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RESEARCH ARTICLE

Identification and expression of adenosine deaminases acting on tRNA (ADAT) during early tail regeneration of the earthworm

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Abstract

Background RNA editing is a widespread phenomenon in all metazoans. One of the common RNA editing event is the chemical conversion of adenosine to inosine (A-to-I) catalyzed by adenosine deaminases acting on tRNA (ADAT). During *D. melanogaster* development, the ADAT1 transcript was found to localize mainly to the central nervous system including brain and ventral nerve cord during brain development. Although an earthworm adenosine deaminases acting on mRNA (ADAR) has been identified and its possible implication in earthworm regeneration has been investigated, there is little accumulated information on ADAT and tRNA editing in the annelid including terrestrial earthworms.

Objective This study aimed to investigate the molecular characteristics and the expression pattern of earthworm *ADAT* during tail regeneration to understand its physiological significance.

Methods Nucleotide sequence of *Ean-ADAT* was retrieved from the genome assembly of *Eisenia andrei* via Basic Local Alignment Search Tool (BLAST). The genome assembly of *Eisenia andrei* was downloaded from National Genomics Data Center (http://bigd.big.ac.cn/gwh/). The alignment and phylogenetic relationship of the core deaminase domains of ADATs and ADARs were analyzed. Its temporal expression during early tail regeneration was measured using real-time PCR.

Results The open reading frame of *Ean-ADAT* consists of 1719 nucleotides encoding 573 amino acids. Domain analysis indicates that Ean-ADAT has a deaminase domain composed of 498 amino acids and a predicted nuclear localization signal at the N-terminal. Its subcellular localization was predicted to be nuclear. The core deaminase region of Ean-ADAT encompasses the three active-site motifs, including zinc-chelating residues and a glutamate residue for catalytic activity. In addition, Ean-ADAT shares highly conserved RNA recognition region flanking the third cysteine of the deaminase motif with other ADAT1s even from the yeast. Multiple sequence alignment and phylogenetic analysis indicate that Ean-ADAT shows greater similarity to vertebrate ADARs than to yeast Tad1p. *Ean-ADAT* mRNA expression began to remarkably decrease before 12 h post-amputation, showing a tendency to gradual decrease until 7 dpa and then it slightly rebounded at 10 dpa.

Conclusions Our results demonstrate that *Ean-ADAT* belongs to a class of ADAT1s and support the hypothesis of a common evolutionary origin for ADARs and ADATs. The temporal expression of Ean-ADAT could suggest that its activity is unrelated to the molecular mechanisms of dedifferentiation.

Keywords Earthworm · Tail regeneration · Adenosine deaminase acting on tRNA · mRNA expression

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Introduction

RNA editing, a post-transcriptional modification of RNA molecules, is a widespread phenomenon in all metazoans. The most common RNA editing event is the chemical conversion of adenosine to inosine (A-to-I) catalyzed by adenosine deaminases acting on RNA enzymes, which act on dsRNA or tRNA substrates (Yoon et al. 2020).

Inosine (6-deaminated adenosine) is a non-canonical nucleoside found in all fields of life. Chemically, it is a guanosine analogue and it only differs from the latter by

the lack of the N2 amino group. Inosine is rarely present in DNA but is often observed in different types of RNAs including double-stranded RNAs, tRNAs and viral RNAs (Grosjean et al. 1996). In RNA, inosine is produced by the deamination of adenosine. Generally, two types of RNA adenosine deaminases exist: adenosine deaminases acting on mRNAs (ADARs) and adenosine deaminases acting on tRNAs (ADATs), the enzymes of each group being specific for specific modification sites (Torres et al. 2014; Gerber and Keller 1999). Inosine is found in tRNAs in all domains of life. It is present mainly at three positions on tRNAs: position 34, which is the first nucleotide of the anticodon (wobble-position), position 37 (following the anticodon), and position 57 (at the TψC-loop) (Auxilien et al. 1996; Torres et al. 2014).

