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Identification and molecular phylogeny of agriculturally important spider mites (Acari: Tetranychidae) based on mitochondrial and nuclear ribosomal DNA sequences, with an emphasis on *Tetranychus*

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Abstract

Tetranychid mites are serious agricultural pests. Identification of species in the Tetranychidae is hampered by their close morphological similarities, especially for species within the genus *Tetranychus*. In this study, we examined the relationships of nine agriculturally important species in the Tetranychidae from China based on mitochondrial (*cytochrome c oxidase subunit 1*) and nuclear (*internal transcribed spacer 1 and 2* of ribosomal RNA gene) sequences. The results confirm the monophyly of the morphologically defined *Tetranychus*, *Panonychus*, *Amphitetranychus* and *Petrobia*. However the position of *Amphitetranychus viennensis* within the Tetranychidae needs to be confirmed. The genetic distances between *Tetranychus truncatus*, *T. turkestani* and *T. urticae* that their taxonomy needs revision. In particular, both *cytochrome oxidase 1* and the *internal transcribed spacers 1* and *2* of rDNA sequences showed large geographical differences within *T. cinnabarinus*, suggesting the existence of cryptic species within this species.

Key words: *COI* , DNA barcoding, *ITS*, phylogeny, species identification, Tetranychidae

Introduction

The Tetranychidae are polyphagous pests that seriously damage vegetables, ornamentals, and agricultural crops throughout cold and temperate zones (Goka & Takafuji 1995). The damage caused by spider mites to agriculture has greatly increased during the past 60 years. Two species that have been given much attention worldwide are the two-spotted spider mite, *Tetranychus urticae* Koch, and the European red mite, *Panonychus ulmi* (Koch) (Helle & Sabelis 1985). The family Tetranychidae is subdivided into two subfamilies: Bryobiinae and Tetranychinae, each being further separated into tribes. The Bryobiinae encompass the tribes Bryobiini, Hystrichonychini and Petrobiini, whereas the Tetranychinae includes the tribes Eurytetranychini, Tenuipalpoidini and Tetranychini (Helle & Sabelis 1985). Morphological characters such as peritreme, aedeagus, tarsus claws and empodium, have been used to identify the species of Tetranychidae, and resolve their phylogenetic relationships.

Distinguishing tetranychid taxa is difficult because of their morphological similarity. In addition, both sexes of many species are often needed to make precise determinations (Ben-David *et al.* 2007). Species in the *Tetranychus* are especially difficult to identify. For example, the two-spotted spider mite *T. urticae* is considered a species complex (Navajas *et al*. 1998) and as many as 44 synonymous names are known (Bolland *et al.* 1998). The question whether red *T. urticae* mites should be considered as a separate species (*T. cinnabarinus* (Boisduval)) has been a subject of debate for many years (Dupont 1979; Gotoh & Tokioka 1996; Zhang & Jacobson 2000). In addition, *T. truncatus* Ehara and *T. turkestani* (Ugarov & Nilolskii) can not be easily distinguished from *T. urticae* by molecular methods (Navajas *et al*. 2003; Ros & Breeuwer 2007). In this study, we provisionally consider *T. urticae*, *T. cinnabarinus*, *T. truncatus* and *T. turkestani* as four different species.

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Because the number of classical mite taxonomists is small and decreasing, molecular methods of taxonomy are becoming increasingly important. Although molecular data also suggest close genetic relationships among some members of the spider mites, they offer a more effective approach to the identification of spider mites (Ben-David *et al.* 2007). With respect to tetranychid mites, DNA-based identification has several advantages over traditional morphological methods. It is more rapid because one can use material from any stage of the life cycle, thus avoiding the need for the time-consuming and risky practice of rearing spider mites (Marrelli *et al.* 2005). It is more versatile because, with PCR amplification, it is possible to use any material such as fragmented insect remains and preserved specimens. It is also simpler because it does not require a comprehensive knowledge of morphology (Leigh *et al*. 2008).

A few studies have used molecular markers for studying genetics in tetranychid mites (reviewed by Navajas & Fenton, 2000). Such markers include allozymes (Hinomoto & Takafuji 1994; Goka & Takafuji 1995; Tsagkarakou *et al.* 1997, 1998 & 1999), microsatellites (Luikart & England 1999; Navajas *et al.* 2002), ribosomal DNA sequences (Navajas *et al.* 1998; Ben-David *et al.* 2007) and mitochondrial DNA sequences (Navajas *et al.* 1996a, 1996b, 1998, 1999; Hinomoto *et al.* 2001, 2007; Toda *et al.* 2000; Xie *et al.* 2006a; Ros & Breeuwer 2007).

