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Discrimination between *Demodex folliculorum* (Acari: Demodicidae) isolates from China and Spain based on mitochondrial *cox1* sequences*

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Abstract: For a long time, classification of *Demodex* mites has been based mainly on their hosts and phenotypic characteristics. A new subspecies of *Demodex folliculorum* has been proposed, but not confirmed. Here, *cox1* partial sequences of nine isolates of three *Demodex* species from two geographical sources (China and Spain) were studied to conduct molecular identification of *D. folliculorum*. Sequencing showed that the mitochondrial *cox1* fragments of five *D. folliculorum* isolates from the facial skin of Chinese individuals were 429 bp long and that their sequence identity was 97.4%. The average sequence divergence was 1.24% among the five Chinese isolates, 0.94% between the two geographical isolate groups (China (5) and Spain (1)), and 2.15% between the two facial tissue sources (facial skin (6) and eyelids (1)). The genetic distance and rate of third-position nucleotide transition/transversion were 0.0125, 2.7 (3/1) among the five Chinese isolates, 0.0094, 3.1 (3/1) between the two geographical isolate groups, and 0.0217, 4.4 (3/1) between the two facial tissue sources. Phylogenetic trees showed that *D. folliculorum* from the two geographical isolate groups did not form sister clades, while those from different facial tissue sources did. According to the molecular characteristics, it appears that subspecies differentiation might not have occurred and that *D. folliculorum* isolates from the two geographical sources are of the same population. However, population differentiation might be occurring between isolates from facial skin and eyelids.

Key words: *Demodex folliculorum*, *cox1* partial sequences, Divergence, Genetic relationship, Phylogenetic tree
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1 Introduction

Demodex is a permanent parasitic mite distributed worldwide, belonging to the mite family Demodicidae of the subclass Acari. The mites infest the hair follicles, sebaceous glands, melbomian glands, ceruminous glands, and internal organs of 11 orders of mammals, including dog, sheep, cat, and pig, etc. For a long time, classification of *Demodex* mites has been based mainly on their hosts and phenotypic characteristics. Since Berger first identified *Demodex* in 1841, 140 species or subspecies have been identified (Li, 2009). Two or more mite species might simultaneously parasitize the same host. It is generally considered that *Demodex* is a host-specific obligate parasite. Presently, *Demodex* cannot be maintained or cultured in vitro so as to parasitize and infect other healthy animal hosts (Zhao *et al.*, 2009a; 2011). However, there are a few reports of cross-infection between humans and animals (Morsy *et al.*, 1995; Wang *et al.*, 1998).

In 1842, Simon first described the multiforms of *Demodex folliculorum*, followed by Wilson (in 1844), Hirst (in 1919), and Fuss (in 1933, 1935, and 1937).

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Akbulatova first described *Demodex brevis* in 1963, but did not name it, because it was then considered as one of two subspecies of *D. folliculorum*, including *D. folliculorum longus* and *D. folliculorum brevis* (Li, 2009). Desch and Nutting (1972) identified *D. folliculorum* Simon 1842 and *D. brevis* Akbulatova 1963, two types of parasitic *Demodex* mites found in humans, by traditional morphological classification methods. Xie *et al.* (1982) proposed a new subspecies, *D. folliculorum sinensis*, a common human hair follicle mite in China, based on its different morphological characteristics. However, their proposal has neither been confirmed nor applied in later studies. The traditional mite classification based solely on phenotype has obvious limitations as it may be affected by the environment and cannot directly reflect the molecular structural characteristics of genomic DNA. This can cause difficulties and indeterminacy in species classification and lineage. The development of molecular biology techniques has made studies of molecular markers and *Demodex* species identification at the molecular level feasible. However, our database searches found only a few reports of effective molecular identification of *Demodex* (Zhao and Cheng, 2009; Toops *et al.*, 2010; Ravera *et al.*, 2011; de Rojas *et al.*, 2012a; 2012b; Zhao and Wu, 2012a; 2012b; Zhao *et al.*, 2009b; 2012a; 2012b; 2012c).

