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Zoological Research

Construction of a cDNA Library from the Testis and Sequence Analysis of the Ubiquitin Gene from *Rana nigromaculata*

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Abstract: A full-length cDNA library from the testis of dark-spotted frogs (*Rana nigromaculata*) was constructed with the SMART (switching mechanism at 5′ end of RNA transcript) technique. Total RNA was extracted from the testis and reverse transcripted into full-length cDNA using PowerScript reverse transcriptase. The first-strand cDNA was amplified using long-distance PCR (LD-PCR). After *Sfi* Ⅰ digestion and fractionation, cDNA (> 500 bp) was ligated to λ TriplEx2 vector and packaged with Gigapack® Ⅲ Gold Packaging Extract. The titers of optimal primary libraries were 2.0×10^6 pfu/mL and 2.4×10^6 pfu/mL and the titers of the amplified libraries were 0.48×10^9 pfu/mL and 3.0×10^9 pfu/mL, respectively. The percentages of recombinant clones of primary libraries and amplified libraries were all over 90%. The libraries were converted into pTriplEx2 plasmids in *E. coli* BM 25.8 strain. The insert sizes were measured by PCR which showed most fragments were over 500 bp and the average length was 1.0 kb approximately. A positive clone of 1 171 bp was sequenced and named RnUb based on sequence similarity with the known ubiquitin genes in GenBank. This sequence was a full-length cDNA with complete coding sequences, which indicated that the library built a base for screening the full-length cDNA. These data showed that this library attained to the requirements of a standard cDNA library. This library provided a useful resource for the functional genomic research of *Rana nigromaculata*.

Key words: *Rana nigromaculata*; Total RNA; SMART; cDNA Library; Ubiqutin

黑斑蛙精巢组织 cDNA 文库的构建及泛素基因序列的分析

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摘要: 采用 SMART(switching mechanism at 5' end of RNA transcript)技术构建了黑斑蛙(*Rana nigromaculata*)精巢组织全长 cDNA 文库。一步法提取成体蛙精巢组织总 RNA, 用 Powerscript™反转录酶逆转录合成第一链 cDNA; 再用 LD-PCR 合成双链 cDNA; 经过 *Sfi* Ⅰ酶切和 Chroma spin-400 柱分离后, 500 bp 以上的片段与 λTriplEx2 载体连接, 再用 Gigapack® Ⅲ Gold Packaging Extract 包装蛋白包装, 即获得原始文库。原始文库进行扩增后得到扩增文库。经检测原始文库的滴度分别为 2.0×10^6 pfu/mL 和 2.4×10^6 pfu/mL, 扩增后的文库滴度分别为 0.48×10^9 pfu/mL 和 3.0×10^9 pfu/mL, 重组率均在 90%以上。通过 *E. coli* BM25.8 菌株将文库转化为 pTriplEx2 质粒, 挑选一阳性克隆进行 PCR 检测, 其插入片段平均长度约为 1.0 kb。挑取一阳性克隆分别从 5′端和 3′端进行测序, 得到一长约 1 171 bp 的序列。经序列分析知, 该序列含有完整的编码框, 可编码 305 个氨基酸, 是一全长 cDNA 序列。提示所建文库是可以用于全长 cDNA 的筛选。结果表明, 所构建的黑斑蛙精巢组织 cDNA 文库的各项指标均满足建库的基本要求。该文库将为蛙类及两栖类的已知或未知的功能基因及新基因的获得及其研究提供可靠资源; 另外, 该文库还将为研究蛙类动物的性别决定和分化相关基因及其表达提供直接的分子资料。

关键词: 黑斑蛙; 总 RNA; SMART 技术; cDNA 文库; 泛素

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To date, sequencing and characterization of complementary DNA (cDNA), which represents a direct link to functional genomics, is a powerful means of identifying genetic polymorphisms and is essential for determination of differential gene expression (Dias et al, 2000; Wiemann et al, 2003; Draper et al, 2002), not only in humans but also in other species. However, full-length cDNAs of very few species can be obtained from the public database currently. So far, there are 1 576 487 expressed sequence tags (ESTs) of Amphibia and 11 756 full-length cDNAs from *Xenopus tropicalis* and *X. laevis*, the model organisms of Amphibia, obtained from the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi and http://www.ncbi.nlm.nih.gov/FLC/getmgc.cgi). Very limited full-length cDNAs from other species of amphibians were found in the public database. To identify more full-length genes of amphibians including characterization-specific expressed, new or unknown genes and study their functions further, construction of cDNA libraries from other species of amphibians is an efficient method.