DNA barcoding is a method proposed to classify animals to the species level using a fragment of mitochondrial DNA, usually from the 5' region of *cytochrome oxidase subunit I* (*COI*) (Hebert *et al.* 2003a). *COI* is well suited for population and species identification because of its high degree of polymorphism (Hebert *et al.* 2003b). Several studies have used the *COI* region to delineate tetranychid species (Lee *et al.* 1999; Hinomoto *et al.* 2001; Navajas *et al.* 1994, 1996a, 1996b, 1998; Toda *et al.* 2000; Xie *et al.* 2006a; Ros & Breeuwer 2007). The *ITS2* (*internal transcribed spacer 2*) sequence has also been used for distinguishing mite species (Navajas 1998; Navajas *et al*. 1998, 1999 & 2001; Vargas *et al.* 2005; Noge *et al*. 2005; Ben-David *et al*. 2007), while the *ITS1* sequence has been used less often (Navajas *et al.* 1996, 1998; Hinomoto *et al*. 2001, 2007; Osakabe *et al.* 2008).

In this study, we attempted to identify the relations among nine spider mite species based on *COI* and *ITS* (*ITS1* and *ITS2*) sequences. The results were compared with morphology-based identifications using keys in Bolland *et al*. (1998) and Wang (1981). We also provide some guidelines for future barcode studies on tetranychid mites.

Material and methods

Taxon sampling. Twenty-seven populations of tetranychid mites used in this study were collected from 22 regions of China. Their locations, host plants and GenBank accession nos. are summarized in Table 1. They were examined carefully by professional Acari taxonomists (Xiao-Feng Xue and Xiao-Yue Hong) in our lab with Zeiss Discovery. V12 and Zeiss Imager. A2 (Göttingen, Germany) research microscope to make sure the identification was correct. Mites were reared in the laboratory on leaf discs of common bean, *Phaseolus vulgaris* L., at 25 °C, 60% RH, and a photoperiod of 16:8 (L: D) h.

Molecular laboratory methods. DNA Extraction. Total DNA was extracted from individual adult females using protocols described by Gomi *et al*. (1997). Mites were individually put into 1.5 ml microcentrifuge tubes with 25 ul of a mixture of STE buffer (100 mM NaCl, 10 mM Tris-HCl, and 1 mM EDTA, pH 8.0) and were homogenized with a sterilized plastic pestle while the tubes were entirely dipped into ice. Proteins were removed with 2 ul of 10 mg/ml proteinase K. These mixtures were incubated at 37°C for 30 min, and proteinase K was inactivated at 95 °C for 5 min. The genomic DNA was briefly centrifuged (4000g) and used immediately for the PCR reaction or stored at -20°C for later use.

PCR was used to amplify partial sequences of *COI*, *ITS1* and *ITS2* using the primer pairs: *COI*- forward 5'-TGATTTTTTGGTCACCCAGAAG-3', *COI*-reverse 5'-TACAGCTCCTATAGATAAAAC-3' (Navajas *et al*., 1996b); *ITS1*-forward 5'-ATATGCTTAAATTCAGCGGG-3', *ITS1*-reverse 5'-GGGTC-GATGAAGAACGCAGC-3' (Hinomoto *et al*., 2001); *ITS2*-forward 5'-GTCGTAACAAGGTTTCCGT-AGG-3', *ITS2*-reverse 5'-TGGCTGCGTTCTTCATCG-3' (Navajas *et al*., 1994). PCR conditions: *COI*: 50 ul volume containing 4 ul of DNA, 28.6 ul of double-distilled H_2O, 5 ul of 10x buffer, 5 ul of 25 mM $MgCl_2$, 4 ul

of dNTPs (10 mM each), 0.4 ul of Taq polymerase (5 U/um, Takara, China-Japan Joint Company, Dalian, Liaoning Province, China), and 1.5 ul each primer (20 uM each). Temperature cycles: 5 min 94°C, 35 cycles of 94°C for 30 s, 51°C for 1 min and 72°C for 1 min, then 72°C for 7 min. *ITS1*: 50 ul volume containing 4 ul of sample, 28.6 ul of double-distilled H_2O, 5 ul of 10x buffer, 5 ul of 25 mM $MgCl_2$, 4 ul of dNTPs (10 mM each), 0.4 ul of Taq polymerase (5 U/um, Takara, China-Japan Joint Company, Dalian, Liaoning Province, China), and 1.5 ul each primer (20 uM each). Temperature cycles: 3 min 94°C, 35 cycles of 94°C for 1 min, 52°C for 1 min and 72°C for 2 min, then 72°C for 10 min. *ITS2*: 50 ul volume containing 2 ul of sample, 30.6 ul of double-distilled H_2O, 5 ul of 10x buffer, 5 ul of 25 mM $MgCl_2$, 4 ul of dNTPs (10 mM each), 0.4 ul of Taq polymerase (5 U/ul, Takara, China-Japan Joint Company, Dalian, Liaoning Province, China), and 1.5 ul each primer (20 uM each). Temperature cycles: 4 min 94°C, 30 cycles of 92°C for 1 min, 51°C for 1 min and 72°C for 1 min, then 72°C for 7 min.

