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ORIGINAL PAPER

Constructing and detecting a cDNA library for mites

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Abstract RNA extraction and construction of complementary DNA (cDNA) library for mites have been quite challenging due to difficulties in acquiring tiny living mites and breaking their hard chitin. The present study is to explore a better method to construct cDNA library for mites that will lay the foundation on transcriptome and molecular pathogenesis research. We selected *Psoroptes cuniculi* as an experimental subject and took the following steps to construct and verify cDNA library. First, we combined liquid nitrogen grinding with TRIzol for total RNA extraction. Then, switching mechanism at 5′ end of the RNA transcript (SMART) technique was used to construct full-length cDNA library. To evaluate the quality of cDNA library, the library titer and recombination rate were calculated. The reliability of cDNA library was detected by sequencing and analyzing positive clones and genes amplified by specific primers. The results showed that the RNA concentration was 836 ng/μl and the absorbance ratio at 260/280 nm was 1.82. The library titer was 5.31×10^5 plaque-forming unit (PFU)/ml and the recombination rate was 98.21 %, indicating that the library was of good quality. In the 33 expressed sequence tags (ESTs) of *P. cuniculi*, two clones of 1656 and 1658 bp were almost identical with only three variable sites detected, which had an identity of 99.63 % with that of *Psoroptes ovis*, indicating that the cDNA library was reliable. Further detection by specific primers demonstrated that the 553-bp Pso c II gene sequences of *P. cuniculi* had an identity of 98.56 % with those of *P. ovis*, confirming that the cDNA library was not only reliable but also feasible.

Keywords Mites · RNA extraction · cDNA library · Construction · Detection

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Introduction

Mites, belonging to Arachnid, Acari, are distributed widely in the natural world with 50,000 known species. They may affect human and animal health and hence have become an important public health problem. However, different mite species can cause different diseases through different pathogenic mechanisms. Constructing a complementary DNA (cDNA) library is an effective approach to study the function genes that are pathogenic related. Nevertheless, the study of pathogenic mechanism of mites has not been well established due to the following difficulties in cDNA library construction. First, obtaining enough mites for RNA extraction is challenging as mites are tiny and culturing them in vitro has not succeeded for most mites, especially for parasitic mites. The second difficulty is to extract RNA from mites, due to the mites' hard chitin. Last but not the least, obtaining enough RNA satisfying molecular biology research is not easy as RNase (an enzyme degrading the RNA) is very common in the environment.

Up to now, although there are some English-language papers on cDNA library construction and function genes in Acari, most are about ticks with a larger body (Oberlander et al. 1995; Gao et al. 2008; Heekin et al. 2013; Lees et al. 2014; Radulović et al. 2014; Kotsyfakis et al. 2015), and only a few are about mites with a tiny body. In mites, the only four English-language papers we found had several drawbacks (Lee et al. 1999; Fischer et al. 2003; Kenyon et al. 2003; Ljunggren et al. 2003). First, only *Psoroptes ovis* and

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Sarcoptes scabiei mites were studied. Second, the mites used for RNA extraction were up to 250–700 mg, which makes it difficult to replicate the study for other species of mites due to the large extraction amount. Last, there is room for improvement on the recombination rate of cDNA library. Among Chinese-language papers on cDNA library construction of mites, only PCR amplification and agarose gel electrophoresis were used for primary identification of cDNA library. As a result, the reliability of the cDNA library was impaired due to the lack of sequencing of positive clones (Liu et al. 2004; Liu et al. 2008; Xiao et al. 2012; Zhang et al. 2013; Bian et al. 2014; Yu et al. 2014). Therefore, it is necessary to explore a method to construct and detect a full-length cDNA library of mites. This will lay the foundation for future studies of function genes that are pathogenic and control related.

In this study, we constructed a full-length cDNA library of *Psoroptes cuniculi* as a representative using switching mechanism at 5′ end of the RNA transcript (SMART) technique. The quality of the library was primarily assessed by the library titer, the recombination rate, and the lengths of expressed sequence tags (ESTs) in electrophoresis. Then, the reliability of the cDNA library was detected by dual methods to confirm whether the inserted cDNA fragment belonged to *P. cuniculi*. One is to sequence the ESTs longer than 500 bp in electrophoresis and conduct the homology searches using the BLAST programs on the NCBI network. Further, a function gene was amplified by specific primers from the *P. cuniculi* cDNA library and then was sequenced and analyzed. This study is aimed to provide a reference for constructing and detecting a cDNA library for other mite species.

Materials and methods

Mite collection and RNA extraction

The pre-experiment of cDNA library construction using *Demodex caprae* being preserved at −80 °C for more than 1 year failed; hence, living *P. cuniculi* mites were used. *P. cuniculi* were collected from naturally infected rabbits from Xi'an, China. Crusts were taken out from the external auditory canal of the rabbits by a sterile forceps and live mites were isolated under 37 °C in thermostat. After being identified as *P. cuniculi* under a microscope, 68 mg mites were collected into a 1.5-ml EP tube. Mites were then washed with 5 % phosphate-buffered saline (PBS; pH=7.4), 1 % SDS, and 5 % PBS (pH=7.4) once, respectively, and assembled by centrifugation. The mites had been ground into powder by liquid nitrogen before total RNA was extracted by TRIzol (Roche, Swiss). The concentration and purity of RNA were measured by an ultraviolet spectrophotometer. The completeness of RNA was estimated by 2 % agarose gel electrophoresis. The RNA sample was then digested by DNase I (Takara, Japan) to eliminate potential genomic DNA (gDNA).

