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Abyss1: A novel *L2*-like non-LTR retroelement of the snakelocks anemone *(Anemonia sulcata)*

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Abstract. Non-LTR retrotransposons are a diverse and taxonomically widely dispersed group of retroelements that can be divided into at least 14 distinguishable clades. Basal metazoans have not been examined in great detail for their retrotransposon content. In order to screen for the presence of reverse transcriptase (RT) related sequences in Cnidaria and Ctenophora, basal phyla of metazoans, PCR with highly degenerate oligonucleotides was performed and an RT-like sequence was identified from the sea anemone species *Anemonia sulcata*. Further screening identified a related element in another anemone species *Actinia equina*. Significant homology to non-LTR retrotransposon RTs was observed, particularly to *L2*-like elements of fish such as *Maui*. The sequence was not detected among other cnidarians and we have designated the *A. sulcata* and *A. equina* elements *Abyss1* and *Abyss2* respectively. Phylogenetic analysis of *Abyss1* compared with members of 14 known non-LTR retroelement clades suggests that the sequence represents a novel *L2* element.

Retrotransposons are mobile genetic elements representing a considerable fraction of the genomes of all eukaryotic species investigated to date (Xiong and Eickbush, 1990; McClure, 1993; Jurka, 1998; Weiner, 2002; Brosius, 2003). In the human genome, long interspersed nuclear elements (LINE-1) are the most abundant non-LTR retroelements representing the major source of RT activity in human cells. LINE-like elements have also been identified in the genomes of such diverse taxa as *Drosophila melanogaster, Bombyx mori* and fish (Di Nocera et al., 1988; Ichimura et al., 1997; Poulter et al., 1999). A common feature of these elements is their capacity to proliferate via reverse transcription of an RNA intermediate and re-insertion of the resulting cDNA into new genomic locations (Moran et al., 1999). Therefore, they are regarded as a potentially powerful evolutionary force in re-shaping the eukaryotic genome (Kidwell and Lisch, 1997; Moran et al., 1999).

Non-LTR retrotransposons can be divided into at least 14 distinct clades (Malik et al., 1999; Lovsin et al., 2001; Permanyer et al., 2003) providing a framework for comparison of novel elements identified from previously uninvestigated species. We have examined cnidarians and one ctenophore for the presence of RT related sequences. While other marine organisms have been examined for non-LTR elements (Poulter et al., 1999; Lovsin et al., 2001; Bouneau et al., 2003) basal metazoans have not. Cnidaria and Ctenophora are animal phyla that originated in the Precambrian period between 1,200 and 1,500 million years ago (Wang et al., 1999). Performing a screen for retroelements in this group has revealed the presence of an RT sequence *(Abyss1)* related to the *L2* elements of pufferfish *Takifugu rubripes* and the zebrafish *Danio rerio*. Of interest, an *Abyss1*-like sequence *(Abyss2)* could be found in a related sea anemone species, *Actinia equina*, but was not detected in the other tested species. Phylogenetic analysis of *Abyss1* suggests that it is a novel *L2* non-LTR retrotransposon.

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Materials and methods

DNA preparation and Southern blot analysis

Samples were hand collected at rock "Strombolicchio" near Stromboli Island, Aeolian Islands Archipelago, by W. Seifarth. High-molecular-weight DNA was prepared according to standard methods (Sambrook et al., 1989). Extractions were prepared from 9 representatives of the phylum Cnidaria, six members of the taxon Zoantharia *(Actinia equina, Anemonia sulcata, Aiptasia mutabilis, Astroides caligularis, Cladocora cespitosa, Parazoanthus axinellae)*, two members of the taxon Octocorallia *(Pteroeides spinosum, Virgularia mirabilis)*, one member of the taxon Scyphozoa *(Cotylorhiza tuberculata)* and one representative of the phylum Ctenophora *(Cestum veneris)*.