Cloning and Sequencing. Each sequence was determined from five female adult individuals (three colones of each individual were picked up and sequenced) to exclude polymerase errors. All the 15 sequences of the same population were the same. Amplified fragments were purified using the Gel Extraction Mini kit (Watson, Shanghai, China). Then, the pure segments were ligated into the pGEM-T vector (Promega, Madison, WI) and introduced into *Escherichia coli* DH5a cells. Bacteria were cultured in LB medium after blue/white selection, and then inserts were sequenced by the biological reagent company called Takara.

Data analysis and Phylogenetic analysis. DNA sequences were submitted to GenBank and compared online with the published sequences by similarity search engines such as BLAST in the NCBI Web, then aligned using the Clustal X computer program (Gene Codes Corporation, Ann Arbor, MI) (Thompson *et al*. 1997). Sequences were aligned and trimmed using the Clustal W algorithm in MEGA version 4 (Tamura *et al*. 2007) with gap opening penalty 15, gap extension penalty 7.5, IUB weight matrix, transition weight 0.5 and delay divergent cut-off 30. DAMBE version 5.1.2 (Xia & Xie 2001) was used to analyze the extent of saturation. Transversions/transitions versus divergence (TN93 genetic distances calculated by DAMBE) were plotted, to assess saturation of sequences. For all the following phylogenetic inference methods, positions containing gaps or missing data were not used. The sequence divergences calculated by MEGA among intraspecific, interspecific species, based on Kimura-2-parameter (K2P) distance, were indicated.

Phylogenetic analyses were conducted by PAUP* version 4.0b10 (Swofford 2002) using Neighbour-Joining (NJ) algorithms (K2P) and Maximum Likelihood (ML) algorithms (TBR heuristics, random addition sequence with ten replicates, reconnection limit of 10). Both PAUP and Modeltest 3.6 (Posada & Crandall, 1998) were used to select the optimal evolution model for the ML analysis. For the NJ analyses robustness of nodes was assessed with 1,000 NJ-bootstrap replicates. For the ML analyses bootstrap support was assessed by performing a NJ bootstrap (1,000 replicates) with distances calculated according to the selected ML model. The NJ tree was built using the Kimura-2-parameter (K2P) model. And the model for ML analysis, selected by Modeltest for the tetranychid dataset, was the General Time Reversible Model with invariable sites and a gamma distribution of rate heterogeneity (GTR+I+G). Implementing the rate class ‘a b a b e f’ significantly improved the likelihood (AIC) and was therefore used for parameter and tree topology estimation (Ros & Breeuwer 2007). Other sequences (see captions of Figs. 3, 4 and 5) obtained from the GenBank were also used for tree building.

Results

Phylogeny estimation using Cytochrome oxidase subunit I sequence data

Fifteen partial sequences of the mitochondrial *COI* gene were obtained and submitted to GenBank (accession nos. GQ141906-141920). Some of the *COI* sequences had 100% similarity to existing GenBank sequences, such as the sequences of *T. cinnabarinus* and *T. urticae* from various populations in China (Xie *et al.* 2006a). The sequenced portion is a 453-bp, AT-rich region from the central part of the mitochondrial *COI* gene. Compared to the standard DNA barcoding fragment (Folmer fragment) of the entire mitochondrial *COI* gene (position 1474–3009), the fragment analyzed in this study is on the position 2173–2625. Base pair numbers

correspond to the *Drosophila melanogaster* Meigen mitochondrial DNA sequence (GenBank accession no U37541). The mean AT percent (74.3%) resembles the result of Xie *et al.* (2006a). Of the 453 bases, 329 (72.6%) were invariant. Of the remaining 124 variable positions, 98 were parsimony informative sites (Table 2).