The widespread dark-spotted frog, *Rana nigromaculata*, is a representative of Ranidae, Anura, Amphbia (Chen, 1991), and plays an important role in the ecological balance of nature. The amphibians represent a bridge in the evolution of vertebrates from aquatic to terrestrial. They have highly specialized morphological and functional characteristics to adapt to different environments (Fei, 1999). The sex determination and differentiation of amphibians were not as typical as other vertebrates. In mammals and birds, sex determination and differentiation are chiefly controlled by genetic factors and many reptiles exhibits typical temperature determination (Zhou et al, 2004). However, it isn't the same in amphibians. There is no difference in sex chromosomes of many amphibians, even no sex chromosomes in some species (Zhou et al, 2004). Sex determination in the majority of amphibians is probably influenced by environmental factors (Zhou et al, 2004), however, in all amphibians, genetic sex determination (GSD) seems to operate (Schmid & Steinlein, 2001). Genetic factors such as DMRT1 and DAX1 may be involved in the differentiation of testis (Shibata et al, 2001; Sugita et al, 2001). Despite the accumulated evidence that GSD is operating in Anura and Urodela, there is little substantial information about how it functions (Schmid & Steinlein, 2001).

On account of background mentioned above, in this paper we isolated total RNA of the testis from adult dark-spotted frogs to construct a full-length cDNA library and expected that this library may present some new molecular materials for this species.

1 Materials and Methods

1.1 Animals and reagents

Adult dark-spotted frogs (*Rana nigromaculata*) were obtained from Wuhu, Anhui, China. A total RNA Extraction kit (Trizol® Reagent) was purchased from Invitrogen Company. A cDNA Library construction kit (SMARTTM cDNA library Construction Kit) was obtained from Clontech Company. The Gigapack®Ⅲ Gold Packaging Extract was the product of Stratagene Company. A 100 bp-ladder marker (from 100 to 2000 bp) was purchased from Vitagene Company.

1.2 Total RNA extraction

Total RNA was isolated from the testis of two adult frogs according to the protocol of Trizol® Reagent and was dissolved in Milli-Q grade water. The purity of total RNA was checked with measuring the absorbance of ultraviolet light at 260 nm (A_{260}) and 280 nm (A_{280}). To examine the integrity and stability of total RNA, a 5-μL sample and another 5-μL sample that was incubated at 37℃ for two hours before electrophoresis, were run on a denaturing formaldehyde agarose gel.

1.3 cDNA library construction

1.3.1 cDNA synthesis and amplification Approximately 0.9 μg of RNA was reverse-transcripted into first-strand cDNA by PowerScript™ reverse transcriptase. Subsequently approximately 2 μL of first-strand cDNA sample was amplified using long-distance PCR (LD-PCR). The product was analyzed by running a 5-μL sample on a 1.1% agarose/EtBr gel alongside DNA size markers.

1.3.2 Ligation of cDNA to λTriplEx2 vector and packaging The double-stranded cDNA (ds-cDNA) was treated with protein K, and followed by *Sfi* Ⅰ digestion and size fractionation. The first four peak fractions containing cDNA (> 500 bp) were pooled together for packaging.

Three parallel ligation reactions were performed using three different ratios of cDNA to the λTriplEx2 vector (1∶0.5, 1∶1, 1∶1.5) to ensure the optimal possible library was obtained. Each of the three ligations was packaged with Gigapack Ⅲ Gold Packaging Extract.

1.4 Titration of the primary library and determining the percentage of recombinant clones

The primary library was diluted by 1∶5, 1∶20, 1∶50 and 1∶100. The number of clones was counted to

calculate the library titer according to the formula: pfu/mL = number of plaques × dilution factor × 10^3 μL/mL (μL of diluted phage plated). The recombination efficiency was identified by blue/white screening in *E. coli* XL1-Blue.

