databases for other species in order to find homologs. *M.* cf. *tardigradum* Rad51 is very similar to the one from the nematode *T. spiralis* (E value 0.0). This result is of interest in light of reports about the closeness between the Tardigrada and Nematoda phyla as part of the Ecdysozoa superclade (Møbjerg *et al.*, 2011). In order to see a clearer relationship between those genes from different species compared with tardigrades, we generated a cladogram. Interestingly, this cladogram (Fig. 3) showed that the origin of the gene from tardigrades is shared with the trematode *C. sinensis* and the nematode *T. spiralis* to tardigrades Rad51. Another cladogram including Rad51 proteins from the vertebrate group [*S. scrofa* (pig), *X. laevis* (frog), *Homo sapiens* Linnaeus, 1758 (human), *Mus musculus* Linnaeus, 1758 (mouse) and *C. pyrroganster* (salamander)] showed a connection with the tardigrades-*Trichinella spiralis*-*Clonorchis cinensis* clade (data not shown), which could explain the proximity that *H. dujardini* and *M.* cf. *harmsworthi* Rad51 showed to Rad51 from the salamander (*C. pyrrhogaster).* All these species share a Rad51 protein identity around 60% (Tab. 2). Also, these new *rad51*/Rad51 sequences belonging to the Tardigrada phylum may complement the studies about the evolution of *recA_RAD51.* This gene should not be used as a molecular marker of species evolution, but its characteristics related to the high functional conservation and diversification into many paralogs and orthologs makes it interesting in the study of its evolutionary history. According to the evolutionary model of the *recA_Rad51* gene family developed by Lin *et al.* (2006), before the separation of archaeans and eukaryotes, the *recA* gene was duplicated producing the gene variants *radα* and *radβ.* Then in eukaryotes another duplication produced the *rad51* and *dmc1* genes and *rad51C*, *xrcc3*, *rad51B*, *rad51D* and *xrcc2* genes, from each gene respectively. During evolution, *Dmc1* and some *Radβ* genes were lost from some insect and nematode species, and several fungal and invertebrate lineages. In the case of *C. elegans* and *D. melanoganster rad51* genes, they tend to form a basal clade outside the plant, animal, and fungal genes, due to the possible high rate of evolution in these specific genes as inferred by larger amount of mutations accumulated (Lin *et al*., 2006). Through another analysis of *rad51* genes and their corresponding proteins performed in the transcriptomic library, the sequence of *xrcc3* gene in *H. dujardini* and *M.* cf. *harmsworthi* (KC152450 and KC152451 respectively) was found, indicating possibly the presence in tardigrades of genes originating from both *radα* and *radβ*, original eukaryotic genes. The proposal of the evolution of moulting protostomes (Ecdysozoa) indicates an initial segregation of metazoans into protostomes and deuterostomes. Afterwards a segregation of protostomes into molting Ecdysozoa and non-molting Lophotrochozoa (Petrov and Vladycheskaya, 2005) may have taken place. This hypothesis could explain a possible *rad51* gene duplication during the evolution of metazoans before the separation of Arthropoda, Nematoda, Platyhelminthes and Chordata.

Finally, the 3D superimposed structures of the Rad51 proteins showed a high similarity between the modelled Rad51 proteins and *S. cerevisiae* Rad51, corroborating our models. In the individual figures it is possible to observe structural differences between proteins from the three tardigrades (Fig. 4). HdRad51 shows an evident

Tab. 2. Identity and similarity percentages of Rad51 sequences, between tardigrades and with respect to other phylogenetically animals. The highest identity values are shown in italic.

Organisms	%	(1)	(2)	(3)	*C. sinensis*	*T. spiralis*	*C. pyrrhogaster*	*X. laevis*	*S. scrofa*
M. cf. *tardigradum* (1)	Id Si	100	64.0 73.0	67.0 79.5	66 78.5	*68.0* 81.4	67.0 79.8	67.0 79.0	61.0 69.0
H. dujardini (2)	Id Si	64.0 73.0	100	*68.0* 77.3	63.0 69.9	62.0 71.2	62.0 69.6	63.0 68.9	*67.0* 79.0
M. cf. *harmsworthi* (3)	Id Si	67.0 79.5	*68.0* 77.3	100	66.0 76.1	64.0 76.4	66.0 76.3	64.0 75.0	*67.0* 77.1

Id, identity; Si, similarity.

β sheet (Fig. 4d) that could correspond to a distinct domain (ENDO3c super family endonuclease III) detected during the DELTA BLAST analysis. Also, another different structure can be detected in MtRad51, denoted with the letter M in the superposed structures (Fig. 4g and 4h). These results demonstrate some of the differences between structures, however, the protein core is highly conserved (section of the protein in darker grey with asterisk shape). This part could be correlated with high identity percentages (around 64-68%) found in the tardigrade alignments (Tab. 2). However, the cladogram and the alignment identity values show a closer similarity/position between HdRad51 and MhRad51, while MtRad51 is located in basal position (Tab. 2; HdRad51 *vs* MhRad51 has 68% identity, and MtRad51 with both of them 64% and 67% identity, respectively). These results may be related to the separate positions of the tardigrade species in the orders Parachela (genus *Macrobiotus* and *Hypsibius*), and Apochela (genus *Milnesium*).

In a previous report, Föster *et al*. (2012) from an analysis of transcriptomic library cDNA sequences suggested the existence of several biochemical pathways for *M. tardigradum* based on sequence homology, and putative DNA repair protein Rad51 was among them. However, the homology between the retrieved putative *rad51* sequence (nucleotide and predicted amino acid sequence; Föster *et al*., 2012) and MTRad51 (sequence obtained in this study) is low and the alignment is very poor. Therefore, the analysis of the sequences in the present study constitute the first report of the complete sequences available in GenBank related to Rad51 from tardigrades *M. tardigradum*, *H. dujardini* and *M.* cf. *harmsworthi*. Thus, the reported fragment sequence by Föster *et al*. (2012) could correspond to a redundant recombinase.

CONCLUSIONS

In this study, while the observed induction of RAD51 protein after radiation in tardigrades may indicate an important role for the recovery after the damage caused by different agents such as radiation, the participation of a non-homologous end-joining mechanism cannot be ruled out. The present identification, *in silico* characterization and the report in GenBank of new putative *rad51* sequences from three different species of tardigrades is important for the DNA repair studies focused on radiation/desiccation tolerance in these animals. This indicates the functional conservation of these proteins among the different kingdoms and animal phyla, based on the presence of domains that are a hallmark of RAD51 proteins.

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