Single-strand and double-strand cDNA synthesis

First, RNA was reverse transcribed to single-strand cDNA (ss cDNA) using reverse transcriptase M-MLV (Takara, Japan) with primers P1 (SMART IV) and P2 (CDS/3′ PCR). Second, double-strand cDNA (ds cDNA) was synthesized by LD-PCR (Takara, Japan) with primers P3 (5′ PCR anchor) and P2 (CDS/3′ PCR) (Table 1). Finally, the PCR products of ds cDNA were separated by 2 % agarose gel electrophoresis, and those longer than 500 bp were isolated and purified by a gel extraction kit (Omega, USA).

Double-strand cDNA cloning

The purified ds cDNA was linked with pMD-19T vector (Takara, Japan), and 2 μl recombination plasmid was transformed into 20 μl *Escherichia coli* DH-5α. Seventy-eight microliters of LB liquid medium (Amp +) was added to make the total volume of 100 μl, which was then smeared evenly on the blue and white Luria broth (LB) medium Amp (+) (10 cm in diameter). After the medium was incubated at 37 °C overnight for 16–18 h, the colonies were observed.

cDNA library quality assessment

The library titer and recombination rate were calculated as follows: library titer=(number of colonies×dilution factor×10^3 μl/ml)/(μl of diluted volume plated), recombination rate=number of white colonies/(number of white colonies+number of blue colonies). Positive clones (white colonies) were selected randomly by a sterile tip for PCR amplification using the universal primers for pMD-19T vector (Table 1). The reaction volume was 12.5 μl and the reaction parameters were as follows: initial denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 63 °C for 30 s, extension at 72 °C for 1 min, and final extension at 72 °C for 10 min. The lengths of inserted fragments were estimated by electrophoretic separation using 2 % agarose gel.

Sequencing and function prediction of positive clones

Positive clones longer than 500 bp in electrophoresis were sequenced by Suzhou GENEWIZ Biotechnology Co. Ltd., China. The length of expressed sequence tag (EST) was calculated as the length of the band—216 bp (59 bp SMART IV+157 bp pMD-19T vector). To verify whether the ESTs belonged to *P. cuniculi*, homology searches were conducted using the BLASTn and BLASTx programs on the NCBI

Table 1 Primers used for cDNA library construction and detection

Target	Primers (5′-3′)	T_m (°C)
ss cDNA	P1:AAGCAGTGGTATCAACGCAGAGTGGCCATTACGGCCGGG P2:ATTCTAGAGGCCGAGGCGGCCGACATG-d(T)30VN	–
ds cDNA	P3:AAGCAGTGGTATCAACGCAGAGT P2:ATTCTAGAGGCCGAGGCGGCCGACATG-d(T)30VN	65
Inserted cDNA fragment	F:CGCCAGGGTTTTCCCAGTCACGAC R:GAGCGGATAACAATTTCACACAGG	63
Pso c II	F1:CCCAATTAAAACTAAAAAATAA F2:CCCAATTAAACTAAAAAATAAT R:GAATACTTTTTAATTTTGAATAAA	49

F forward, *R* reverse, T_m melting temperature

V=A, G, or C; N=A, G, C, or T

network. The open reading frames (ORFs) were found by ORF finder. The sequences of *Psoroptes* were aligned by Clustal X1.8 and the sequence identity was computed. Neighbor-joining (NJ) tree across Psoroptidia based on known ESTs was reconstructed by MEGA 5.0. The tree confidence was assessed by 1000 bootstraps.