PCR, cloning, sequencing and Southern blot

Primers and PCR conditions used to identify RT related sequences are described in Seifarth et al. (2000). PCR products were cloned into the pUC19 vector and transformed into competent *E. coli* cells according to Sambrook et al. (1989). DNA was prepared for sequencing using a Plasmid Midiprep kit (Qiagen) according to manufacturer's instructions.

Alternatively, PCR was performed with an Expand Taq High Fidelity system (Roche) in a 50-µl final volume with approximately 100 ng template, 40 pmol each primer, 0.2 mM each dNTP. Reactions were cycled with a 5-min 94 °C denaturation step followed by 40 cycles at 94 °C for 30 s, 50 °C for 1 min, 72 °C for 1 min with a 10-min final extension at 72 °C. Cloning of products was performed by ligating PCR products into the pGEM-T vector system (Promega) following the manufacturer's protocol. Competent bacteria were transformed by electroporation. Screening for clones containing the correct insert was done by colony PCR in 30-µl volumes using M13 forward and reverse primers and a *Taq* DNA polymerase master mix system (Qiagen). Twenty cycles of PCR were performed (5-min 94 °C denaturation step followed by 20 cycles of 94 °C for 30 s, 50 °C for 1 min, 72 °C for 1 min with a 10-min final extension at 72 °C). Positive clones were identified by agarose gel electrophoresis of a 5-µl aliquot of the colony PCRs. Positive clone PCR products were purified of PCR primers and reagents using a Qiaquick PCR purification kit (Qiagen) according to the manufacturer. Purified products were submitted for DNA sequencing with the T7 and SP6 primers.

For Southern blotting, 10 µg of each DNA sample was digested with *Taq*I, blotted and hybridized with ^{32}P-labeled 111-bp *A. sulcata* cloned RT DNA. Hybridization was performed under moderately stringent conditions (5× SSC, 60 °C). Filters were exposed to X-ray film for 6 and 48 h and developed.

Rapid amplification of cDNA ends (RACE)

To obtain additional flanking sequences beyond the 111-bp region isolated from *A. sulcata* with the degenerate primers, a RACE (rapid amplification of cDNA ends) strategy was employed according to the protocol of Sorensen et al. (1999). Primers used were as follows: Dynal Dynabeads M-280 Streptavidin primers, all shown 5′ to 3′: (CAGTTCAATCGGATCCAGGAATTCNNNNNNNGGCCT, CAGTTCAATCGGATCCAGGAATTCNNNNNNNGCGCT, CAGTTCAATCGGATCCAGGAATTCNNNNNNNCCGGT, CAGTTCAATCGGATCCAGGAATTCNNNNNNNCGCGT), Biotin-labeled primers: (CAGTTCAATCGGATCCAGGAATTC, GAGAGGATCCCAATGTTCTGTAACACTTGGGAGGTC, GAGAGGATCCCTGGGTCCTTTGCTTTTTAGCATATAC), primers located within the original 111-bp *A. sulcata* RT isolate: (GAGAGGATCCGTATATGCTAAAAAGCAAAGGACCCAG, GAGAGGATCCGACCTCCCAAGTGTTACAGAACATTG). Multiple fragments from both 5′ and 3′ flanking regions were cloned and sequenced to generate a 1.3-kb RT fragment of the *A. sulcata* RT gene that we have called *Abyss1*. Additional primers for screening *A. equina* DNA for the presence of an *Abyss1* homolog were as follows: (L1-ACCGTTTGCCCGTGACCTGT, R1-GTAGATTGAAGGAGAGCAGC, R2-TCCCAAAGATGATGAGTTTAG). The *A. sulcata* and *A. equina* sequences have been deposited in GenBank (accession numbers AY392529 and AY392530 respectively).

