

Sequencing for complete rDNA sequences (18S, ITS1, 5.8S, ITS2, and 28S rDNA) of *Demodex* and phylogenetic analysis of Acari based on 18S and 28S rDNA

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Abstract Due to the difficulty of DNA extraction for *Demodex*, few studies dealt with the identification and the phyletic evolution of *Demodex* at molecular level. In this study, we amplified, sequenced, and analyzed a complete (*Demodex folliculorum*) and an almost complete (D12 missing) (*Demodex brevis*) ribosomal DNA (rDNA) sequence and also analyzed the primary sequences of divergent domains in small-subunit ribosomal RNA (rRNA) of 51 species and in large-subunit rRNA of 43 species from four superfamilies in Acari (Cheyletoidea, Tetranychioidea, Analgoidea, and Ixodoidea). The results revealed that 18S rDNA sequence was relatively conserved in rDNA-coding regions and was not evolving as rapidly as 28S rDNA sequence. The evolutionary rates of transcribed spacer regions were much higher than those of the coding regions. The maximum parsimony trees of 18S and 28S rDNA appeared to be almost identical, consistent with their morphological classification. Based on the fact that the resolution capability of sequence length and the divergence of the 13 segments

(D1–D6, D7a, D7b, and D8–D12) of 28S rDNA were stronger than that of the nine variable regions (V1–V9) of 18S rDNA, we were able to identify *Demodex* (Cheyletoidea) by the indels occurring in D2, D6, and D8.

Introduction

Eukaryotic ribosomal DNA (rDNA), the gene encoding the ribosomal RNA (rRNA) in cell nucleus, comprises a big multi-gene family in the form of tandem multi-copy. Each copy is composed of structural genes including large-subunit (LSU) 28S rDNA, 5.8S rDNA, and small-subunit (SSU) 18S rDNA and nonstructural genes including non-transcribed and transcribed spacer regions. With variable evolution rates, different rDNA portions can be used for phylogenetic study at different taxonomic category levels. Compared with the internal transcribed spacers 1 and 2 (ITS-1 and ITS-2), 18S and 28S rDNA share lower evolution speeds and are made up of conserved regions and divergent domains (variable regions in 18S rDNA and expansion segments in 28S rDNA). Both 18S and 28S rDNA have been the extraordinarily helpful molecular markers for phylogenetic analysis until now (Baverstock and Johnson 1990).

Demodex mites belong to the class Arachnida, Acariformes, Cheyletoidea, and Demodicidae. Since Simon's report on *Demodex* in 1842, there have been 140 *Demodex* species or subspecies identified from 11 orders of mammals (dog, sheep, cat, pig, mouse, etc.), residing in hair follicles, sebaceous glands, meibomian glands, and internal organs. *Demodex folliculorum* and *Demodex brevis* are two types of human *Demodex* (Desch and Nutting 1972). For a long time, the

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classification and phylogenetic study of *Demodex* have been based on the hosts and phenotype features of the mites, which may lead to phylogenetic indeterminacy and difficulties. Therefore, it is necessary to study *Demodex* at the gene level.

However, there are only a few studies on *Demodex* at a molecular level (Toops et al. 2010; Zhao and Wu 2011, 2012; De Rojas et al. 2012), and there is no report on the molecular data of its rDNA. The reasons are threefold. First, it is hard to obtain standardized mites for experiments. *Demodex* mites are species-specific to their hosts. Other than a few reports about cross-infection between human and animals (Morsy et al. 1995; Wang et al. 1998), the mites have not been maintained in vitro successfully (Zhao et al. 2009a, 2010). Second, *Demodex* DNA extraction is difficult due to the tiny body and thick chitin of the mites (Zhao et al. 2009b). Third, it is hard to get sufficient number of *Demodex* mites from the same host for genomic DNA extraction. A large number of mites would be needed for the gDNA extraction at a time, i.e., 1,500 mites/host for *D. folliculorum* and 2,000 mites/host for *D. brevis* (Zhao and Cheng 2009). Despite these difficulties, we successfully ampli-

fied and sequenced the complete rDNA sequences (18S, ITS-1, 5.8S, ITS-2, and 28S) of the two human *Demodex* mites, which were further analyzed, for the first time in this study. And the primary sequence analysis of nine variable regions in SSU rRNA and the 13 expansion segments in LSU rRNA for four superfamilies (Cheyletoidea, Tetranychioidea and Analgoidea in Acariformes, and Ixodoidea in Parasitiformes) in Acari were conducted, in terms of sequence lengths, evolution rates, and divergences. The findings in the present study have provided molecular data for *Demodex* study at the gene level.