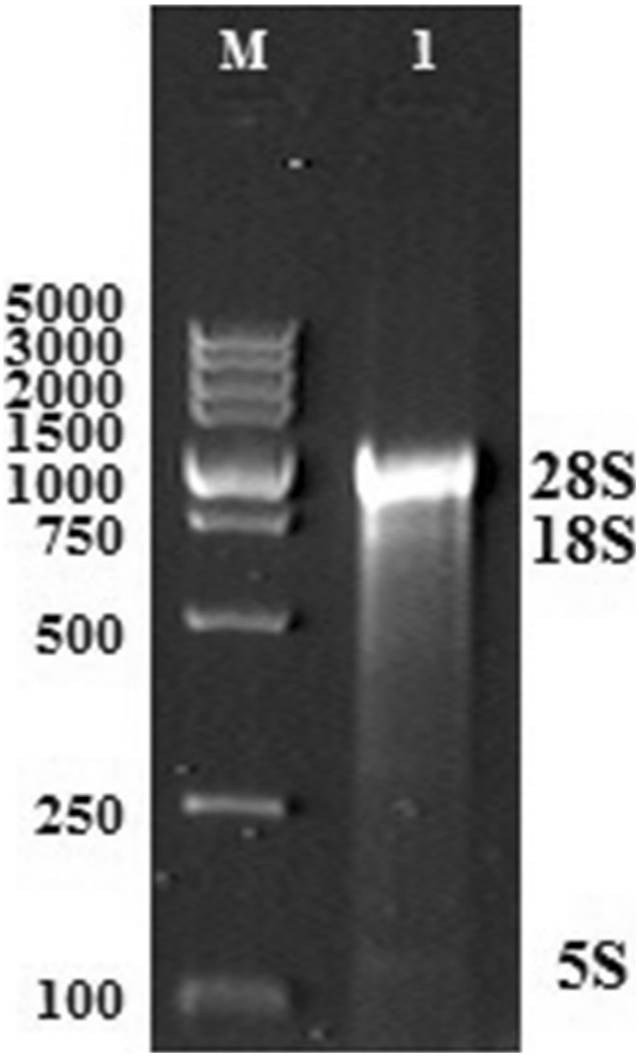

Fig. 1 Electrophoretogram of total RNA of *P. cuniculi*. *Lane M* DL5000 DNA marker, *lane 1* total RNA

PCR amplification and detection by specific primers

In order to further verify the reliability of the library, specific primers were designed according to the two allergen II sequences of *P. ovis* from GenBank (two forward primers were designed as there were several different bases at the 5′ terminal of the two sequences) (Table 1) for PCR amplification using the bacteria liquid of *P. cuniculi* cDNA library as template. The sequences of *P. cuniculi* and *P. ovis* were aligned by

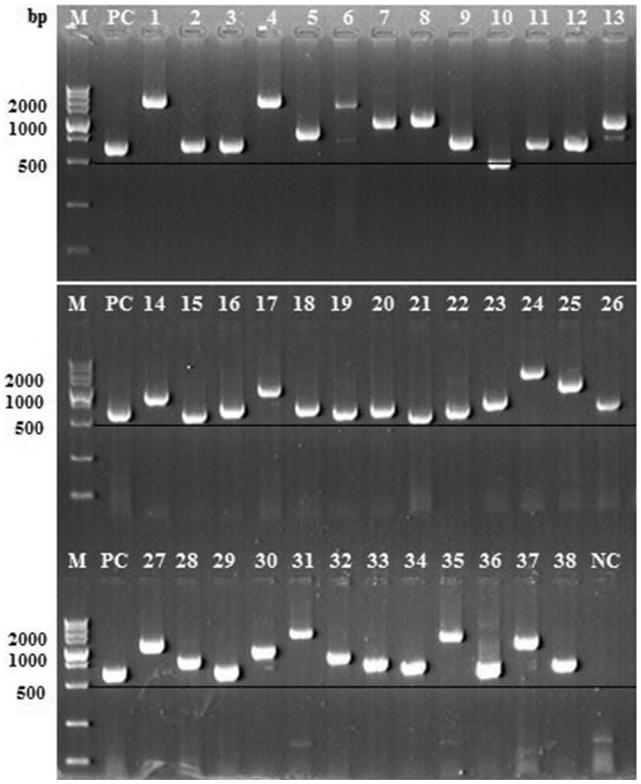

Fig. 2 PCR products of positive clones of the *P. cuniculi* cDNA library. *Lane M* DL5000 DNA marker, *lanes 1–38* positive clones, *PC* positive control of 500 bp, *NC* negative control

Table 2 Thirteen clones with identified characteristics

Clone	Nuc. (bp)	Accession no.	BLASTn/x	Homologue with	Accession no.	Putative function	Location	Identity (%)	Query cover (%)	Total score	*E* value	ORF (bp)
7	745	JZ875758	n	*A. mellifera*	XM_006569579	TF IIB	Nucleus	74	93	437	1e−118	732
			x	*A. mellifera*	XP_006569641			82	94	387	3e−131	
1	1658	JZ875752	n	*P. ovis*	JQ001154	SRP54	Cytoplasm	99	81	2471	0.0	1494
			x	*P. ovis*	AFJ22205			99	81	925	0.0	
4	1656	JZ875755	n	*P. ovis*	JQ001154	SRP54		99	81	2453	0.0	762
			x	*P. ovis*	AFJ22205			99	81	928	0.0	
8	873	JZ875759	n	*Lyc. singoriensis*	EU247223	CB		68	78	210	3e−50	648
			x	*Lyc. singoriensis*	ABX75522			63	79	285	4e−92	
6	1189	JZ875757	n	*La. hesperus*	HQ005864	ARP P0	Ribosomal 60S	72	73	466	4e−127	798
			x	*La. hesperus*	ADV40160			67	69	456	4e−128	
2	431	JZ875753	n	*Lys. testaeipes*	AY961527	ARP P1		72	65	152	3e−33	111
			x	*Lys. testaeipes*	AAX62429			79	29	74.3	4e−14	
3	430	JZ875754	n	*Lys. testaeipes*	AY961527	ARP P1		72	66	152	3e−33	111
			x	*Lys. testaeipes*	AAX62429			79	29	74.3	4e−14	
5	593	JZ875756	n	*La. hesperus*	HQ005867	SD	Mitochondria	79	43	226	3e−55	240
			x	*La. hesperus*	ADV40163			76	66	224	5e−69	
9	447	KR072484[a]	n	*P. ovis*	AF270816	ITS1-5.8S		98	78	609	2e−170	–
10	312	KR072485[a]	n	*D. pteronyssinus*	GQ205607	5.8S-ITS2-28S	Ribosomal	97	100	527	4e−146	–
11	409	KR080455[b]	n	*B. pseudomallei*	CP004369	NG	Chromosome	74	53	199	3e−28	–
12	501	KR080456[b]	n	*E. coli*	CP010585	NG		99	100	899	0.0	–
13	654	KR080457[b]	n	*S. marcescens*	AP013063	NG		90	96	911	0.0	–