Alignments and phylogenetic analysis

Sequences for *Takifugu rubripes* and *Danio rerio* were found by screening the WGS trace database of NCBI with the *A. sulcata* 1.3-kb sequence using the blastx algorithm (Altschul et al., 1990). The pufferfish screen yielded the *Maui* element as the most significant match. Screening of the zebrafish genome yielded two similar sequences (accession numbers AL591213 and AF086712). Representative members of the 11 non-LTR clades (Malik et al., 1999) were chosen for alignment and match those in Fig. 2 of Malik et al. (1999). The *NeSL* (Z82058) and the *Xiphophorus Rex1* (AF155728) elements were also added. Additional *CR1* elements were included such as *Sam6* (Z82275) and *T1* (M93689). An *L2* element from *Bombyx mori (samurai)* was also included (AB055391) as were two group II intron RT sequences from *Calothrix sp.* (X71404) and *Neurospora crassa* (S07649). Protein sequences were aligned using ClustalX (Thompson et al., 1997) and manually adjusted to improve the alignment and to constrain residues to those identified as conserved sequence blocks of the fingers/palm and thumb subdomains in the RT gene of non-LTR retrotransposons (Malik et al., 1999). Given the significant divergence of the various non-LTR clades from one another, a second alignment was generated for comparative purposes using Gblocks to identify regions of the alignment most suitable for phylogenetic analysis (Castresana, 2000). Low stringency conditions were employed and blocks identified on the Gblocks server (http://woody.embl-heidelberg.de/phylo/). Alignments are available from the authors by request.

Phylogenetic analysis was performed using Paup 4.0b. (Swofford, 2002) for both alignments. Neighbor-joining method (Saitou and Nei, 1987) and maximum-parsimony heuristic searches were implemented to generate phylogenetic trees. *Calothrix sp.* and *Neuospora crassa* group II intron RT sequences were used as outgroups and 1000 bootstrap replicates performed for both analyses.

Results

A degenerate primer pair mix was applied to DNA from 9 cnidarians and 1 ctenophoran. The expected product size was small (approximately 150 bp). Products were cloned and sequenced. Of the cloned sequences, only *A. sulcata* yielded an RT-like sequence. It bears similarity to non-LTR retrotransposon RT sequences. We have designated the sequence *Abyss1*. The sequence was used to probe a Southern blot of genomic DNAs (Fig. 1a). A signal was detected from *A. sulcata* and another anemone species *A. equina*. The remaining species were negative though faint bands were detected in *Cestum veneris, Cotylorhiza tuberculata* and *Cladocora cespitosa*. However, PCR methods did not reveal RT-like sequences with the degenerate primers employed, so the bands could be non-specific.

To better characterize the relationship of the *A. sulcata Abyss1* element to other non-LTR retrotransposons, a longer fragment was obtained by RACE PCR (Sorensen et al., 1999). Employing this method, a 1.3-kb fragment of the RT gene was isolated. As DNA from this sample was limited, further sequence determination was not attempted.

Two primer pairs based on the 1.3-kb sequence and conserved regions in other known RTs were used to screen *A. equina* DNA to confirm the Southern blot results. A 111- and a 230-bp fragment were obtained and clones sequenced (Fig. 1b). *Pteroides spinosum* DNA was also tested and was negative consistent with the Southern blot results. From the sequences obtained from *A. equina*, we conclude that a related element exists in *A. equina* that is undetectable in other tested species. The *A. equina* element we have designated *Abyss2*. However, we cannot exclude the possibility that the element exists in all specimens and has diverged to the point that it is not detectable by hybridization or with the various primers, including the degenerate primer mix, used in PCR experiments for the other lineages.