ADAR family present only in metazoans appears to have evolved from ADAT, a critical protein present in all eukaryotes, via the addition of a double-stranded RNA binding domain (Grice and Degnan 2015). It is thought that ADATs involved in the modification of tRNA have a common evolutionary origin with ADARs involved in pre-mRNA editing (Gerber et al. 1998). Unlike ADARs, only a few ADATs have been identified in eukaryotes including yeasts (Gerber et al. 1998), octopus (XP_029641018), drosophila (Keegan et al. 2000), and human (Maas et al. 1999). The phenotypic consequences of the lack of inosine modifications on tRNAs in metazoans have been almost completely unexplored. During *D. melanogaster* development, the ADAT1 transcript which is specific for adenosine 37 of $tRNA^{ala}$ was found to localize mainly to the central nervous system including brain and ventral nerve cord (Keegan et al. 2000), suggesting a possible role for ADAT1 and I37 modification of $tRNA^{Ala}$ during brain development (Torres et al. 2014).

Earthworms show a wide spectrum of regenerative potential capable of re-constructing body parts lost due to injury. Among the earthworm species, *Eisenia andrei* has powerful regenerating capacity and can completely regenerate an amputated tail about within a month. It is generally believed that earthworm regeneration is an epimorphosis, which is characterized by the dedifferentiation of adult tissue to form a highly proliferating cell mass called a blastema, followed by its re-specification into appropriate cell types (Ribeiro et al. 2018). In *E. andrei*, blastema forms beneath wounded dermis at 1–3 day post-amputation (dpa), and segmentation occurs within 7 dpa, when redifferentiation is not yet dynamic (Park et al. 2013; Shao et al. 2020). Between 7 and 10 dpa, the blastema continues to grow and elongates, but there are no external signs of segmentation. After 7 dpa, outgrowth of the regenerating tissue was observable under low magnification, after which the redifferentiation of various tissues actively takes place (Park et al. 2013).

Very recently, an earthworm ADAR, *Pex-ADAR*, has been identified and its spatiotemporal expression implies that this RNA editing enzyme could be implicated in muscle redifferentiation (Yoon et al. 2020). However, in the annelid including terrestrial earthworms, there is little accumulated information on ADAT and tRNA editing. Through the genome sequence of *E. andrei*, we identified a full-length cDNA sequence showing significant homology to mammalian *ADAT1*. To our knowledge, this is the first report on the molecular characterization of an annelid ADAT and its expression analysis in earthworm tail regeneration, which should help to elucidate the physiological significance of ADAT, which is conserved from yeast to human.

Materials and methods

Animals and computational sequence analysis

Sexually mature *E. andrei* obtained from a commercial source (Seoul, Korea) were reared by the method previously described (Park et al. 2017). Before being used, the earthworms were placed in Petri dishes lined with filter paper moistened with earthworm saline for 48 h to purge the gut contents. Nucleotide sequence of *Ean-ADAT* was retrieved from the genome assembly of *Eisenia andrei* via Basic Local Alignment Search Tool (BLAST). The genome assembly of *Eisenia andrei* was downloaded from National Genomics Data Center (http://bigd.big.ac.cn/gwh/) under accession code: GWHACBE00000000. The open reading frame (ORF) was determined using the ORF finder on the server of National Center of Biotechnology Information (NCBI). The subcellular localization of Ean-ADAT was predicted by PSORT II (Nakai and Horton 1999).

Comparative and phylogenetic analyses

Amino acid sequences of ADAT1s and ADARs were retrieved from the GenPept Database via BLASTP and UniProt (http://www.uniprot.org/). Amino acid sequence alignment was carried out by MUltiple Sequence Comparison by Log-Expectation (MUSCLE) algorithm and phylogenetic analysis was performed by the maximum likelihood method, using MEGA X software (https://www.megasoftware.net/). Bootstrap analysis was performed with 100 replications. The phylogenetic tree was built with ADAT1 orthologs of metazoan animal models and yeasts (Tad1p), [Lophotrochozoa: *Eisenia andrei*, *Crassostrea gigas* (XP_011432605), *Octopus sinensis* (XP_029641018), *Pomacea canaliculata* (XP_025115922) and *Mizuhopecten yessoensis* (XP_021375838); Ecdysozoa: *Araneus ventricosus* (A0A4Y2QEA4) and *Drosophila melanogaster* (Q9V3R6); Deuterostomia: *Homo sapiens* (Q9BUB4), *Rattus norvegicus* (D4ADL5), *Mus musculus* (Q9JHI2), *Gallus gallus*