Nuc nucleotide, *A Apis*, *P Psoroptes*, *Lyc Lycosa*, *La Latrodectus*, *Lys Lysiphlebus*, *D Dermatophagoides*, *B Burkholderia*, *E Escherichia*, *S Serratia*, *TF IIB* transcription factor IIB, *SRP54* signal recognition particle protein 54 k, *CB* cathepsin B, *Arp* acidic ribosomal protein, *SD* succinate dehydrogenase, *NG* nuclear genome

[a] Genomic sequences of mites

[b] Genomic sequences of bacteria

Clustal X1.8, and the sequence identity was computed. NJ tree across Psoroptidia was reconstructed by MEGA 5.0. The tree confidence was assessed by 1000 bootstraps.

Results

RNA quality

The quality of the obtained RNA is good. The concentration of total RNA was 836 ng/μl. The OD260/280 and OD260/OD230 were 1.82 and 2.08, respectively, indicating high purification. Figure 1 shows three bands of 28S, 18S, and 5S with the 28S significantly lighter than 18S, indicating good integrity of RNA.

cDNA library quality

There were 1062 bacterial colonies in the plate of cDNA library with a recombination rate of 98.21 % (1043/1062). The library titer was 5.31×10^5 plaque-forming unit (PFU)/ml ($1062/2 \times 10^{-3}$). Thirty-eight randomly selected clones were identified to be longer than 500 bp in the electrophoresis, including 4 clones of 1500–2000 bp, 8 clones of 1000–1500 bp, and 26 clones of 500–1000 bp (Fig. 2). The cDNA library was preliminarily proved to be successfully constructed.

Sequencing and function prediction

The 38 clones identified above were sequenced. Two sequences were identified as ribosomal genes of mite and three were identified as genome of bacteria. The other 33 clones were identified as ESTs of *P. cuniculi* which was made up of 6.06 % (2/33) of known genes, 18.18 % (6/33) of putative genes, and 75.76 % (25/33) of novel genes. The lengths of ESTs ranged from 319 to 1658 bp (average 711 bp).

Table 2 presented the 13 sequences with known characteristics, of which 8 sequences (clones 1–8) were ESTs of *P. cuniculi*. The protein function predicted by BLASTn and BLASTx was the same, demonstrating that the blast results were reliable. The clones 1 and 4 (1656 and 1658 bp, respectively) were almost identical with only three variable sites detected. The total score of BLASTn for clones 1 and 4 was

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P. cuniculi1. China GACCGCATTA CGGTCGCTGG GTAATGCAAC AATCATCAAT CAAGAAGTAT TGGATGGCCT ACTCAAAGAA ATATGTACAG CCCTAATTGA ATCCGATGTG AATATTATGT TGGTCAAAAA  [ 120]
P. cuniculi2. China .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... [ 120]
P. ovis. JQ001154    .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... [ 120]

P. cuniculi1. China ATTACGTGAA AATGTTCGAA ATGTTATTGA TTTTGATGAA ATGGCTGCGG GCCTAAACAA ACGCCGCATG ATTATGCGTG CCGTATTTCA AGAACTGTGT AAACTACTTG ATCCAGAAGT  [ 240]
P. cuniculi2. China .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... [ 240]
P. ovis. JQ001154    .......... .......... .......... .......... .........A. .......... .......... .......... .......... .......... ...T...... .......... [ 240]

P. cuniculi1. China GAAAGCATGG CAACCTGTTA AAGGCAAGAC TAATGTTATT ATGTTTGTCG GCTTGCAAGG TATCGGTAAG ACTACGACCT GTACAAAAAT GGCTTATTAT TATATGAAAA AAGGATGGAA  [ 360]
P. cuniculi2. China .......... .......... .......... .......... .......... .......... .........A. .......... .......... .......... .......... .......... [ 360]
P. ovis. JQ001154    .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... [ 360]

P. cuniculi1. China ATGTGCGCTG GTTTGTGCCG ATACATTTCG TGCTGGTGCA TTCGATCAGC TCAAACAAAA TGCAACCAAA GCTCGAATAC CTTTTTATGG TTCTTATACA GAATCGGACC CGGTTGTAAT  [ 480]
P. cuniculi2. China .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... [ 480]
P. ovis. JQ001154    .......... .......... ...........C.......... .......... .......... .......... .......... .......... .......... .......... .......... [ 480]

P. cuniculi1. China CGCTTCGGAA GGTGTGGAAA AATTTACAAA TGAAAATTTT GAAATCATCA TTGTCGACAC TAGTGGTCGC CATAAACAAG AAAGTTCATT GTTCGAAGAA ATGTTGGCCA TTCAAGCGGC  [ 600]
P. cuniculi2. China .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... [ 600]
P. ovis. JQ001154    .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... [ 600]