Blastx searches of *Abyss1* sequence consistently yielded high similarity scores for an *L2*-like element in zebrafish *(Danio re-*

a

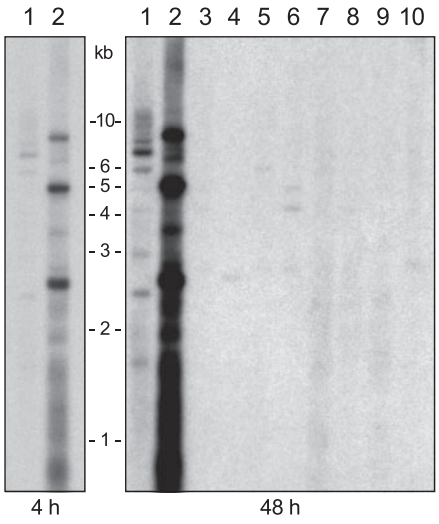

Fig. 1. (**a**) Southern blot analysis of cnidarian and ctenophore genomic DNA samples derived from (1) *Actinia equina,* (2) *Anemonia sulcata*, (3) *Astroides caligularis*, (4) *Cestum veneris*, (5) *Cotylorhiza tuberculata*, (6) *Cladocora cespitosa*, (7) *Virgularia mirabilis*, (8) *Parazoanthus axinellae*, (9) *Aiptasia mutabilis*, (10) *Pteroeides spinosum*. The 111-bp *A. sulcata* fragment originally isolated using the multiplex oligo primer mix was used as a probe of *Taq*I digested genomic DNA. The left panel is a 6-hour exposure of lanes 1 and 2, the right panel a 48-hour exposure of the entire blot. (**b**) *Actinia equina* clone sequences for a 111- and 230-bp segment of the *Abyss2* element detected by Southern blot in **a**. The *Abyss1* element was used as a reference. Dots represent identity to the reference sequence. Gaps are represented by the symbol "–". There was an amino acid difference between the *Abyss1* sequences derived from the small and large fragments (not shown). In addition, there is some clone variation in the *A. equina* sequences. Though this could represent polymerase errors, it could also represent different copies of the *Abyss2* elements that have slightly diverged.

b

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Abyss1          GGTGTGACACAAGGAAGTATACTGGGTCCTTTGCTTTTTAGCATATACATCAATGACCTCCCAAGTGTTACAGAACA
A. equina 1a    .....AC....-........CT....A..A.................T..A..C..T........C...C.T.....
A. equina 2a    .....AC....G........CT.......A.................T..A..C..T........C...C.T.....
A. equina 3a    .....AC.....-......CT....A..A.................T..A..C..T........C...C.T.....
A. equina 1b    .....AC....G........CT.......A.................T..A..C..T........C...C.T.....
A. equina 2b    .....AC....G........CT.......A.................T..A..C..T........C...C.T.....
A. equina 3b    .....AC....G........CT.......A.................T..A..C..T........C...C.T.....

Abyss1          TTGCACAACTCAATGTTACGTGGATGACACCAAACTGCTGCTCTCCTTCAATCTACAGGACCAGGCATGTACTGTG
A. equina 1a    ...T..T.............T..C.........T.
A. equina 2a    ...T..T.............T..C.........T.
A. equina 3a    ...T..T.............T..C.........T.
A. equina 1b    ...T..T.............T..C.........T..T....TA.T........C........ACA..A..T...T
A. equina 2b    ...T..T.............T..C.........T..T....TA.T........C........ACA..A..T...T
A. equina 3b    ...T..T.............T..C.........T..T....TA.T........C........ACA..A..T...T

Abyss1          TCAAGACTTAATCAAGACCTCCACAGAATAACTATCTGGACTCTTGACAATTACCTACTGCTAAACCCTGACAAAA
A. equina 1a
A. equina 2a
A. equina 3a
A. equina 1b    ..................TTT...........AT.....A..C.............T.....T..C.......
A. equina 2b    ..................TTT...........AT.....A..C.............T.....T..C.......
A. equina 3b    ..................TTT...........AT.....A..C.............T.....T..C.......
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rio) and the *Maui* element of pufferfish *(Takifugu rubripes)*. Screening of the NCBI WGS tracefiles yielded sequences equivalent in length to the *A. sulcata* RT gene for both fish species. The fish sequences and *A. sulcata* sequences were aligned to representative members of non-LTR retrotransposons and to group II intron RTs from *Calothrix sp.* and *Neurospora crassa*. An alignment of the amino acid residues used in the phylogenetic analysis for the *L2* elements compared to *Abyss1* and a *CR1* element is shown in Fig. 2. For the full dataset alignment, the representative groups chosen match those used in Malik et al. (1999) and Permanyer et al. (2003). Similarly, the alignment was constrained to the most conserved amino acids of the identified conserved peaks of the fingers-palm subdomains and thumb subdomains of RT identified by Burke et al. (1999) and Malik et al. (1999) that were included in the *A. sulcata* sequence obtained (Fig. 2). A second alignment was generated by submitting the alignment to Gblocks analysis (Castresana, 2000). This program allows for the identification of phylogenetically useful sites and elimination of positions that contribute noise. The age and divergence of the RT sequences necessarily reduced the number of sites significantly (81 amino acids remained in the resulting alignment compared to 377 in the original alignment).