1.5 Library amplification and conversion of cDNA library to plasmid

The primary libraries were amplified on 40 plates of 12 cm diameter. The titer and recombinant efficiency were calculated using the same method as the above. A small quantity of *R. nigromaculata* cDNA library was converted into plasmid in *E. coli* BM 25.8 strain.

One hundred clones were randomly picked to check the insert fragments by PCR using 5′ sequencing primer and 3′ sequencing primer. Sequencing analysis was completed with an ABI 3730 automatic DNA sequencer.

1.6 cDNA cloning of *R. nigromaculata* ubiquitin gene and comparison to other homologues

One full-length cDNA of *R. nigromaculata* ubiquitin gene was gained using random sequencing analysis and was identified using the BLAST in the NCBI database in order to find homologues from other species. All the deduced amino acid residues were aligned using Clustal-X (Thompson et al, 1997).

2 Results and Analysis

2.1 Total RNA extraction

The key to construction of an excellent quality cDNA library is to prepare good quality RNA first. The ratio of the readings at A_{260} and A_{280} (A_{260}/A_{280}) was approximately 1.82. The concentration of RNA was approximately 0.9 μg/μL according to the absorbance of ultraviolet light at 260 nm. As shown in Fig. 1, two bright bands of 18*S* rRNA and 28*S* rRNA can be seen clearly, indicating that the total RNA is integrated and stable enough for cDNA library construction. The stability of RNA was verified by incubating a small sample at 37℃ for two hours. There was little difference between the incubated and the fresh samples, appeared in Fig. 1. If the RNA appeared to be unstable, the incubated sample will be weaker than the unincubated sample. The total RNA isolated from the testis of frogs of *R. nigromaculata* was pure, integrated and stable for cDNA library construction.

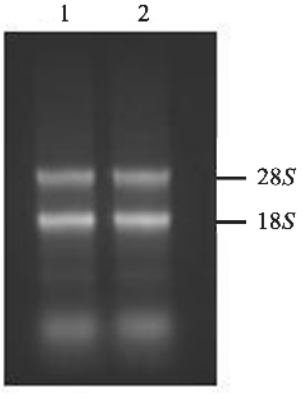

Fig. 1　Total RNA from testis of *Rana nigromaculata*
Lane 1: a sample of 5-μL total RNA; Lane 2: a sample incubated at 37℃ for 2 h.

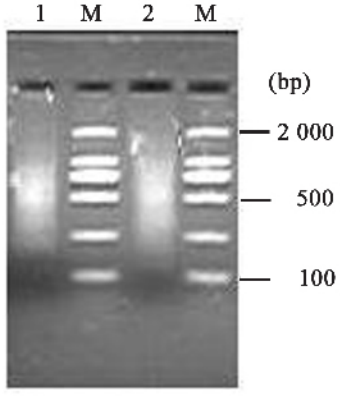

Fig. 2　The products of LD-PCR
M: marker; Lane 1: the product of LD-PCR with 25 cycles; Lane 2: the product of LD-PCR with 22 cycles.

2.2 Synthesis of cDNA

The SMART technique was used to reverse transcript RNA into cDNA, which was gained by LD-PCR with the adaptor primers. Incomplete cDNA and cDNA transcribed from poly A- RNA will lack the SMART anchor and not be amplified.

As shown in Fig. 2, the ds-DNA appeared as a smear of bands of 0.5 – 2 kb on the gel. The product of 25 cycles appeared as an intense low-molecular-weight (< 500 bp) smear and subsequently the product of 22 cycles was chosen for next step. Fragments smaller than 500 bp were eliminated by cDNA fractionation to avoid that the library had a preponderance of very small inserts or/and apparently non-recombinant clones.

2.3 Characterization of cDNA library

As shown in Tab. 1, the titers of three primary libraries constructed from three different ratios of cDNA to vector (1∶0.5, 1∶1, 1∶1.5) were (1) 0.8×10^6 pfu/mL, (2) 2.0×10^6 pfu/mL, (3) 2.4×10^6 pfu/

mL, which showed that the optimal ratio was 1∶1.5. The capacity of the three libraries was as follows (1) 0.4×10^6 clones, (2) 2.0×10^6 clones, (3) 2.4×10^6 clones. The primary libraries with titers of over 1.0×10^6 pfu/mL were both amplified. The titers of amplified libraries were 0.48×10^9 pfu/mL and 3.0×10^9 pfu/mL, respectively. The recombination efficiencies of the primary and amplified libraries were all over 90%, appeared in the Tab. 1. The insert ratio and the average length of inserted fragments were measured by PCR, as shown in Fig. 3. The average size was approximately 1.0 kb and majority inserts were all over 0.5 kb.