Materials and methods

Demodex collection and DNA extraction

In this study, ethical permission was not required because the mite collection method was a noninvasive sampling technique routinely used in pathogen. All the sufferers were from Xi'an, China. Cellophane tape method was applied to

Table 1 Primer design

| rDNA | Primers | Model species | Primer design | | | Sequence lengths (bp) | | | |
|------------|----------------|---------------------------------|---|-------------|--------------|-----------------------|--------------|--------------|--|
| | | | Sequence (5'–3') | Length (bp) | GC (%) | T _m (°C) | <i>D. f.</i> | <i>D. b.</i> | |
| 18S rDNA | 18S1 | <i>C. thomsoni</i> , AF255703 | F: TACCTGGTTGATCCTG R: ATGCTTTCGCAGTAGT | 16 16 | 50.0 43.8 | 48.0 | 955 | 966 | |
| | 18S2 | <i>C. thomsoni</i> , AF255703 | F: CGGGGGCATTAGTATT R: CCTTCCGCAGGTTTCAC | 16 16 | 50.0 62.5 | 48.0 | 927 | 931 | |
| ITS region | ITS1-5.8S-ITS2 | <i>C. thomsoni</i> , AF255703 | F: TCGTAACAAGGTTTTTCG R: GTTCGCTATCGGTCTCT | 17 17 | 41.2 52.9 | 50.0 | 882 | 1,017 | |
| | | <i>D. melanogaster</i> , M21017 | F: GCATATCAATAAGCGGA R: GTTCGCTATCGGTCTCT | 17 17 | 41.2 52.9 | 48.0 | 309 | 309 | |
| 28S rDNA | 28S0 | <i>D. melanogaster</i> , M21017 | F: GTGGTAAACTCCATCTAA R: GCATAGTTCACCATCTT | 18 17 | 38.9 41.2 | 52.0 | 544 | 544 | |
| | 28S2 | <i>T. kanzawai</i> , AY750691 | F: CCGAAAGATGGTGA ACTA R: CTTAGGACCGACTGACCC | 18 18 | 44.4 61.1 | 50.0 | 828 | 805 | |
| | 28S3 | <i>T. kanzawai</i> , AY750691 | F: ATCTTGGTGGTAGTAGCA R: AGAGCCAATCCTTATCC | 18 17 | 44.4 47.1 | 54.0 | 536 | 503 | |
| | 28S4 | <i>T. kanzawai</i> , AY750691 | F: CGCAGCAGGTCTCCA R: GTTCCCTTGGCTGTG | 15 15 | 66.7 60.0 | 50.0 | 748 | 550 | |
| | 28S5 | <i>T. kanzawai</i> , AY750691 | F: GCCAAATGCCTCGTC R: AGGTTTCTGTCCTCACTG | 15 18 | 60.0 50.0 | 50.0 | 705 | 584 | |
| | 28S6 | <i>T. kanzawai</i> , AY750691 | F: GGGAGTTTACTGGGG R: CTTAGAGGCGTTCAGGC | 16 17 | 62.5 58.8 | 50.0 | 523 | 521 | |
| | 28S7 | <i>T. kanzawai</i> , AY750691 | F: GGGAGTTTACTGGGG R: CTGCTCTAATAAGTACATCACC | 16 22 | 62.5 40.9 | 52.0 | 857 | – | |
| | | | <i>P. polyxenus</i> , HM165066 | | | | | | |

F forward primer, R reverse primer, *D. f.* *Demodex folliculorum*, *D. b.* *Demodex brevis*

sample the isolates of *D. folliculorum* and *D. brevis* from the sufferers' faces. Mite samples were collected using a self-made needle under a 10×4 microscope and preserved in Eppendorf tubes at −20 °C. The mites were then put into liquid nitrogen for 1 min of freeze thawing, followed by a 10-min milling, and this procedure was repeated four times. After that, DNA extraction was conducted using a DNA extraction kit (Omega) obeying the manufacturer's protocol (Zhao et al. 2009b).