TABLE 1. Spider mites used in this study: their location, host plant, *COI*, *ITS1* and *ITS2* accession numbers in the Genbank

Species	Population	N*	Host plant	Accession No.		
				COI	ITS1	ITS2
Tetranychus cinnabarinus (Boisduval)	Zhengzhou, Henan	5	Cotton (*Gossypium hirsutum* L.)	GQ141906	GQ141950	GQ141922
	Anqing, Anhui	5	Cotton (*G. hirsutum* L.)	DQ437552**	GQ141945	DQ512869**
	Gaoxiong, Taiwan	5	Eggplant (*Solanum melongena* L.)	DQ437555**	GQ141947	DQ517301**
	Huhhot, Neimenggu	5	Bean (*Phaseolus vulgaris* L.)	DQ437545**	GQ141949	DQ515789**
	Fuzhou, Fujian	5	Bean (*P. vulgaris* L.)	DQ437554**	GQ141946	DQ517300**
	Yinchuan, Ningxia	5	Bean (*P. vulgaris* L.)	DQ437567**	***	DQ523843**
	Yulong, Yunnan	5	Bean (*P. vulgaris* L.)	GQ141907	GQ141948	GQ141921
T. urticae Koch	Shanghai	5	Pipal (*Ficus religiosa* L.)	DQ016523**	GQ141954	DQ778067**
	Tianshui, Gansu	5	Bean (*P. vulgaris* L.)	GQ141909	GQ141951	GQ141924
	Kunming, Yunnan	5	Eggplant (*S. melongena* L.)	DQ016516**	GQ141953	DQ778064**
	Ji'nan, Shandong	5	Apple (*Malus pumila* Mill.)	GQ141908	GQ141958	GQ141923
	Meixian, Shaanxi	5	Apple (*M. pumila* Mill.)	DQ016518**	GQ141952	DQ680171**
	Xingcheng, Liaoning	5	Apple (*M. pumila* Mill.)		GQ141955	
	Xuzhou, Jiangsu	5	Apple (*M. pumila* Mill.)		GQ141957	
	Zhengzhou, Henan	5	Apple (*M. pumila* Mill.)		GQ141956	
T. truncatus Ehara	Shihezi, Xinjiang	5	Bean (*P. vulgaris* L.)	GQ141912	GQ141939	GQ141926
T. turkestani (Ugarov & Nikolskii)	Shihezi, Xinjiang	5	Bean (*P. vulgaris* L.)	GQ141913	GQ141938	GQ141927
T. kanzawai Kishida	Fuzhou, Fujian	5	Bean (*P. vulgaris* L.)	GQ141911	GQ141937	GQ141928
Amphitetranychus viennensis (Zacher)	Ji'nan, Shandong	5	Apple (*M. pumila* Mill.)	GQ141910	GQ141936	GQ141925
Panonychus citri (McGregor)	Wuhan, Hubei	5	Sweet orange (*Citrus sinensis* (L.))	GQ141914	GQ141940	GQ141929
Pa. ulmi (Koch)	Xuzhou, Jiangsu	5	Apple (*M. pumila* Mill.)	GQ141915	GQ141941	GQ141930
Petrobia harti (Ewing)	Beijing	5	Creeping oxalis (*Oxalis corniculata* L.)	GQ141916	GQ141942	GQ141931
	Nanjing, Jiangsu	5	Creeping oxalis (*O. corniculata* L.)		GQ141944	
	Nantong, Jiangsu	5	Creeping oxalis (*O. corniculata* L.)	GQ141920	***	GQ141934
	Hangzhou, Zhejiang	5	Creeping oxalis (*O. corniculata* L.)	GQ141918	***	GQ141935
	Luoyang, Henan	5	Creeping oxalis (*O. corniculata* L.)	GQ141919	***	GQ141933
	Haikou, Hainan	5	Creeping oxalis (*O. corniculata* L.)	GQ141917	GQ141943	GQ141932

*N: sample size; ** These numbers mean that the sequences were previously submitted to the Genbank by our lab. The others are reported for the first time and submitted by Hong XY and Li GQ this time; ***Although we did not obtain *ITS1* sequences of *T. cinnabarinus* from Yinchuan, and *Petrobia harti* from Nantong, Hangzhou and Luoyang because of unsuccessful PCR experiments and very limited number of samples, we supplemented *ITS1* sequences of *T. urticae* from Xingcheng, Xuzhou and Zhengzhou, and *Petrobia harti* from Nanjing to make our data more convincing. Most important of all, these adjustments have not influenced results and conclusions.

TABLE 2. Summary of the genetic markers used.