Phylogenetic analysis consistently grouped *Abyss1* with *Maui*, zebrafish, and the *Bombyx mori samurai* element (Fig. 3). Bootstrap support was generally low as has been previously observed with the phylogenies based on non-LTR RTs (Malik et al., 1999). Due to the reduced number of sites in the Gblocks alignment overall phylogenetic resolution was substantially reduced and bootstrap support did not reach statistical significance (not shown). Using *CRE2* as an outgroup (Malik et al., 1999) produced similar results to the full alignment but little

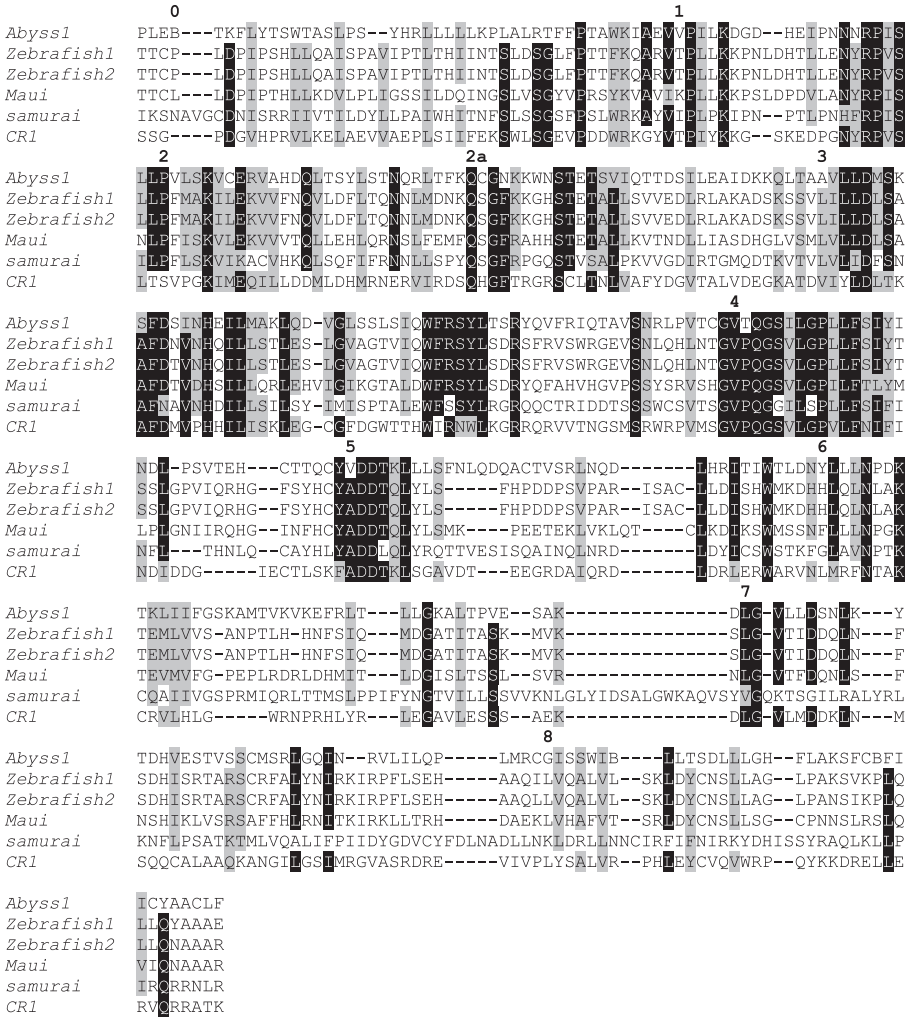

Fig. 2. Multiple sequence alignments of the amino acids used in phylogenetic analysis for the *L2* elements examined in this study. A *CR1* element is included as a reference. Highly conserved sites (in at least 75 % of the sequences) are shaded in black, sites with high similarity in grey and non-conserved sites are unshaded. Numbers above the sequence refer to conserved peaks defined by Malik et al. (1999).

improvement in bootstrap values (data not shown). However, the results of neighbor-joining analysis showed a clear association of *Abyss1* with other *L2* clade members consistent with the blastx results (Fig. 3).

Discussion

The *Abyss1* non-LTR retroelement is the first described retrotransposon from cnidarians. It is of interest that the distribution is not uniform. *Abyss1* was not clearly detectable by PCR or hybridization methods in any other cnidarian or in the one ctenophore sample tested. A similar element was found only in the related anemone species *Actinia equina* which was designated *Abyss2*. Related elements are found in fish, sea urchin and molluscs (Lovsin et al., 2001). Some investigators have suggested the possibility