Primer design

Specific primers were designed respectively for the two segments of 18S rDNA, ITS region, and the eight segments of 28S rDNA according to the corresponding conserved regions identified in the alignments of referred sequences from GenBank (Table 1). The primers were synthesized in Beijing AuGCT Biotechnology Co., Ltd. The model species and the designed primers are listed in Table 1.

PCR amplification, cloning, and sequencing

All PCR reactions were conducted on AB Applied Biosystems 2720 Thermal Cycler. Each 12.5-μL reaction volume contained 1 μL of DNA template, 1 μL of each primer (1 μmol/L), 6 μL of 2× Premix Taq buffer (pH 8.3, 0.05 U/μL Taq DNA polymerase, 3 mmol/L MgCl₂, and 0.4 mmol/L each dNTP), and 3.5 μL of double-distilled water. The PCR conditions were as follows: an initial denaturation of DNA for 5 min at 94 °C, followed by 40 cycles of 1-min denaturation at 94 °C, annealing for 1 min at proper temperature (Table 1) and extension for 2 min at 72 °C, and finally extension for 10 min at 72 °C. The PCR products were separated by electrophoresis in 2 % agarose gels and visualized under UV light using 100 bp DNA ladder (TaKaRa) as the marker.

The PCR products were purified with Omega Gel Extraction Kit, recombined with pMD18-T vector

(TaKaRa), and then cloned into *Escherichia coli* (DH5α), which was followed by ampicillin selection. After the plasmids were extracted, PCR amplified, and identified, the bacterial liquid with positive plasmids was chosen for sequencing in Beijing AuGCT Biotechnology Co., Ltd.

Sequence alignments and analysis

After being aligned in DNAssist version 1.0, the amplified sequence segments (Table 1) were combined to form complete rDNA sequences for *D. folliculorum* and *D. brevis* separately. *Apis mellifica* and *Drosophila melanogaster* (Insecta: Endopterygota) were taken as outgroups. Species from four Acari superfamilies (Cheyletoidea, Tetranychoida, Analgoidea, and Ixodoidea), whose 18S rDNA and 28S rDNA were available in GenBank, were chosen to reconstruct 18S and 28S rDNA maximum parsimony (MP) phylogenetic trees under Maximum Composite Likelihood model in MEGA 4.0 (Tamura et al. 2007). Next, based on the alignment results obtained from ClustalX (version 1.8; Thompson et al. 1997), the five regions of rDNA sequences (Table 2) as well as the divergent domains of SSU rDNA and LSU rDNA were identified in line with that of *A. mellifica* (Insecta: Hymenoptera) (Gillespie et al. 2006), with small adjustments according to the predicted secondary structures (see our follow-up research paper). Finally, the lengths of the divergent domains and their divergences compared to *D. folliculorum* were calculated.

Results and discussion

Complete rDNA sequence

In this study, 11 gDNA samples were extracted successfully from 11 isolates of three *Demodex* species (five *D. folliculorum*, three *D. brevis*, and three *Demodex canis*). However, only one complete *D. folliculorum* rDNA sequence (6,397 bp) and one