2.4 Sequence analysis of the ubiquitin gene

Tab. 1 Characterization of the primary and amplified cDNA libraries of *R. nigromaculata*

Number of primary libraries	Ligation ratio of cDNA to vector	Titer of primary libraries (pfu/mL)	Capacity of primary libraries (clones)	Recombination efficiency of primary libraries (%)	Titer of amplified libraries (pfu/mL)	Capacity of amplified libraries (clones)	Recombination efficiency of amplified libraries (%)
(1)	1∶0.5	0.8×10^6	0.4×10^6	97.4	–	–	–
(2)	1∶1	2.0×10^6	1.0×10^6	95.6	0.49×10^9	3.2×10^{11}	90.3
(3)	1∶1.5	2.4×10^6	1.2×10^6	98.7	3.0×10^9	9.6×10^{11}	92.7

Capacity of library = Titer of library × Volume of library.

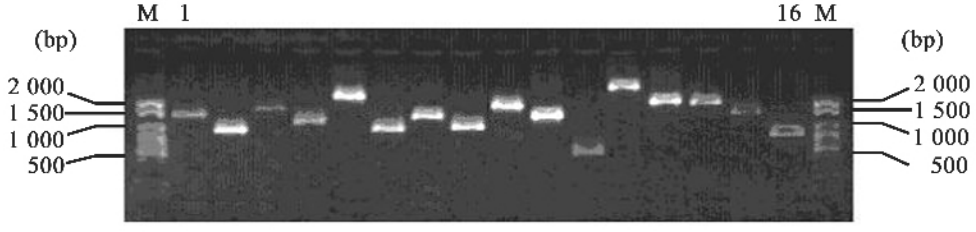

Fig. 3 Part of the inserted cDNA fragments randomly selected from the amplified cDNA library
M: marker; Lane 1 – 16: 16 inserted cDNA fragments.

A random positive clone was sequenced and a fragment of 1 171 base pairs (bp) was obtained (GenBank accession number DQ520795 in the NCBI database). The sequence information is shown in Fig. 4. The fragment has four initial codons of ATG, a termination codon TAA, a poly-A tail of 30 As and a signal sequence of AATAAA for adding the poly-A tail. The open reading frame (ORF) of 918 bp (from the140th bp to the 1 058th bp) encodes 305 amino acids (aa). Four repeated units (r1 from the 140th to the 367th; r2 from 368th to 595th; r3 from 596th to 823rd and r4 from 824th to 1 058th) were shown in the sequence, of which the first three units are all 228 bp encoding 76 aa and the last unit consists of 234 bp encoding 77 aa. The sequence was a full-length cDNA, with a complete coding sequence (CDS), indicating that the library constructed in this paper provided a physical resource for full-length cDNA clones.

The four nucleotide repeats in Fig. 4 show similarity with each other: r1 and r2 show 91% nucleotide homology; r3 has 35 different nucleotides from r1 and they have 85% homology; r4 and r1 are 77% homologous differing at 43 sites. The nucleotide changes among the four repeats are at the second or/and third nucleotide position that are silent variations, but the base 'T' at position 650 of r3 is different from the base 'C' in other repeats results in the amino acid change at position 19 (Ser not Pro in repeat 3). It is unclear if this is due to experimental error or if it is a specialized change in this species.