(Q5ZI16), *Xenopus laevis* (A0A1L8GF60) and *Danio rerio* (F1R076); yeasts, *Saccharomyces cerevisiae* (P53065) and *Schizosaccharomyces pombe* (O42912)], ADAR1 orthologs [Homo sapiens (P55265) and *Mus musculus* (Q99MU3)], and ADAR2 orthologs [*Homo sapiens* (P78563) and *Mus musculus* (Q91ZS8)].

Quantitative real time PCR

Total RNA was isolated from the tail regenerates of *E. andrei* during regeneration using TRIzol (Ambion, Austin, TX, USA) at the times indicated. We selected mRNA from total RNA using oligo (dT) primers (Promega, Madison, WI, USA) and then reverse transcribed the mRNA into cDNA (SuperScript II First-Strand Synthesis System for RT-PCR, Invitrogen, Waltham, MA, USA). Quantitative reverse-transcription PCR (qRT-PCR) was performed using WizPure™ qPCR Master (SYBR) (Wizbiosolutions, Korea) with specific primer pairs on an Applied Biosystems Stepone plus real-time PCR System. The sequences of primer pairs were as follows: *Ean-ADAT* (forward) 5′-GCAGCACCGGAT GGAGTTAG-3′ and (reverse) 5′-ATCTGGCCTTGGACG AGTCA-3′; and *Ean-β-actin* (forward) 5′- CATCCATCG TCCACAGGAAGTG-3′ and (reverse) 5′-CGTGTTCAT CTCAGGAGGCAGA-3′. Relative quantification of mRNA was conducted using the comparative $2-\Delta\Delta Ct$ method with *β-actin* as the reference gene. All data are expressed as means ± SEM and analyzed by using GraphPad Prism 6.01 (GraphPad Software, Inc.). Differences between groups were assessed using a student's t-test.

Results and discussion

Sequence and domain analyses

The nucleotide and predicted amino acid sequences of *Ean-ADAT* found in the earthworm *E. andrei* are shown in Fig. 1. The open reading frame of *Ean-ADAT* consists of 1719 nucleotides encoding 573 amino acids with a calculated molecular mass of 62.7 kDa. Domain analysis indicates that Ean-ADAT has a deaminase domain composed of 498 amino acids and a predicted nuclear localization signal at the N-terminal. Its subcellular localization was predicted to be nuclear.

Since it is thought that the differences between the sequences of the deaminase core region is a reliable index to determine the ortholog's relationship to newly sequenced RNA-editing enzymes (Keegan et al. 2004), the alignment of core deaminase domains of Ean-ADAT with other ADAT and ADAR family members is carried out (Fig. 2).

Like the other ADATs, the core region of Ean-ADAT encompasses the three active-site motifs, including zinc-chelating residues (histidine or cysteine in black boxes) and a glutamate (E) residue in the first active-site motif that is thought to be involved in proton transfer in the deaminase active site. It is also noted that Ean-ADAT shares highly conserved RNA recognition region flanking the third cysteine of the deaminase motif with other ADAT1s even from the yeast, indicating that Ean-ADAT belongs to a class of ADAT1s. However, the predicted Ean-ADAT adenosine deaminase domain shows 49.2% identity and 69.0% similarity to the human ADAR1 protein, and 52.0% identity and 65.9% similarity to the human ADAR2, while it shows 31.7% identity and 54.2% similarity to the *S. cerevisiae* Tad1 protein. The fact that Ean-ADAT shows greater similarity to vertebrate ADARs than to yeast Tad1p, supporting the hypothesis of a common evolutionary origin for ADARs and ADATs (Gerber et al. 1998; Keegan et al. 2000).