Mitochondrial DNA sequence data have been widely used in phylogenetic studies among animal taxa because of their matrilineal inheritance, lack of extensive recombination, and accelerated nucleotide substitution rates. Recently, we conducted mitochondrial 16S rDNA partial sequence analysis of five *D. folliculorum* isolates from the facial skin of Chinese individuals and two *D. folliculorum* isolates from Spain (one from facial skin and the other from eyelids) to identify intraspecies variation between the two geographic *D. folliculorum* isolates (Spain and China). The results indicated that there were no differences among the isolates from the two geographic areas or from the two tissue sources (Zhao and Wu, 2012b). However, protein coding genes (such as cytochrome oxidase subunit 1 (*cox1*), cytochrome oxidase subunit 2 (*cox*2), nicotinamide adenine dinucleotide phosphate dehydrogenase subunit I (*NDI*), and nicotinamide adenine dinucleotide phosphate dehydrogenase subunit S (*NDS*)), which have more rapid evolution rates than mitochondrial 16S rDNA, are more useful for the phylogenetic analysis of closely related species, subspecies and different geographic populations. In this study, we analyzed sequences of the partial mitochondrial *cox1* gene to characterize *D. folliculorum* isolates from China and to compare them with those of specimens from Spain, to investigate their taxonomic status. The *cox1* partial sequences of one *D. brevis* isolate and one *D. canis* isolate were also included and analyzed to establish a reference frontier for intraspecies and interspecies variations in *D. folliculorum*, because of the lack of a standard for intraspecific identification of *D. folliculorum* from different populations using mitochondrial *cox1*.

2 Materials and methods

2.1 *Demodex* mite collection

Five *D. folliculorum* isolates and one *D. brevis* isolate (Xi'an, China) were sampled by the cellophane tape method from sufferers' facial skin, and one *D. canis* isolate (Xi'an, China) was sampled by scraping from the infection focus of a Tibetan mastiff. The adult of each isolate was collected separately using home-made needles, after being identified microscopically (magnification 10×4) (Li, 2009), and then preserved in Eppendorf tubes (EPs) containing 20 μl dilution buffer.

The mitochondrial *cox1* fragments of two *D. folliculorum* isolates (one from facial skin and the other from eyelids) from Spain were provided by Manuel DE ROJAS.

2.2 DNA extraction

Genomic DNA of individual mites was extracted according to Zhao *et al.* (2012b). DNA release additive (0.5 μl) was added to each tube and vortexed. After being incubated at room temperature for 5 min, the reagents were placed in 98 °C for 2 min, and finally preserved in EPs at −20 °C.

2.3 Polymerase chain reaction (PCR) amplification of *cox1* partial sequence

Specific primers (sense: 5′-GATTTTTTGGTC ACCCAGAAG-3′; anti-sense: 5′-AGTGGAAGTG GGCTACGAC-3′) of the mitochondrial *cox1* partial sequence were designed according to the correlated sequences (GenBank accession Nos. FN424247 and

FN424248, Spain) and the primers were synthesized by Beijing AuGCT Biotechnology Co., Ltd., China. Extracted DNAs of *D. canis*, *D. brevis*, and *D. folliculorum* (Table 1) were amplified using an Applied Biosystems 2720 thermal cycler in 12.5 μl reaction volume containing 6 μl of 2× Premix Taq buffer, pH 8.3 (0.05 U/μl Taq DNA polymerase, 3 mmol/L $MgCl_2$, 0.4 mmol/L each dNTP), 1 μl of 1 μmol/L of each primer, 1 μl of DNA template, and 3.5 μl of double distilled water. The amplification followed the thermal profile: pre-degeneration at 94 °C for 4 min; then 40 cycles of degeneration at 94 °C for 1 min, annealing at 58 °C for 1 min, and extension at 72 °C for 2 min; and finally extension at 72 °C for 10 min in a thermal cycler. The PCR products were separated by electrophoresis in 2% agarose gels (0.02 g/ml), prestained with ethidium bromide, at 120 V for 20 min in tris-borate-ethylenediaminetetraacetic acid (TBE) buffer, and visualized under UV light with 100 bp DNA ladder (TaKaRa) as the marker.