Table 2 Information of complete rDNA sequence of *Demodex folliculorum* (*D. folliculorum*) and almost complete rDNA sequence of *Demodex brevis* (*D. brevis*)

| rDNA | Species | Location | Length (bp) | GC (%) | Divergence (%) |
|------|------------------------|--------------------------|-------------|--------|----------------|
| 18S | <i>D. folliculorum</i> | 1–1,809 | 1,809 | 43.7 | 3.8 (68/1,824) |
| | <i>D. brevis</i> | 1–1,824 | 1,824 | 43.4 | |
| ITS1 | <i>D. folliculorum</i> | 1,810–1,999 | 190 | 48.4 | 60.7 (142/234) |
| | <i>D. brevis</i> | 1,825–2,058 | 234 | 58.1 | |
| 5.8S | <i>D. folliculorum</i> | 2,000–2,151 | 152 | 47.4 | 25.3 (39/154) |
| | <i>D. brevis</i> | 2,059–2,212 | 154 | 58.4 | |
| ITS2 | <i>D. folliculorum</i> | 2,152–2,339 | 188 | 51.1 | 62.0 (171/276) |
| | <i>D. brevis</i> | 2,213–2,488 | 276 | 67.4 | |
| 28S | <i>D. folliculorum</i> | 2,340–6,397 | 4,058 | 40.2 | |
| | <i>D. brevis</i> | 2,489–5,837 (D12 missed) | 3,349 | 46.8 | |

GC guanine–cytosine content

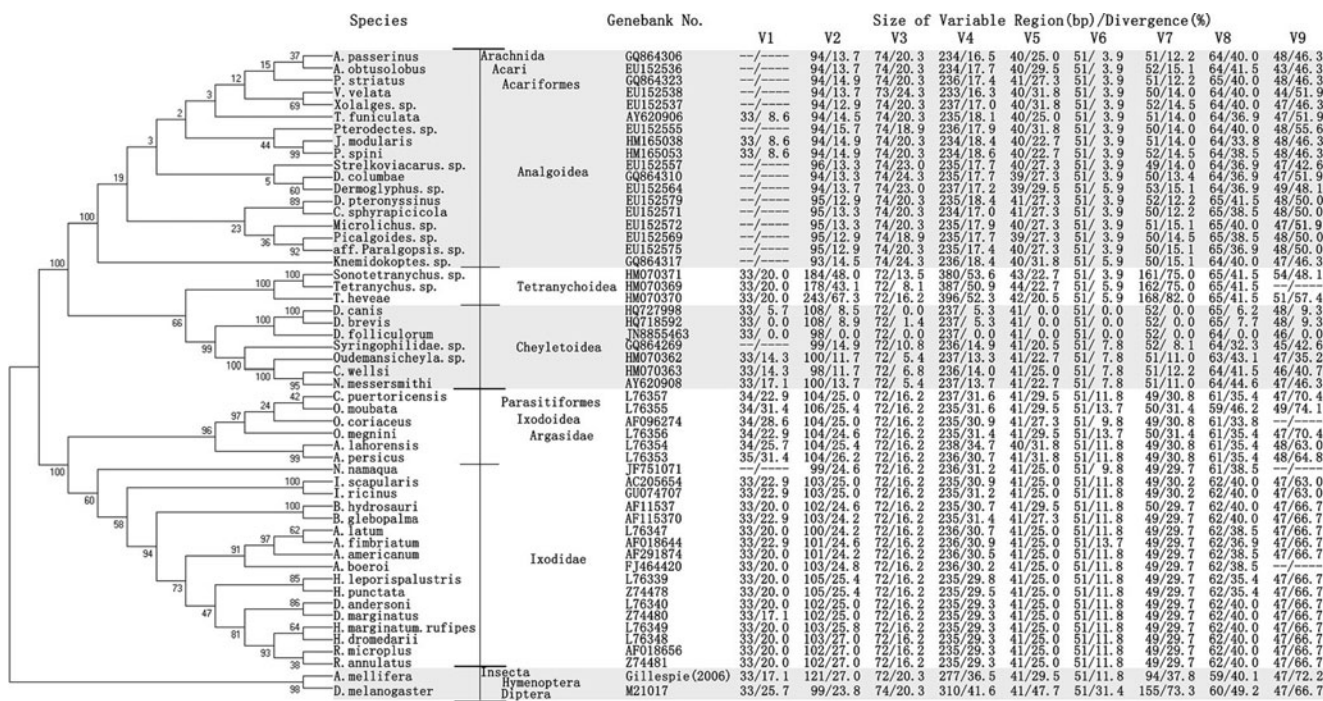


Fig. 1 MP tree based on 18S rDNA complete or almost complete sequences from 51 Acari species and two outgroups and lengths and divergences between *D. folliculorum* and any other species for each variable region (V1–V9)