Furthermore, phylogenetic analysis exhibits that Ean-ADAT could be grouped together with other ADAT1s and it has greater similarity to vertebrate ADAR1s and ADAR2s than to yeast Tad1p (Fig. 3), supporting the evolutionary hypothesis that pre-mRNA editing may have evolved when an original ADAT acquired dsRNA-binding domains and a new set of targets in pre-mRNA (Keegan et al. 2000).

Temporal expression of *Ean-ADAT* mRNA during early tail regeneration

The expression level of *Ean-ADAT* mRNA during the early tail regeneration of *E. andrei* was determined using real-time qRT-PCR (Fig. 4). During early tail regeneration, *Ean-ADAT* mRNA expression began to remarkably decrease before 12 h post-amputation, showing a tendency to gradual decrease until 7 dpa and then it slightly rebounded ($p < 0.01$) at 10 dpa. The qRT-PCR analysis of the temporal expression of *Ean-ADAT* mRNA indicated that its transcription was inactivated in the early stages (1–7 dpa) when blastemal cells proliferate and the central nerve cord is reconstructed. Expression then slightly rebounded at subsequent stages (10 dpa) when diverse cell types or tissues were regenerated in each segment. This could suggest that Ean-ADAT activity is unrelated to the molecular mechanisms of dedifferentiation. Its slight but significant rebound at 10 dpa might be attributable to the partial restoration of amputated region or be implicated in redifferentiation.

In *D. melanogaster*, the expression pattern of *ADAT1* transcript localized mainly to the central nervous system possibly suggests that its activity could be associated with brain and/or ventral nerve cord development (Torres et al. 2014). However, the high level of *ADAT1* expression in the