P. cuniculi1. China TATTCAACCA AATCTCGTTA TTTACGTTAT GGACGCATCG ATTGGTCAAG CTTGTGAATC GCAAGCACGT GCATTCAAAT CAAAAGTCGA TGTCGGTGCC ATAATCGTCA CCAAATTAGA  [ 720]
P. cuniculi2. China .......... .......... .......... .......... ....-..... .......... .......... ....-..... .......... .......... .......... .......... [ 720]
P. ovis. JQ001154    .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... [ 720]

P. cuniculi1. China TGGCCATGCT AAAGGTGGTG GCGCCCTCAG TGCTGTCGCA GCAACCAAAT CACCAATCAT TTTCATTGGT ACCGGTGAAC ATATTGATGA ATTTGAACCA TTCAAAGTAC GTCCATTCAT  [ 840]
P. cuniculi2. China .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... [ 840]
P. ovis. JQ001154    .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... [ 840]

P. cuniculi1. China ATCGAAATTG TTGGGTATGG GCGATATTGA AGGTTTGATT GACAAGGTAA ATGAACTGAA ACTGGATGAT AATGAAGAAT TGATCGAGAA AATTAAACAT GGGGAATTCA CCATTCGTGA  [ 960]
P. cuniculi2. China .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... [ 960]
P. ovis. JQ001154    .......... .......... .......... .......... ....G..... .......... .......... .......... .......... .......... .......... .......... [ 960]

P. cuniculi1. China TATGTATGAA CAGCTAACGA ACATAATGAA AATGGGTCCA TTCAATCAAA TTCTAAACAT GATTCCTGGA TTCGGTGCCG AACTGGCCAA ATGTGCATCC GAAGCTGAAT CGATGTCACG  [1080]
P. cuniculi2. China .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... [1080]
P. ovis. JQ001154    .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... [1080]

P. cuniculi1. China ATTAAAGCGG ATGATGACCA TAATGGATTC AATGAGTGAC TCGGAACTGG ATTCACGTGA AGGCGCCAAG CTATTTTCTA AACAACCAAC ACGTATTGTT CGTGTATCAC GTGGTGCTGG  [1200]
P. cuniculi2. China .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... [1200]
P. ovis. JQ001154    .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... [1200]

P. cuniculi1. China CTGCACACAA CGTGAAGTAC AAGAACTACT ATCCCAATAT ACTAAATTTG CGGCTGTCGT CAAAAAGATG GGCGGAATTA AAGGTCTGTT TAAAAGTGGT GATTTGGCTA AAAATGTTAA  [1320]
P. cuniculi2. China .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... [1320]
P. ovis. JQ001154    .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... [1320]

P. cuniculi1. China TCCAGCACAA ATGGCCAAAT TGAATCAACA  [1350]
P. cuniculi2. China .......... .......... ..........  [1350]
P. ovis. JQ001154    .......... .......... ..........  [1350]
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Fig. 3 Alignments of three SRP54 sequences of *Psoroptes*

high with SRP54 of *P. ovis* (2471 and 2453, respectively), and the nucleotide identified was as high as 99.63 % (5/1350) (Fig. 3), indicating that both clones 1 and 4 should be SRP54, and *P. cuniculi* and *P. ovis* should be of the same species. The phylogenetic tree based on SRP54 sequences also showed that *P. cuniculi* clustered with *P. ovis* first, and then gathered with *Chorioptes* and *Otodectes* as a clade of Psoroptidae, and finally gathered with Turbinoptidae, Pterolichidae, and Pyroglyphidae as Psoroptidia (Fig. 4). This was in accordance with morphological classification. Therefore, the constructed cDNA library of *P. cuniculi* was reliable.

Detection by specific primers

The Pso c II gene fragments of *P. cuniculi* were successfully amplified by the two pairs of primers (Fig. 5) and sequenced to be 553 bp (JZ875784, JZ875785). The sequence identified of *P. cuniculi* and *P. ovis* (BQ834604, AF187083) was 98.56 % (8/554) (Fig. 6), indicating that *Psoroptes* mites parasitizing in rabbit and sheep were of the same species. The phylogenetic tree also showed that *P. cuniculi* and *P. ovis* gathered as a clade between Pyroglyphidae and Acaridae (Fig. 7). Hence, the cDNA library can satisfy the need for amplification.