almost complete *D. brevis* sequence (5,837 bp, lacking 28S terminal with D12) were obtained. For *D. canis*, only the complete 18S rDNA sequence and a large part of 28S rDNA sequence were obtained, but no sequence of the ITS regions was obtained. Therefore, only one *D. folliculorum* isolate and one *D. brevis* isolate were included in *Demodex* sequence alignments. The alignment results and the division of divergent domains can be found in Supplementary Material 1.

We also showed that, for *D. folliculorum*, V1–V9 covered 38.5 % (697/1809) of its 18S rDNA, and D1–D12 covered 43.0 % (1,743/4,050) of its 28S rDNA. For *D. brevis*, V1–V9 accounted for 38.9 % (710/1824) of the 18S rDNA, and D1–D11 took 34.0 % (1,135/3,340) of the 28S rDNA (D12 lacked; Table 2).

Table 2 shows that the complete 18S rDNA sequences of *D. folliculorum* and *D. brevis* were the most

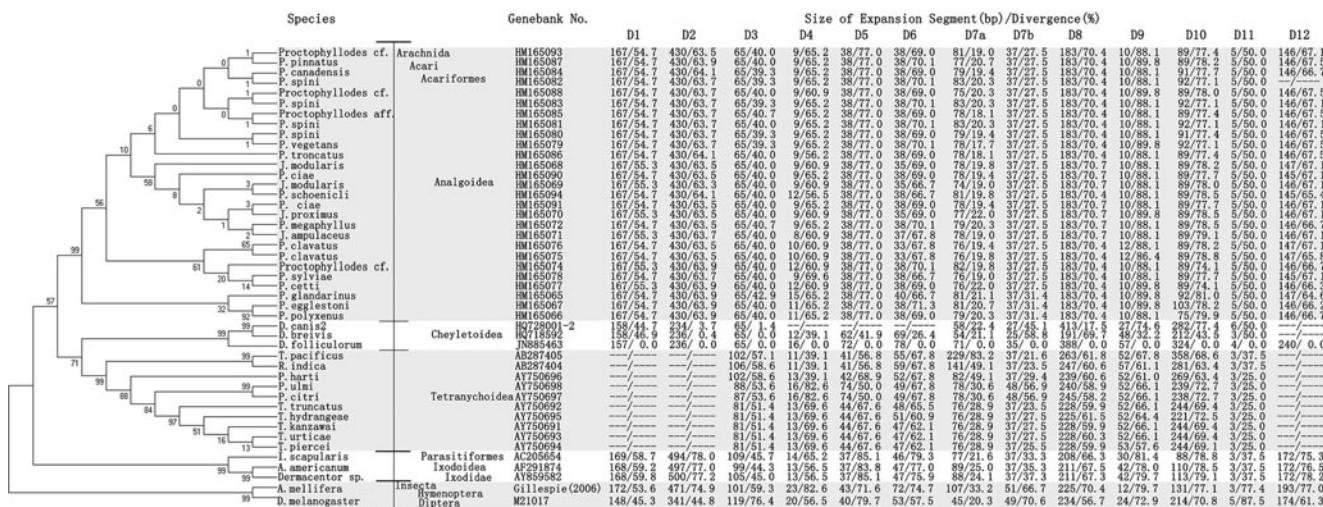


Fig. 2 MP tree based on 28S rDNA complete or almost complete sequences from 43 Acari species and two outgroups and lengths and divergences between *D. folliculorum* and any other species for each expansion segment (D1–D12)

conserved, with the lowest divergence and most approximate GC contents, and they were followed by less conserved 5.8S rDNA and then 28S rDNA. ITS-1 and ITS-2 shared the fastest evolution rates. It was indicated that different portions of rDNA might be used in phylogeny analysis at different taxonomic category levels due to discrepant sequence variances.