that non-LTR retrotransposons are capable of horizontal transfer among distantly related taxa (Volff et al., 2000). However, *Abyss* related elements are not necessarily absent from the other cnidarian genomes. For example, the *Zebulon* element of the pufferfish *Tetraodon nigroviridis* was not detected by Southern blot analysis in other related fish taxa (Bouneau et al., 2003). It was found in Southern blot negative species by screening genome databases that have substantial portions of the genome sequenced. No such databases exist for cnidarians at present. Rather than postulate multiple loss or gains of this element, it is more likely *Abyss1* and *Abyss2* have been more recently or historically active in the anemones than other cnidarians. The result of this activity is a higher copy number and perhaps greater sequence homology which thus allowed for the detection of both *Abyss1* and *Abyss2* with the methods employed in this study. Less active or non-active elements in other cnidarians may have been lost or diverged to the point that they are not easily detectable by PCR or hybridization methods.

The non-LTR retroelements can be aligned such that conserved fingers/palm and thumb subdomains of the right hand structure of the retroviral RT are found in most members of each clade (Malik et al., 1999). The fingers/palm subdomains and part of the thumb domain were covered in the fragment of the RT gene retrieved for *Abyss1*. All conserved blocks of the subdomains could be found when comparing the *L2* elements to one another and showed substantial conservation among the different elements.

Another feature of *Abyss1* is that it is most similar to the pufferfish *Maui* element and a *L2*-like element from zebrafish.