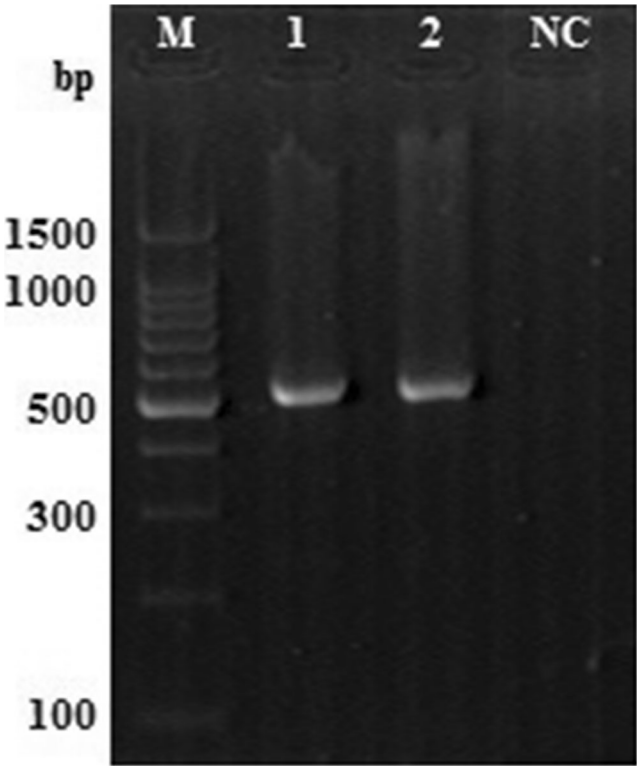

Fig. 5 PCR products of Pso c II of *P. cuniculi. Lane M* 100 bp DNA ladder marker, *lane 1* Pso c II (F1), *lane 2* Pso c II (F2), *NC* negative control

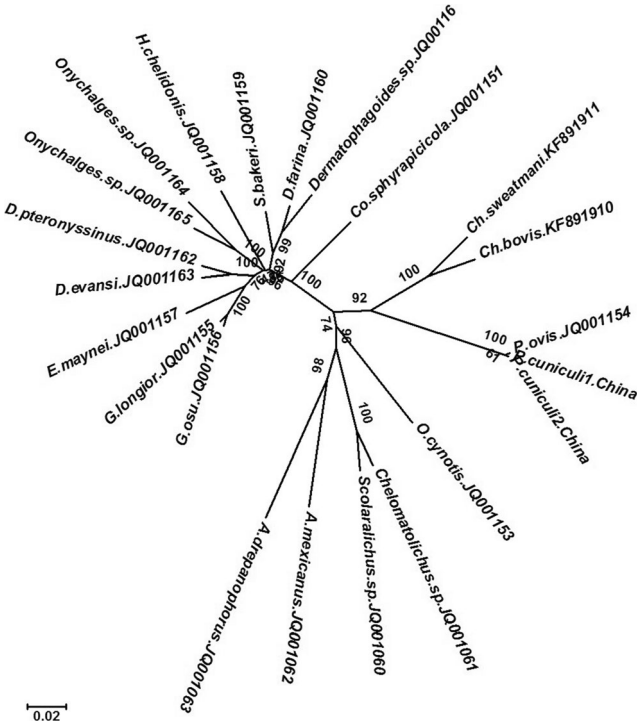

Fig. 4 Radiative NJ phylogenetic trees across Psoroptidia inferred from SRP54. *P Psoroptes*, *Ch Chorioptes*, *O Otodectes*, *A Aniacarus*, *Co Congocoptes*, *D Dermatophagoides*, *H Hirstia*, *S Sturnophagoides*, *E Euroglyphus*, *G Gymnoglyphus*

Discussion

At present, the traditional cDNA library and the full-length cDNA library are the two main types of cDNA library. The full-length cDNA library could satisfy the requirement for large-scale, high throughput, and highly efficient studies of functional genomics due to its advantages over traditional cDNA library, including long-fragment cloning, high proportion of full-length cDNAs, less invalid cloning, etc. The construction of cDNA library becomes more appealing after the introduction of a new and more efficient method, namely SMART (Chenchik et al. 1996). It has been widely used in microbial pathogens (anisopliae, monascus, etc.) (Zhang et al. 2007; Zhu et al. 2009) and parasites (roundworm, tapeworms, *Giardia lamblia*, and others) (Wei et al. 2007; Zhang et al. 2009; Guo et al. 2012; Zhou et al. 2011). However, it is rarely used in medical mites because of the difficulties in mite collection and RNA extraction.

In this study, we successfully constructed the full-length cDNA library of *P. cuniculi* by optimizing experimental conditions, as well as improving experimental design and operation. *P. cuniculi* was selected as the study object to facilitate comparison with the same species, *P. ovis*, whose cDNA library has been constructed. The mites were ground into powder by liquid nitrogen and RNA was extracted

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P. cuniculi1. China CCCAATTAAA ACTAAAAAAT AATTTTAAAA AAATCAAAAT GATGAAAACT TTGGTAGTTC TCGCCATCAC TTTGGCTGTT GTATCAGCTG GCAAAGTCAA ATTCTAAGAC TGTGGAAAAG [120]
P. cuniculi2. China ........... -......... .......... .......... .......... .......... .......... .......... .......... ....C..... .......... [120]
P. ovis. BQ834604   ........... .......... .......... .......... .......... .......... ....T..... .......... .......... .......... ....C....G .......... [120]
P. ovis. AF187083   ........... .......... .......... .......... .......... .......... .......... .......... .......... .S..R.... R..YC..... .......... [120]

P. cuniculi1. China GGGAAGTTGA ATCTCTTGAA GTTGAAGGCT GTTCAGGTGA TTACTGCGTC ATTCACAAAG GTAAAAAAAC TTGATTTAGC CATCAGTGTA ACATCGAACC AAGATTCAGC CAATTTGAAA [240]
P. cuniculi2. China ........... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... [240]
P. ovis. BQ834604   .A......... .......... .......... .......... .......... .......... .......-.. .......... .......... .......... .......... .......... [240]
P. ovis. AF187083   .R......... .......... .......... .......... .......... .......... .......-.. .......... .......... .......... .......... .......... [240]