2.4 Cloning and sequencing of *cox1* partial sequence

Expecting that the quantity of DNA extracted was too poor to be sequenced directly, cloning of PCR product was extended to obtain more DNA. *cox1* fragments were purified using an OMEGA gel extraction kit, cloned after being linked with pMD18-T vector (TaKaRa), and then transformed into *Escherichia coli* (DH5α). Ampicillin screening and PCR after plasmid extraction were applied for the identification of clones containing *cox1* fragments. At least two clones screened from six identical *cox1*-positive clones per sample were sequenced by Beijing AuGCT Biotechnology Co., Ltd., China. The two sequences obtained were each aligned with the template used for primer design to confirm their consistency and reliability. If the sequencing failed or the sequence consistency was poor, the cloning and sequencing procedure was repeated.

2.5 DNA sequence analysis

The nucleotide alignments of the *cox1* sequences of the nine *Demodex* isolates obtained were conducted using ClustalX software version 1.8 (Thompson *et al.*, 1997) in the multiple alignment mode. The gene divergence of the pairwise *cox1* partial sequences was estimated by DNAStar. Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0 (Tamura *et al.*, 2007) was employed to analyze the nucleotide composition, variable sites, parsim-info sites, and singleton sites, and to compute genetic distances based on the Maximum Composite Likelihood Model.

2.6 Base composition bias and skew measures of the third-position nucleotides

MEGA 4.0 was further used to analyze transition/transversion and nucleotide composition of the third-position nucleotides. CG and AT content percentages, GC-skew (G−C)/(G+C) and AT-skew (A−T)/(A+T) (Perna and Kocher, 1995) were computed and graphical representations of GC-skew and AT-skew were drawn in Excel 2003.

2.7 Phylogenetic analysis

Demodex brevis (one isolate) and *D. canis* (one isolate) were taken as outgroups for the *D. folliculorum* isolates (five Chinese and two Spanish). A total of nine *cox1* partial sequences from the *Demodex* were used to reconstruct the phylogenetic tree by maximum likelihood (ML) method in PhyML 3.0. Other methods (neighbour-joining (NJ), minimum evolution (ME), and maximum parsimony (MP)) were explored in MEGA 4.0 to confirm the reliability of phylogenetic relationships in the nine isolates of three *Demodex* species. Information on the *cox1* sequences is listed in Table 1.

Table 1 Information of nine *cox1* sequences

Mite species	Nation	GenBank acc. No.
Demodex folliculorum 1*	China	JF784002
Demodex folliculorum 2*	China	HQ844222
Demodex folliculorum 3*	China	JF784003
Demodex folliculorum 4*	China	JF784004
Demodex folliculorum 5*	China	JF784005
Demodex folliculorum 6	Spain	FN424248
Demodex folliculorum 7	Spain (eyelids)	FN424247
*Demodex canis**	China	JF731345
*Demodex brevis**	China	HQ844223

* *Demodex* mites were collected in this study

3 Results

3.1 PCR amplification and cloning

The mitochondrial *cox1* fragments of the seven isolates of three *Demodex* species from Xi'an, China were amplified successfully, and shown by sequencing

to be 429 bp long. We submitted the sequences to GenBank, and obtained the accession Nos. JF784002, HQ844222, JF784003, JF784004, JF784005, JF731345, and HQ844223.

3.2 *cox1* sequence analysis

The *cox1* partial sequences (429 bp) of the seven *Demodex* isolates (five *D. folliculorum*, one *D. brevis* and one *D. canis*) were aligned with those of the two *D. folliculorum* isolates from Spain (Fig. 1). DNAStar analysis showed a 97.4% (418/429) sequence identity and a 2.6% divergence in the five *D. folliculorum* isolates from the facial skin of Chinese individuals, and MEGA 4.0 analysis showed 418 identical sites and 11 variable sites (8 singleton and 3 parsim-info sites). The average contents of A, C, G, and T were 33.9%, 23.8%, 13.8%, and 28.5%, respectively, and the nucleotide frequencies were biased toward A+T, averaging 62.4%. The mean G+C contents of five *D. folliculorum* isolates from the facial skin of Chinese individuals and one from a Spanish individual were 37.6% and 36.8%, respectively, a 0.8% difference. The G+C contents of one *D. folliculorum* isolate from the eyelids of a Spanish individual, one *D. canis* isolate, and one *D. brevis* isolate were 37.3%, 38.0%, and 41.5%, respectively.