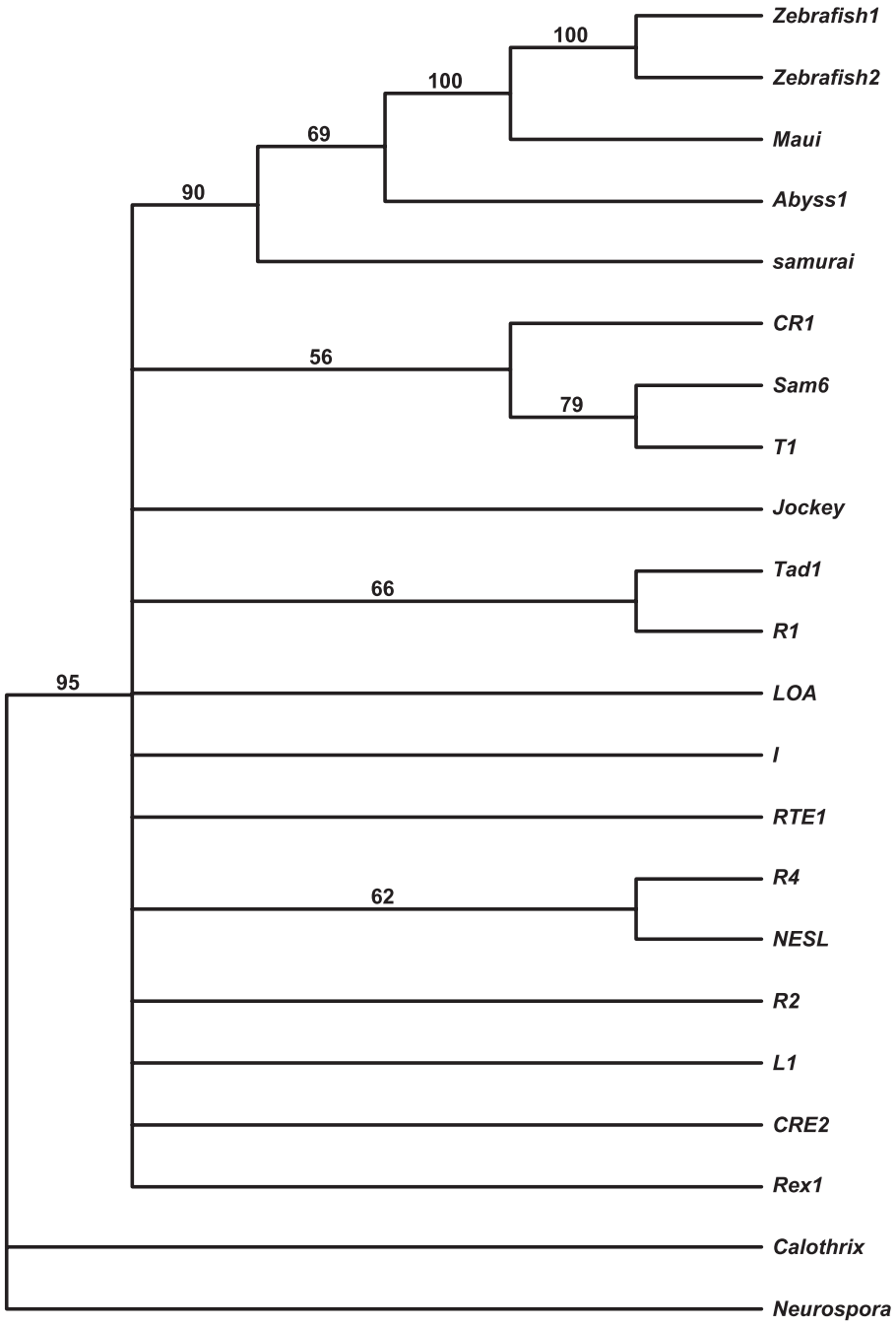

Fig. 3. Phylogeny of the *Abyss1, Maui*, zebrafish, and *B. mori samurai L2* elements in relation to representatives of known classes of non-LTR retrotransposons. A neighbor-joining tree is shown with bootstrap support of 50 % or greater indicated. The tree was rooted with *Calothrix sp.* and *Neurospora crassa* group II intron RT sequences.

The *Maui* element shares many features with *CR1* elements from other organisms such as absence of a poly-A tail (Poulter et al., 1999). However, *Maui* has recently been assigned to the *L2* clade of non-LTR retrotransposons (Lovsin et al., 2001; Permanyer et al., 2003). Phylogenetic analysis of the *Abyss1* element suggests that it groups with the *L2* clade. Statistical support was seen for this grouping, but strong support for an association of *Abyss1* and other *L2* elements with *CR1* over other related clades such as *Jockey* and *Tad1* was not found. In addition, blastx searches do not group *Abyss1* with another family of *CR1*-like elements in fish, the *Rex1* type retroelements (Volff et al., 2000). Thus, *Abyss1* is a *L2* non-LTR retroelement.

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