P. cuniculi1. China CTCGATATTG TTGCCGATAT CAACGGTGTA CAAATTGAAG TTCCTGGCGT TGATCATGAT GGTTGCCATT ACGTCAAATG TCCAATCAAG AAAGGCCAAC ACTTTGACGT CAAATACACA [360]
P. cuniculi2. China ........... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... [360]
P. ovis. BQ834604   ........... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... [360]
P. ovis. AF187083   ........... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... [360]

P. cuniculi1. China TACAGCATTC CAGCAATCTT GCCAACTACC AAAGCTAAAA TCATTGCTAA AATTATTGGT GATAAAGGTC TTGGTGGTTG TATCGTAATC AATGGTGAAA TTCAAGACTA AATCAATAAA [480]
P. cuniculi2. China ........... .......... .......... .......... .......... .G........ .......... .......... .......... .......... .......... ....G..... [480]
P. ovis. BQ834604   ........... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... [480]
P. ovis. AF187083   ........... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... [480]

P. cuniculi1. China AACCTAA-AA ATATTTTGAT GAAATTAGAT TTGTTATTTT TATTTCTCAT TTTATTCAAA ATTAAAAAGT ATTC [554]
P. cuniculi2. China ......CT.. .......... .......... .......... .......... .......... .......... .... [554]
P. ovis. BQ834604   .......-.. .......... .......... .......... .......... .......... .......... .... [554]
P. ovis. AF187083   .......-.. .......... .......... .......... .......... .......... .......... .... [554]
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Fig. 6 Alignments of four allergen II sequences of *Psoroptes*

with TRIzol to ensure complete lysis of tissue and cells. SMART technique was selected to construct a full-length library. Instead of phage vector TriplEx2 and expression vector ZAP, plasmid vector pMD-19T was used to increase the recombination rate and sequencing convenience. As a result, the quality of RNA satisfied the requirement for cDNA library construction. The concentration of RNA was 836 ng/μl, and the absorbance ratios of the RNA at 260/280 and 230/260 nm were 1.82 and 2.08, respectively. The mites needed for RNA extraction significantly reduced from 250 to 700 mg to 68 mg. The library quality was significantly improved: the library titer increased from 2.80×10^5 PFU/ml (Lee et al. 1999) to 5.31×10^5 PFU/ml, and the recombination rate increased from 76 to 89 % (Lee et al. 1999; Kenyon et al. 2003) to 98.21 %, consistent with the quality standard proposed by Clontech company (USA) where primary library titer ≥5×105 PFU/ml and recombination rate ≥90 %. In this way, the mites used for RNA extraction were decreased and the quality of cDNA library was improved significantly.

It is important to detect the reliability of the library with two independent methods conducted. In this study, first, the clone 1 and 4 sequences of *P. cuniculi* showed 99.63 % identity with the SRP54 gene sequence of *P. ovis*, which not only confirmed that both clones were SRP54 gene but also indicated that *P. cuniculi* and *P. ovis* were of the same species, in line with the result obtained by Pegler based on ribosomal ITS2 (Pegler et al. 2005). Second, the bacteria liquid of *P. cuniculi* cDNA library was used as a template for PCR amplification using specific primers designed according to allergen II gene sequences of *P. ovis*. These sequences of *P. cuniculi* and *P. ovis* showed 98.56 % identity, further indicating that the constructed cDNA library was reliable.

It is a common practice among Chinese scholars to assess the cDNA library only by PCR amplification and electrophoresis of positive clones (Liu et al. 2004; Liu et al. 2008; Tao et al. 2011; Xiao et al. 2012; Zhang et al. 2013; Bian et al. 2014; Hu et al. 2014; Yu et al. 2014). However, that simple assessment is hardly satisfactory as the electrophoretic bands could suggest only the length of the inserted fragments, but not their nature. The inserted fragments might be the cDNA/gDNA of the studied mites, the gDNA of bacteria carried by the studied mites, or even the gDNA/cDNA of the host. In this study, according to the blast results of the 38 clones which were longer than 500 bp identified by electrophoresis (Table 2), clones 9–10 were highly homologous with ribosomal ITS1-5.8S gene of *P. cuniculi* and ribosomal 5.8S-ITS2-28S gene of *Dermatophagoides pteronyssinus*, respectively, while clones 11–13 showed 74, 99, and 90 % identities with *Burkholderia pseudomallei*, *E. coli*, and *Serratia marcescens*, respectively. Therefore, we suggested that sequencing long fragments (usually ≥500 bp) and/or PCR amplification with specific primers should be applied to confirm if the cDNA library was successfully constructed.

It should be noted that the actual length of ESTs is usually shorter than the bands in electrophoresis (≥500 bp). In this study, the length of ETSs is equal to the length of the band minus 216 bp (SMART IV+pMD-19T vector). Therefore, the length of the shortest EST is 319 bp.