3.3 Divergence

Table 2 shows that in *D. folliculorum* species, the average pairwise sequence divergence was 1.24% among five isolates from Xi'an, China, 0.94% between one isolate from the facial skin of a Spanish individual and five from Chinese individuals, and 2.15% between one isolate from the eyelids and six isolates from the facial skin. The average pairwise sequence divergence of all seven *D. folliculorum* isolates was 1.43%. The interspecies divergence between the *D. canis* and *D. folliculorum* isolates was 14.53%, and between the *D. brevis* and the *D. folliculorum* isolates was 23.10%. Thus, interspecific divergence was significantly larger than intraspecies divergence in *D. folliculorum*.

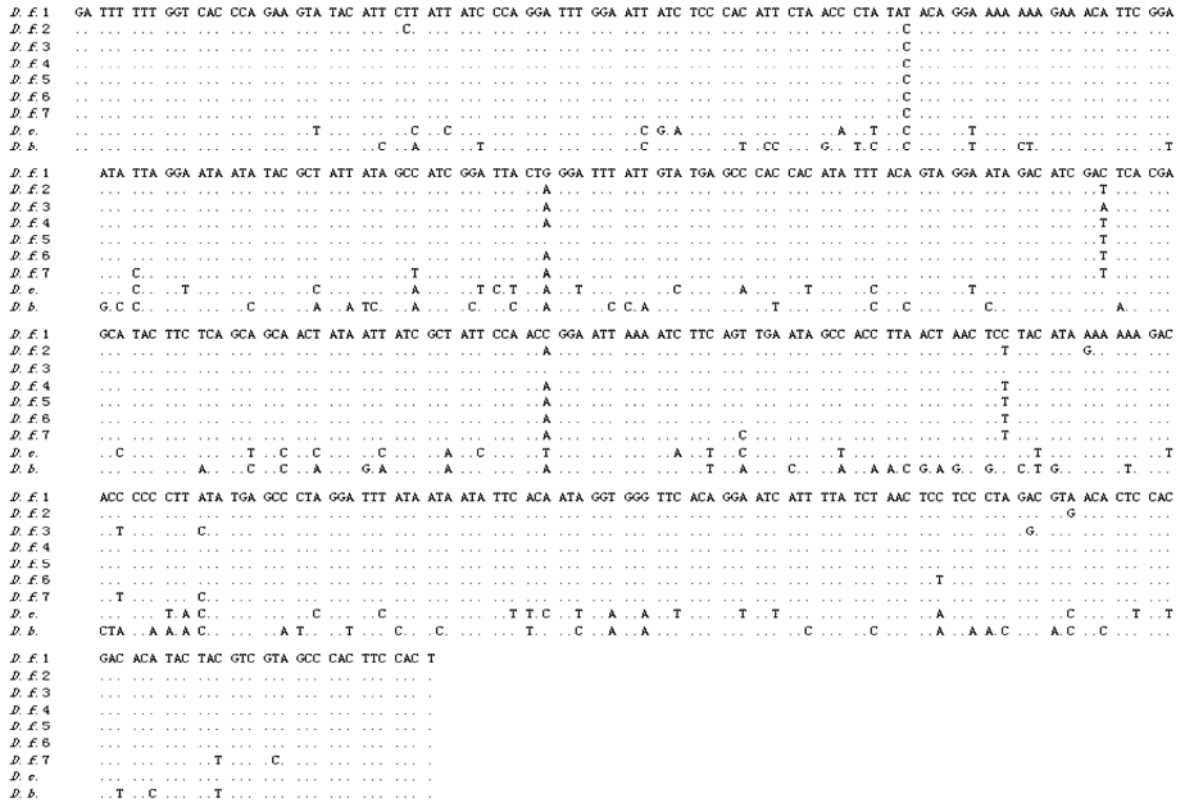


Fig. 1 Alignments of the nine *cox1* fragments of the three *Demodex* species

The nucleotide data were grouped into triplets corresponding to the codons. *D.f.*: *D. folliculorum*; *D.c*: *D. canis*; *D.b*: *D. brevis*