As shown in Fig. 5, the RnUb protein has similar characteristics and shows at least 96% similarity with polyubiqutin of other species, which indicates that the ubiquitin gene is conserved through the process of evolution. Except the last aa, the predicted amino acid sequences of ubiquitin of the dark-spotted frog, human, mouse, the western clawed frog and the salmon trout only differ at the 171st aa (that is the 19th aa in repeat 3 shown in Fig. 4), where a serine exists in RnUb while a proline exists in the other four species. This may infer that the change of nucleotide at 650th position in RnUb is due to experimental error. We assume that there would be strong selective pressure in the evolution of the ubiquitin gene, as most substitutions are silent variations at the nucleotide level and few are amino acid replacement substitutions.

```
5′ UTR                                                    1 GCAGGACAAGGAAGGTATC 19
          20 CCTCCAGATCAGCAGAGGCTGATTTTTGCTGGCAAGCAGCTAGAAGATGGCCGCACCCTC 79
          80 TCTGACTACAATATCCAGAAGGAATCCACTCTGCACTTGGTGTTGCGTCTGAGGGGTGGG 139
           1 M  Q  I  F  V  K  T  L  T  G  K  T  I  T  L  E  V  E  P/S S 20
(r1)     140 ATGCAAATCTTTGTCAAGACCCTAACTGGCAAAACTATTACTTTGGAAGTTGAGCCAAGT 199
(r2)     368 ----------------------------------------------------------CCA--- 427
(r3)     596 ----------T----A--A--G--C----------G--G---TCC--- 655
(r4)     824 ---------A-----G------T-------T-----C------C-A--------ACCA--C 883
          21 D  T  I  E  N  V  K  A  K  I  Q  D  K  E  G  I  P  P  D  Q 40
(r1)     200 GACACAATTGAGAACGTCAAAGCTAAAATCCAGGACAAGGAAGGTATCCCTCCAGATCAG 259
(r2)     428 --------------T--------------T--------A--------T------------ 487
(r3)     656 --------------T--------------T--------A--------T------------ 715
(r4)     884 -----T-----A--T--G--G--C-----T--A--T--A-----C--T------------ 943
          41 Q  R  L  I  F  A  G  K  Q  L  E  D  G  R  T  L  S  D  Y  N 60
(r1)     260 CAGAGGCTGATTTTTGCTGGGAAACAGCTAGAAGATGGCCGCACCCTCTCTGACTACAAT 319
(r2)     488 ----------------A--C----------------T--------T--A--------C 547
(r3)     716 --A--A----------A--C----------------T--------T--A--------C 775
(r4)     944 --A-----T--C-----A--A--------G----------T--TT-G--A--T--T--C 1003
          61 I  Q  K  E  S  T  L  H  L  V  L  R  L  R  G  G  F  stop
(r1)     320 ATCCAGAAGGAATCCACTCTGCACTTGGTGTTGCGTCTGAGGGGTGGG             367
(r2)     548 -----A----------T-A--TC-T--CC-C--A-----A------              595
(r3)     776 --------A-----T--C--C--TC-T--TC-C-----A--A--A--A            823
(r4)    1004 --T-----A-----T----------C-T--C-----C----------TTTTTAA      1057
3′ UTR  1058 GATGACAATCAGCAGTACTGCTTTTCCTTGCATTTAAGTTTATGCTTCTCTTGCACTTTT 1117
        1118 GTTTCAATAAAGTAATTGCATTCCAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
```

Fig. 4　Full-length cDNA sequence and deduced amino acid sequence of ubiqutin gene of *R. nigromaculata*

5′ UTR: 5′ untranslated region of 139 bp; 3′ UTR: 3′ untranslated region of 84 bp; r1, r2, r3 and r4 indicate the four repeats; '-': the short lines means the same nucleotide as the first r1; ATG: initiation codon; TAA: termination codon; AATAAA indicates a polyadenylation signal sequence; P/S means 'P' or 'S' at position 19 in the aa sequence; The aa of the 19th position are encoded by CCA of r1, r2 and r4 or by TCA of r3; CCA encodes the P (Pro) while TCA encodes S (Ser).