High-quality RNA is an important prerequisite of cDNA library construction. To extract high-quality RNA, you should make sure of the following issues: First, to guarantee the completeness of RNA, use living mites for RNA extraction because RNA would degrade rapidly in dead mites. Second, to ensure the RNA releases completely, use liquid nitrogen grinding for complete homogenate. Third, to avoid RNase contamination, execute the operation skillfully and quickly under a clean, unpolluted environment. Last, to avoid DNA or protein residual, suck up RNA from the upper water phase accurately and then treat RNA with DNase. In our pre-

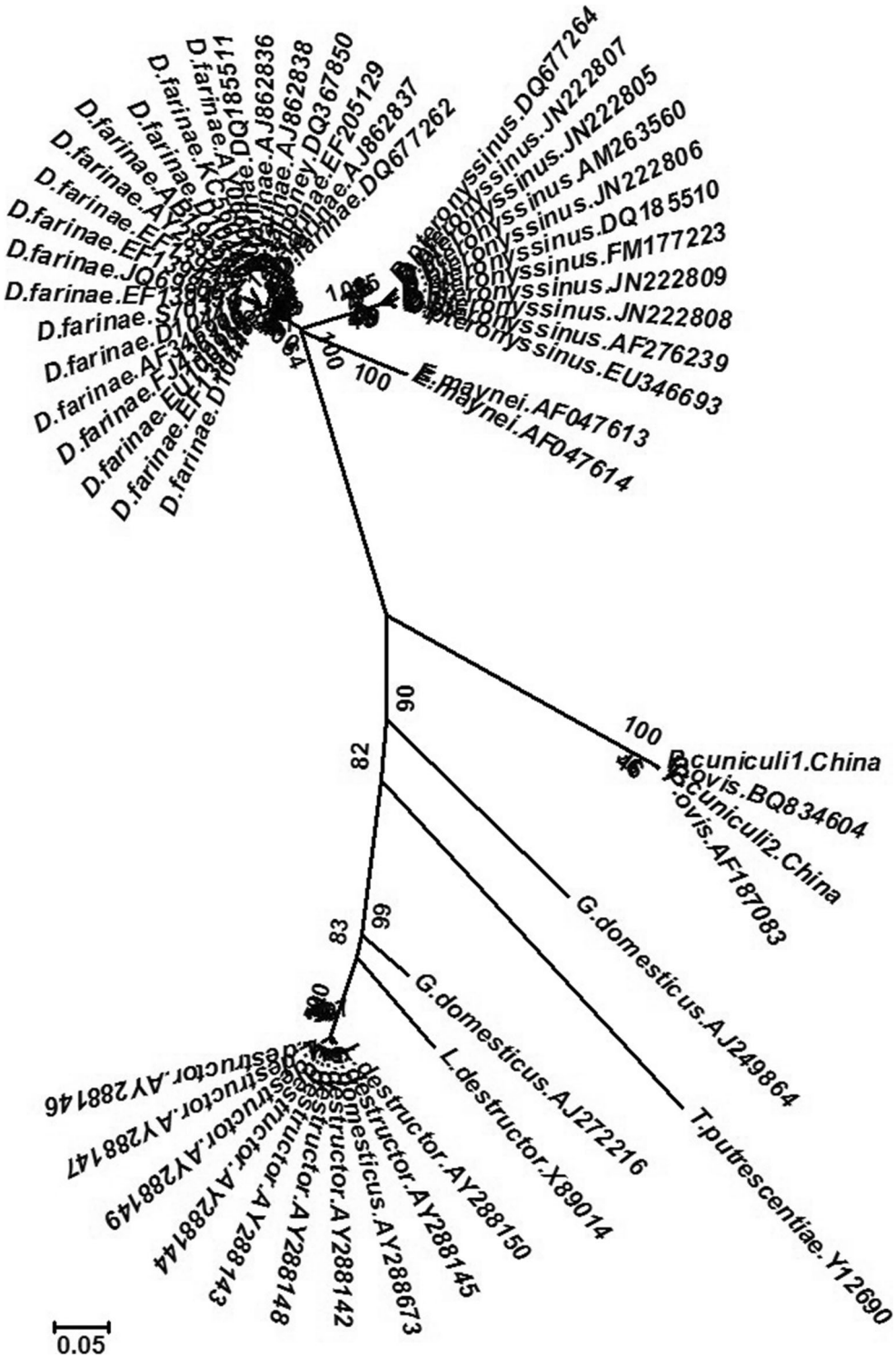

Fig. 7 Radiative NJ phylogenetic trees across Psoroptidia inferred from allergen II. *P Psoroptes*, *G Gymnoglyphus*, *T Tyrophagus*, *L Lepidoglyphus*, *E Euroglyphus*, *D Dermatophagoides*

experiment of cDNA library construction using *D. caprae*, the reason for failure was probably that the mites had been dead and RNA was degraded as they were cryopreserved for more than a year.

In the study, the successful attempt to construct a full-length cDNA library using *P. cuniculi* will provide a valuable experience for cDNA library construction of other mite species. The six function genes obtained play important roles in protein synthesis, processing and metabolism, immune evasion, as well as growth and development. Those genes are essential for protein expression, pathogenic mechanism study, and preventive vaccine development.

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