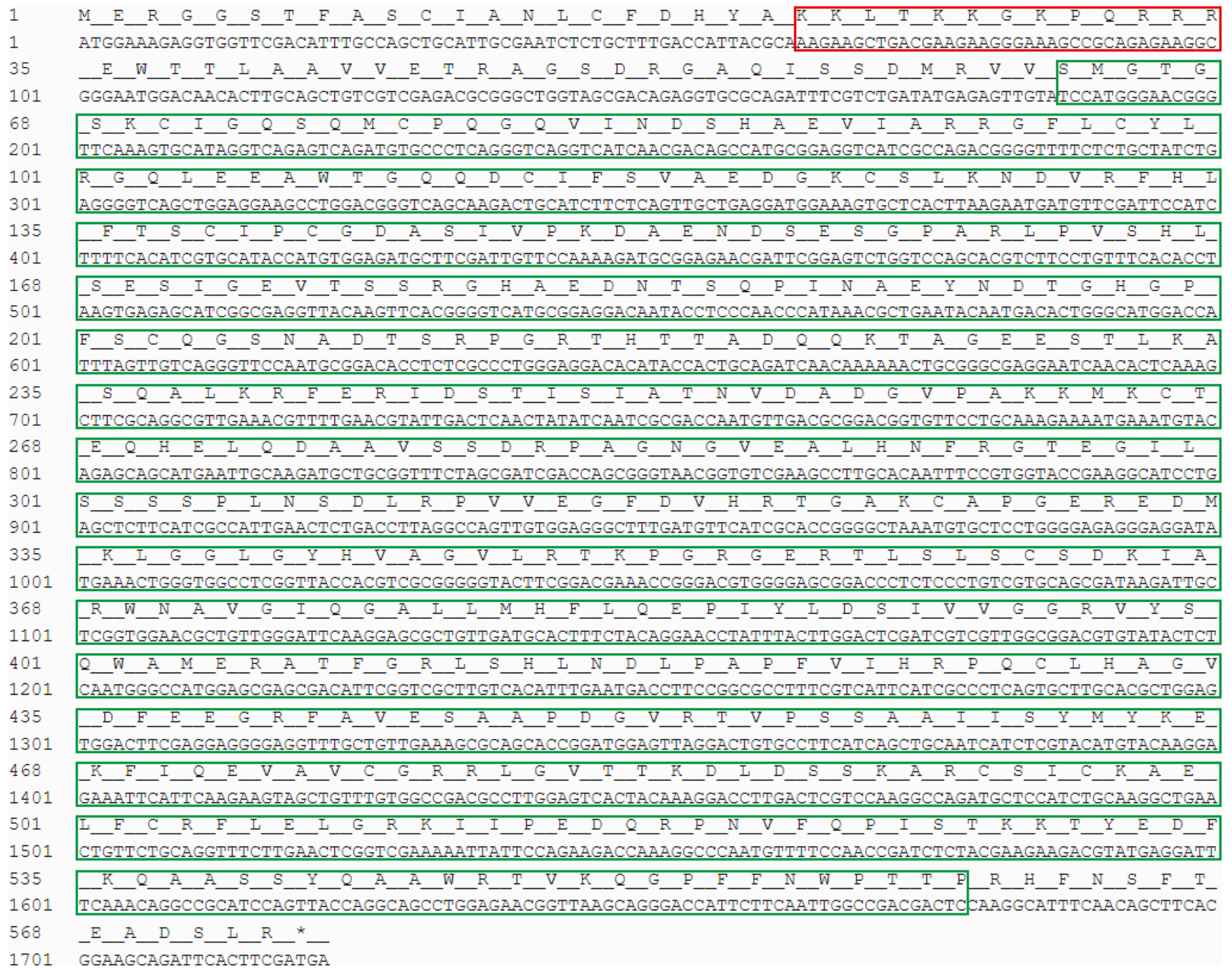

Fig. 1 Nucleotide and deduced amino acid sequences of earthworm *Ean-ADAT*. The open reading frame of *Ean-ADAT* gene consists of 1719 nucleotides encoding 573 amino acids. Predicted nuclear localization signal and deaminase domain are presented in the red and green boxes, respectively. The stop codon is indicated by an asterisk. Residue numbers for nucleotides and amino acids are indicated to the left row (colour figure online)

nervous system is controversial. Considering the high degree of homology between dADAT1 and the ADAR enzymes, it is possible that the embryonic expression of *dAdat1* is found in the central nervous system and is reminiscent of rRED2 (a brain specific member of the RNA-specific adenosine deaminase family) expression which is confined to the brain (Melcher et al. 1996; Keegan et al. 2000). As the function of ADATs during development and differentiation has been explored to a limited extent, the earthworm regeneration system would be helpful to elucidate the molecular mechanism behind how ADATs activities are involved in the nervous system.