3　Discussion

Conventionally-generated cDNA libraries contain a high percentage of 5′-truncated clones (Wellenreuther et al, 2004). Fortunately, the SMART technique can overcome it. There are four advantages of using this method to construct full-length libraries at least: First, the terminal transferase activity of PowerScript reverse transcriptase adds a few additional nucleotides, primarily deoxycytidine, to the 3′ end of the cDNA, when the RT reaches the 5′ end of the mRNA. The SMART Ⅳ Oligo, with an oligo (G) at its 3′ end, base-pairs with the deoxycytidine stretch, creating an extended template. Second, cDNA synthesis employs long-distance PCR for generating full-length cDNA with two adaptor primers. Incomplete cDNAs and cDNA transcribed from poly A- RNA will lack the SMART anchor and not be amplified (Chenchik et al, 1998). Third, fragments smaller than 500 bp were eliminated by cDNA fractionation. Fourth, asymmetrical *Sfi* Ⅰ (A & B) restriction enzyme sites at the adaptor primers at the 5′ and 3′ cDNA ends are extremely rare in mammalian DNA; therefore, all the cDNAs remain intact after *Sfi* Ⅰ digestion Methylation steps are eliminated; valuable internal restriction sites are preserved. The vector λTriplEx2 containing the asymmetrical *Sfi* Ⅰ sites in the multiple cloning site (MCS), eliminates adaptor ligation and facilitates directional cloning. Actually the SMART method has become one of the conventional methods to obtain the full-length cDNA (Chenchik et al, 1998).

There are three chief aspects that identify the quality of a cDNA library. One is the capacity of the library. The capacity should be more than 1.7×10^5