Table 2 Pairwise divergence and genetic distance in the *cox1* partial sequence between *Demodex* species*

Mite species	*D.f.*1	*D.f.*2	*D.f.*3	*D.f.*4	*D.f.*5	*D.f.*6	*D.f.*7	*D.c*	*D.b*
*D.f.*1		1.9	1.4	1.2	0.9	1.4	2.9	14.4	23.3
*D.f.*2	0.019		2.1	0.7	0.9	0.9	2.4	15.0	23.7
*D.f.*3	0.014	0.022		1.4	1.7	1.7	2.1	14.4	23.0
*D.f.*4	0.012	0.007	0.014		0.2	0.2	1.7	14.4	23.0
*D.f.*5	0.009	0.009	0.017	0.002		0.5	1.9	14.7	23.3
*D.f.*6	0.014	0.009	0.017	0.002	0.005		1.9	14.4	23.0
*D.f.*7	0.029	0.024	0.022	0.017	0.019	0.019		14.4	22.4
D.c	0.167	0.175	0.167	0.167	0.171	0.167	0.167		26.0
D.b	0.309	0.313	0.304	0.304	0.308	0.304	0.293	0.354	

* Above the diagonal: pairwise divergence; Below the diagonal: pairwise genetic distance. Divergence=1–%identity. *D.f.*: *D. folliculorum*; *D.c*: *D. canis*; *D.b*: *D. brevis*

3.4 Genetic distance

The pairwise genetic distances were computed by MEGA 4.0 (Table 2). In *D. folliculorum*, the average pairwise genetic distance of five isolates from the facial skin was 0.0125 among Chinese individuals, 0.0094 between one isolate from the facial skin of a Spanish individual and five isolates from Chinese individuals, and 0.0217 between one isolate from the eyelids and six isolates from the facial skin. The average intraspecific genetic distance among the seven isolates was 0.014. The genetic distance between isolates from different species was 10 times larger than that of isolates from the same species. Interspecific/intraspecific genetic distance was 12.05 (0.169/0.014) between *D. folliculorum* and *D. canis*, and 21.79 (0.305/0.014) between *D. folliculorum* and *D. brevis*.

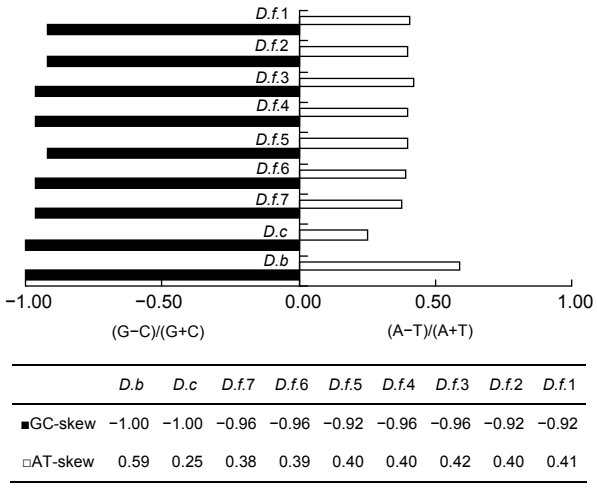
3.5 The third-position nucleotides analysis

Of the 142 third-position nucleotides (Fig. 1) in *D. folliculorum* species, the mean transition/transversion rate was 2.7 (3/1) among five isolates from the facial skin of Chinese individuals, 3.1 (3/1) between one isolate from the facial skin of a Spanish individual and five isolates from Chinese individuals, and 4.4 (3/1) between one isolate from the eyelids and six isolates from the facial skin. The transition/transversion rate between the *D. folliculorum* and *D. canis* isolates was 1.2 (8/7) and between the *D. folliculorum* and *D. brevis* isolates was 0.7 (7/9). The G+C contents of seven *D. folliculorum* isolates are shown in Table 3. GC-skew and AT-skew data are presented in Fig. 2.