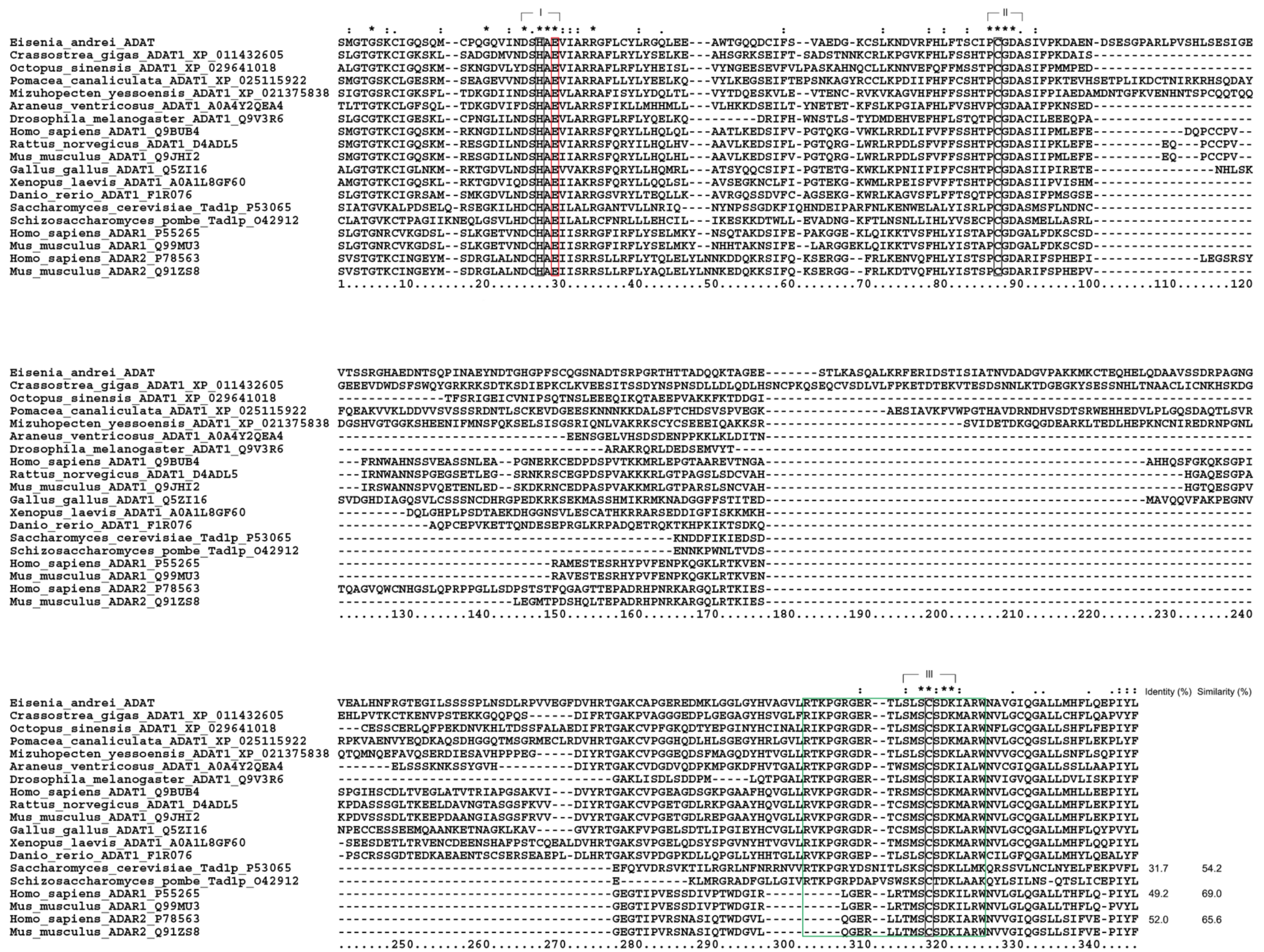

Fig. 2 Alignment of core deaminase domains from ADAT1 and ADAR family, showing relatively well conserved RNA recognition region of ADATs compared to vertebrate ADARs. The three active site motifs are bracketed, within which zinc-binding residues (histidine or cysteines) are represented in closed black boxes. A glutamate residue in the first active-site motif for catalytic activity is presented in the red box. A potential RNA recognition region flanking the third cysteine of the deaminase motif is presented in green box. The accession number of each sequence is denoted after the species name. Conserved residues are indicated with an asterisk (*), while (:) and (.) indicate conservative and semi-conservative substitutions. Sequence identity (%) and similarity (%) were expressed in comparison with human ADAR1, ADAR2 and *S. cerevisiae* Tad1p (colour figure online)