Since the complete 28S rDNA sequences were limited to just four superfamilies in Acari, only the 18S rDNA of 51 species from the same taxa was chosen to contrast the resolution capacity of 28S sequenced with that of the 18S rDNA sequences. It was found that the MP trees derived from the 18S rDNA and the 28S rDNA (Figs. 1 and 2) were identical at superfamily level and above. The species of each superfamily would form a cluster first, then the taxa of Acariformes and Parasitiformes would form two distinct branches and gathered later, forming the Acari branch that would eventually gathered with *A. mellifica* and *D. melanogaster*, the outgroup species. These results were in line with the traditional classification based on phenotype characteristics, demonstrating an important role of both 18S rDNA and 28S rDNA primary sequences in phylogeny across Acari.

V1–V9 and D1–D12 regions

Compared to complete rDNA sequences, the divergent domains shared higher evolution rates, suggesting a greater significance of these domains in phylogenetic study. In this study, in 18S rDNA, in terms of sequence length, in the inter- and intra-superfamilies in Acari, except the obvious insertions in V2, V4, and V7 in Tetranychoida, slight indels (usually less than 10 bp) were found in V1, V3, V5, V8, and V9 and none in V6 in all the studied species including the outgroups *A. mellifica* and *D. melanogaster*. In terms of divergences, the pairwise sequence divergences between *D. folliculorum* and any other species from the four Acari superfamilies were in accordance with the phylogenetic relationship shown in the MP trees. However, the V3 divergences between Cheyletoidea and Ixodoidea (Parasitiformes) were smaller than those between Cheyletoidea and Analgoidea (Acariformes). For the inter-superfamilies, the divergence between Demodicidae (Cheyletoidea) and others reached the highest in V9 (35.2–74.1 %) and V8 (32.3–44.6 %) and was lowest in V6 (3.9–11.8 %) and V3 (8.1–24.3 %). Further investigation showed that V2, V4, and V7 were the most valuable regions for discrimination of 18 rDNA. The divergences of *D. folliculorum* (Cheyletoidea) compared with the species in the other three superfamilies were all within the range of 11.0 to 34.7 % (Fig. 1), except for the larger divergences compared with Tetranychoida (48.0–82.0 %) due to insertions.

Much more differences in sequence length and divergence were identified in 28S rDNA compared with 18S

rDNA. A deletion of about 200 bp occurred in *Demodex* in D2, and an insertion of about 20–30 bp occurred in D6 in *Demodex* as well as in *A. mellifica*. In D8, *D. folliculorum* and *D. canis* had an insertion of about 200 bp, and the divergences between *D. folliculorum* or *D. canis* and other superfamilies were high. There was no insertion in *D. brevis*. The D12, which was absent in some species, was much longer in *D. folliculorum*. It was worth noticing that D3, D5, D9, and D10 were expanded to various extents in Tetranychoida, and D3 and D7a of *Tetranychus pacificus*, *Raoiella indica*, and *Petrobia harti* were more significantly expanded. Between Demodicidae and any other taxon, the most different segments were D9 (57.6–89.8 %) and D10 (63.4–81.0 %), followed by D6, D5, D2, D8, D12, D1, D4, D11, D3, and D7b in descending order. D7a was the segment with the smallest divergences (17.7–30.6 %), except in *T. pacificus*, *R. indica*, and *P. harti* of Tetranychoida due to insertions.

Conclusions

In this study, we have amplified, cloned, sequenced, and analyzed a complete and an almost complete rDNA sequence of 18S, ITS-1, 5.8S, ITS-2, and 28S of the two human *Demodex*, *D. folliculorum* and *D. brevis*. Then, we analyzed the sequences of all the divergent regions in the four Acari superfamilies whose 18S rDNA and 28S rDNA were both available in GenBank. It can be inferred that 28S rDNA is more valuable than 18S rDNA in molecular discrimination at the gene level.

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