Table 3 Base composition bias of the third-position nucleotides

Mites	T (%)	C (%)	A (%)	G (%)	A+T (%)	G+C (%)
*D.f.*1	19.0	34.5	45.1	1.4	64.1	35.9
*D.f.*2	19.7	33.1	45.8	1.4	65.5	34.5
*D.f.*3	19.0	33.8	46.5	0.7	65.5	34.5
*D.f.*4	19.7	33.1	46.5	0.7	66.2	33.8
*D.f.*5	19.7	33.1	45.8	1.4	65.5	34.5
*D.f.*6	20.4	32.4	46.5	0.7	66.9	33.1
*D.f.*7	21.1	31.7	46.5	0.7	67.6	32.4
D.c	24.7	34.5	40.8	0.0	65.5	34.5
D.b	12.7	38.0	49.3	0.0	62.0	38.0

D.f.: *D. folliculorum*; *D.c*: *D. canis*; *D.b*: *D. brevis*



	D.b	*D.c*	*D.f.*7	*D.f.*6	*D.f.*5	*D.f.*4	*D.f.*3	*D.f.*2	*D.f.*1
■GC-skew	−1.00	−1.00	−0.96	−0.96	−0.92	−0.96	−0.96	−0.92	−0.92
□AT-skew	0.59	0.25	0.38	0.39	0.40	0.40	0.42	0.40	0.41

Fig. 2 Graphical representation of GC-skew and AT-skew of the third-position nucleotides
D.f.: *D. folliculorum*; *D.c*: *D. canis*; *D.b*: *D. brevis*

3.6 Phylogenetic analysis

The phylogenetic trees were reconstructed for the nine *cox1* partial sequences from isolates of three *Demodex* species. The topological structures of NJ, ME, and MP trees were nearly the same as that of the ML tree (Fig. 3). Two geographical *D. folliculorum* isolate groups, five isolates from the facial skin of Chinese individuals and one from a Spanish individual, were gathered first, then one isolate (*D. folliculorum* 7) from the eyelids of a Spanish individual, and finally *D. brevis* and *D. canis*.

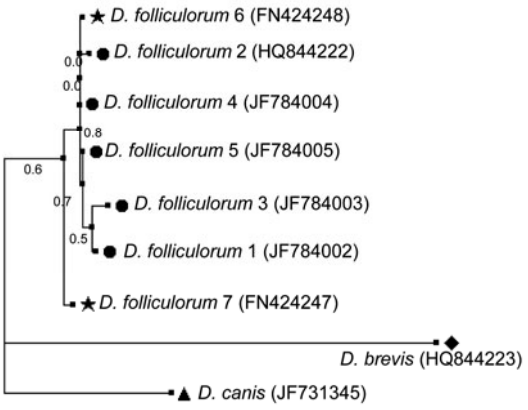


Fig. 3 Maximum likelihood (ML) tree for nine isolates of *Demodex* species with *D. brevis* and *D. canis* as outgroups
The numbers on each node represent the bootstrap supports. *D. folliculorum* isolates from China (●); *D. folliculorum* isolates from Spain (★); *D. canis* (▲); *D. brevis* (♦)

4 Discussion

A subspecies is a category ranking immediately below a species and designates a population of a particular geographic region or host. It is genetically distinguishable from other such populations of the same species and capable of interbreeding with them. A species will either be recognized as having no subspecies at all, or having two or more, but never only one.

Based on the definition of a subspecies, we hold doubts about the validity of the facial *D. folliculorum* subspecies proposed by Xie *et al.* (1982). First, *D. folliculorum* is distributed worldwide, and few reports of polymorphism in *D. folliculorum* resulting from geography or race of human hosts can be found. There are ample studies containing the term "*D. folliculorum*" in PubMed but none with "*D. folliculorum folliculorum*", and no reports on *D. folliculorum sinensis* can be found in the China National Knowledge Infrastructure (CNKI), suggesting that the subspecies of *D. folliculorum* has not been acknowledged. Second, the validity of their results and conclusions was impaired because of the scarcity of hosts and samples. The mite samples in their study were collected from a limited number of hosts, which might not reflect the general morphological characteristics of the mites in China or serve as being representative of "*D. f. sinensis*". Therefore, we suspect that the differences in morphological characteristics between "*D. f. folliculorum*" and "*D. f. sinensis*" proposed by Xie *et al.* (1982) might reflect polymorphism in geographically isolated populations but not differences between subspecies. Whether subspecies, based on their morphological differences, exist in *D. folliculorum* remains to be confirmed.