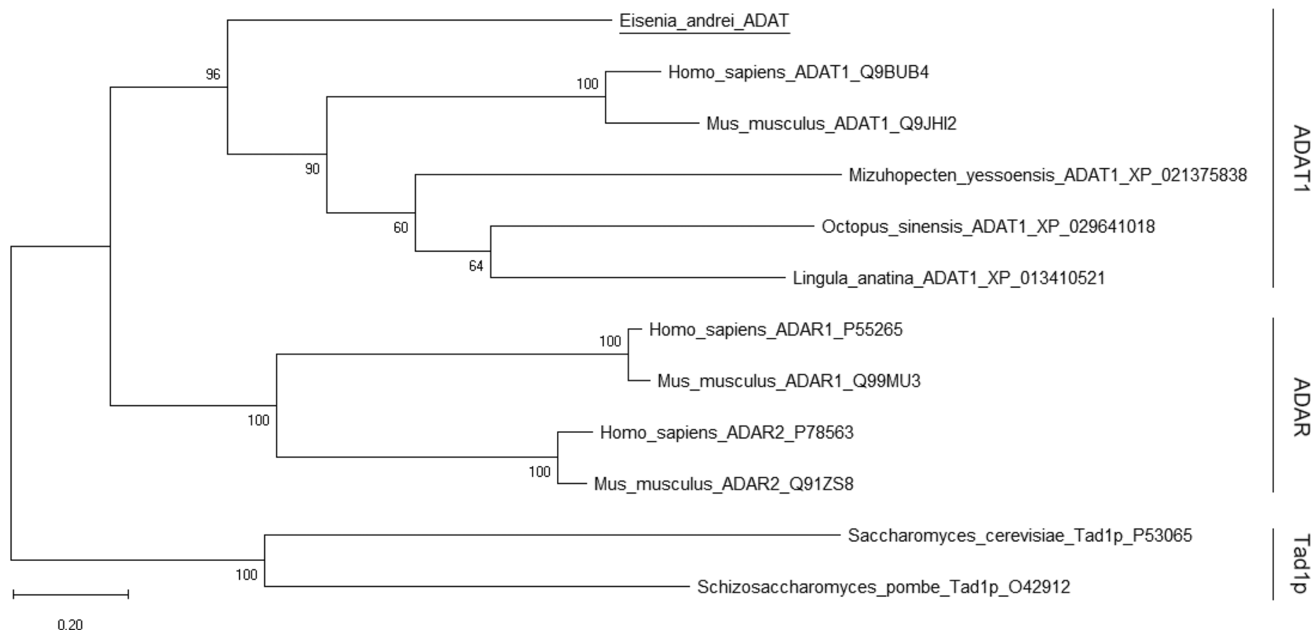

Fig. 3 Phylogenetic relationship among deaminase domains of ADAT1s, ADARs and Tad1ps based on the Maximum likelihood method. Phylogenetic analysis indicates that Ean-ADAT could be clustered with ADAT1s from other animal species employed, separately grouped from ADAR1s and ADAR2s of vertebrates (*Homo sapiens* and *Mus musculus*). The phylogenetic tree shows that Ean-ADAT has greater similarity to vertebrate ADARs than to yeast Tad1ps. The numbers at the nodes are scores from 100 bootstrap resamplings of the data

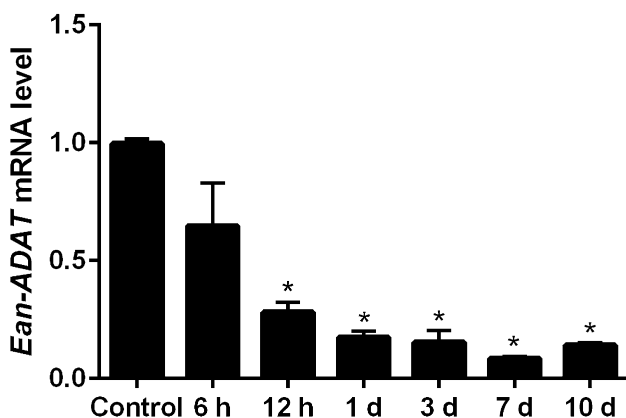

Fig. 4 Temporal expression analysis of *Ean-ADAT* mRNA using real time qRT-PCR during early tail regeneration of *E. andrei*. In the regenerating tail, *Ean-ADAT* mRNA expression began to decrease before 12 h post-amputation, showing the minimal expression around 7 dpa. The relative level was normalized to β-actin. The data, obtained from three independent experiments, are expressed as mean ± SEM. *Indicates statistical significance ($p < 0.01$) compared with un-amputated control

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Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interest regarding the publication of this.

Ethics approval and consent to participate Not applicable.

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