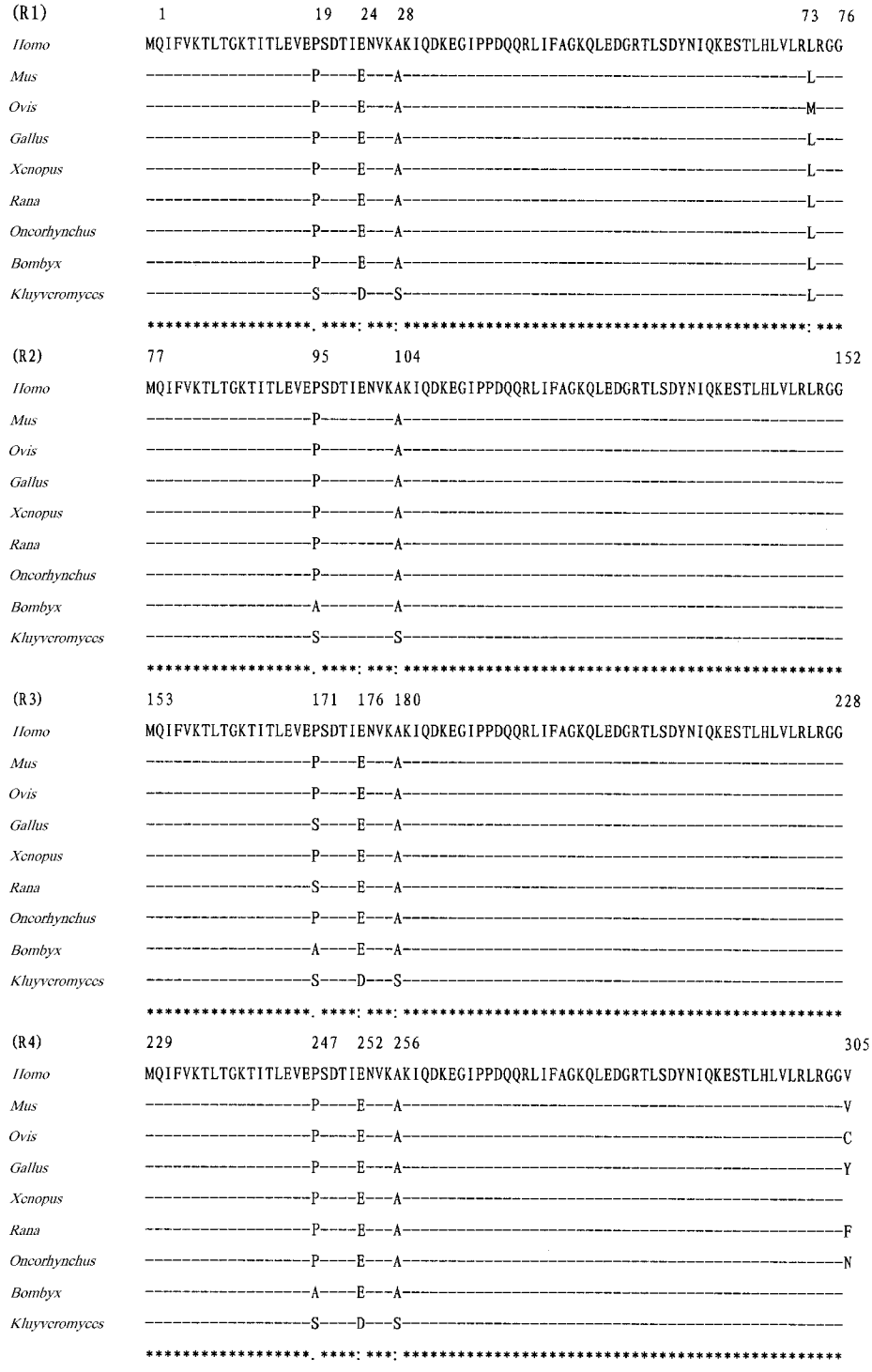

Fig. 5 Similarity of putative amino acid sequences of RnUb with the ubiqutin gene from nine other species

The asterisk indicates identical aa residues, and '.' or ':' indicate strongly positive or weakly positive residues. The 'S' is the only amino acid by which the frog, human, mouse and western clawed frog ubiquitin gene differed. The four repeat units were marked with (R1), (R2), (R3) and (R4). The following eight species have been used in this figure: three mammals, *Homo sapiens* (accession number AAH08955), *Mus musculus* (AAH21837), *Ovis aries* (AAB92373); a bird, *Gallus gallus* (XM_415847); one amphibian, *Xenopus tropicalis* (AAH74652); a fish, *Oncorhynchus mykiss* (AAK51460); one insect, *Bombyx mori* (BAA76676); and a yeast *Kluyveromyces lactis* (CAB50898).

clones to ensure that the low-abundance mRNA would be present in the library (Sambrook & Russell, 2001; Li et al, 1998). The primary library had no less than 0.4×10^6 clones, which in principle was sufficient for inclusion of most rare mRNA. The high recombination efficiency is another index of good quality library (Li et al, 2006). In this paper, the recombination efficiency of primary and amplified libraries was all over 90%. The third aspect is that the average length of inserted cDNA should be no less than 1.0 kb to ensure the integrity of cDNA (Jin et al, 2004; Tanaka et al, 1996; Yang et al, 2004). The average size of inserted fragments in this study was 1.0 kb approximately. The full-length cDNA library constructed from the dark-spotted frog conformed to the requirements of a standard library. This library provided a useful resource for the functional genomic research of *R. nigromaculata*.

cDNA libraries are widely used to identify genes and splice variants, and as a physical resource for full-length clones (Wiemann et al, 2003). The regions encoding proteins of full-length cDNA are usually conserved, and may have some similarities with closely related regions, which can help us to identify and analyze the protein encoding genes (Gracey et al, 2001). For example, the RnUb obtained in the library as a full-length cDNA showed similar characteristics with ubiquitin of other species. In the previous reports, ubiquitin, a 76-amino acid protein, is one of the most conserved proteins known in eukaryotic cells, with only three amino acid changes from yeast to humans and it can be covalently attached to a variety of cellular proteins and plays a pivotal role in many aspects of eukaryota (Amerik & Hochstrasser, 2004; Deng, 2000; Johnson, 2002; Laney & Hochstrasser, 1999; Muller & Schartz, 1995; Pickart, 2000; Pickart, 2001; Ravid & Hochstrasser, 2004; Schwartz & Hochstrasser, 2003; Sun & Chen, 2004; Vijay-Kumar et al, 1987; Weissman, 2001; Wilkinson et al, 1986; Wilkinson, 2000). It can be expressed as polyubiquitin, or as a fusion with other proteins that are usually 52-aa or 76 – 81-aa ribosomal proteins (Jentsch et al, 1991). Polybiquitin genes have been cloned from various species and can be classified by the number of repeated ubiquitin monomers as follows: nine or more, three or four, and monomeric ubiquitin, which are referred to as human UbC, UbB and UbA, respectively (Wiborg et al, 1985; Martin et al, 2002). In this paper, RnUb, a polyubiquitin with four identical monoubiquitin repeats, is a homologue of UbB.

This full-length cDNA library will be useful not only in obtaining the targeted full-length cDNA, identification of characterization-specific expressed, new or unknown genes and their functions in *R. nigromaculata* but also in studying the genetic diversity of the frogs and even amphibians. And further, it provided a useful resource for the expressional and functional research of sex determination and differentiation genes in this species.

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