Here we provide the first comparison of *cox1* partial sequences from nine isolates of three *Demodex* species. Subspecies differentiation of *D. folliculorum* from the facial skin has not been found at the molecular level, which is in accord with our deduction of polymorphism in geographical isolates. The extent of sequence divergence used to establish intraspecific differentiation in animals is rarely greater than 2% and mostly less than 1% (Avise and Walker, 1999; Hebert *et al.*, 2003; Tsao and Yeh, 2008). In *D. folliculorum* from the facial skin, the average pairwise divergence between an isolate from Spain and five isolates from China was 0.94%, smaller than that (1.20%) of five isolates from Xi'an China. The average pairwise genetic distance between one isolate from Spain and five isolates from China was 0.0094, which was not only smaller than that (0.0125) of five isolates from Xi'an, China, but also in accordance with the standard that interspecific genetic distance is rarely less than 0.020 (Liu *et al.*, 2010). Of the 142 third-position nucleotides, the mean transition/transversion rate among five isolates from China was 2.7 (3/1), and between one isolate from Spain and five isolates from China was 3.1 (3/1), both larger than the intraspecies standard of 2.0 (Kumar *et al.*, 2008; Zhou *et al.*, 2010). All the results indicate that no subspecies differentiation exists in *D. folliculorum* from the facial skin of these two geographical groups.

However, a population difference was found between *D. folliculorum* isolates from facial skin and eyelids. The average pairwise divergence between one isolate from eyelids and six from facial skin was 2.15%, larger than the intraspecies standard of 2.0%. The average pairwise genetic distance between them was 0.0217, larger than the intraspecies standard of 0.020, both results indicating that a difference might exist between the two *D. folliculorum* populations from different facial tissues (eyelids and facial skin). The distinct local internal environment of *D. folliculorum* may be related to this differentiation. Our results are in accordance with the proposal of de Rojas *et al.* (2012a) that *cox1* gene sequences can help to identify different populations (in the facial skin and eyelids in this study), which were morphologically very close and difficult to separate by classical methods. However, as the same *D. folliculorum* sample from the eyelids of a Spanish individual was used, the general applicability of our results was impaired because of the scarcity of samples, and therefore needs to be confirmed.

Further examination of *cox1* partial sequences using ML phylogenetic tree analysis (Fig. 3) showed that three *Demodex* species gathered separately, suggesting that they are three independent species. In *D. folliculorum* species, six isolates from the facial skin gathered together and did not form sister clades. This result supported our conclusion that the *D. folliculorum* isolates from the facial skin were from the same population and no geographical differences existed. One isolate from the eyelids of a Spanish individual formed sister clades with six isolates from the facial skin, supporting our conclusion that differentiation may exist between populations of *D. folliculorum* from eyelids and facial skin.

From the present study, we conclude that population differences in *Demodex* may be related to the parasitic host and the source tissue, but not to geographical sources or races of human hosts. No subspecies differentiation exists in the *cox1* genetic sequences of the two geographical isolates from the facial skin of Chinese and Spanish individuals, while population differentiation may exist in *D. folliculorum* from two source tissues (eyelids and facial skin). Although only a limited number of *Demodex* isolates were studied due to the deficiency of molecular data in GenBank, this study has identified that *cox1* can be an effective molecular marker for distinguishing intraspecies and interspecies variation in *Demodex*. Therefore, when traditional taxonomy shows indeterminacy in species classification, it might be helpful to look for evidence at the DNA level.

Compliance with ethics guidelines

Ya-e ZHAO, Jun-xian MA, Li HU, Li-ping WU, and Manuel DE ROJAS declare that they have no conflict of interest.

This article does not contain any studies with human or animal subjects performed by any of the authors.

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