Gene	*COI*	*ITS1*	*ITS2*
Number of taxa	23	23	23
Alignment length	453	662	751
Parsimony informative sites	98	355	316
Polymorphic sites	124	419	404
Invariant sites	329	223	311
Singleton variable sites	26	61	85
*Distances within *T. urticae*	0.005	0.018	0
*Distances within *T. cinnabarinus*	0.05	0.039	0.0214
*Distances within *Petrobia harti*	0.001	0	0.008

* Average Kimura-2-parameter distances within species based on *COI*, *ITS1* and *ITS2* respectively.

TABLE 3. Kimura-2-parameter distances between nine species based on differences of the *COI* nucleotide sequences.*

	1	2	3	4	5	6	7	8	9
[1]*P. ulmi*									
[2]*P. citri*	0.0890								
[3]*T. cinnabarinus*	0.1172	0.1295							
[4]*T. turkestani*	0.1066	0.1220	0.0856						
[5]*T. urticae*	0.1283	0.1415	0.0839	0.0690					
[6]*T. truncatus*	0.1272	0.1377	0.0839	0.0646	0.0143				
[7]*T. kanzawai*	0.1170	0.1351	0.1102	0.0940	0.0925	0.0915			
[8]*A. viennensis*	0.1220	0.1272	0.1368	0.1015	0.1262	0.1194	0.1066		
[9]*Petrobia harti*	0.1228	0.1383	0.1569	0.1523	0.1717	0.1685	0.1627	0.1357	

* Low divergence (0.0143) was observed between *T. urticae* and *T.truncatus*. All other comparisons ranged from 0.0646 to 0.1717.

After statistical calculation in MEGA 4, Kimura-2-parameter distances between nine species based on *COI* nucleotide variation are shown in Table 3. The inter-genus nucleotide divergence between *Tetranychus* and *Petrobia* was 0.1627 (Table 4), which was much higher than the intra-genus distance for *Tetranychus* and *Petrobia*. However, because the genera *Amphitetranychus* and *Petrobia* contain only one species, we could not compare the interspecific nucleotide divergences. The intraspecific nucleotide divergence ranged from 0.001 (*Petrobia harti* (Ewing) between five populations) to 0.05 (*T. cinnabarinus* between seven populations), and the mean distance between five populations within *T. urticae* from the *COI* sequences was 0.005. The extent of saturation was assessed by plotting the number of transitions and transversions versus TN distance of *CO1* gene in pairwise comparisons between all samples in spider mites (Fig. 1). From the figure, we can see that both transversions and transitions had not reached saturation not only for all codon positions, but also only for the third codon position, demonstrating that *COI* sequences can be used for barcoding and phylogenetic analysis of tetranychid species.

A phylogenetic tree of the 23 sequences plus 13 *COI* sequences of spider mites from other countries showed that as the out-groups, *Brevipalpus obovatus* Donnadieu and *Cenopalpus pulcher* (Canestrini & Fanzago) separated first from tetranychid taxa at the base of the tree (Fig. 4a, 4b). The NJ tree showed a similar topology as the ML tree, bootstrap support values were slightly lower in the ML tree. The phylogenetic relationship among the taxa was not well resolved. This is probably due to the strongly biased nucleotide composition (Ros & Breeuwer 2007). Several clades emerge, although the exact branching order remains unresolved. The species *T. urticae* and presumably *T. cinnabarinus* have been widely sampled together with the data of Xie (2006a), we found that intraspecific variation of *T. cinnabarinus* is substantial.

The positions of the Henan and Yunnan populations from the *T. cinnabarinus* remain ambiguous. These may be the misidentification of the specimens. However, the same species whose sequences were obtained from both GenBank and this study were almost at the same positions on the two trees, thus demonstrating the reliability of this study. And the phylogenetic tree agreed well with a tree of other spider mites from families, genera and species previously defined by morphological criteria (Bolland *et al*. 1998).

TABLE 4. Kimura-2-parameter distances within genera and between genera based on differences of the *COI* nucleotide sequences. NA, not applicable because the genus had only one species.

	Panonychus	*Tetranychus*	*Amphitetranychus*	*Petrobia*	Intra-genus
[1]*Panonychus*					0.0890
[2]*Tetranychus*	0.1274				0.0678
[3]*Amphitetranychus*	0.1246	0.1277			NA
[4]*Petrobia*	0.1306	0.1627	0.1357		NA

TABLE 5. Summary of *ITS1* and *ITS2* length variation.

Species and specimen	Lengths of PCR product	
	ITS1	*ITS2*
Tetranychus urticae	582	645
T. turkestani	582	645
T. truncatus	582	645
T. cinnabarinus	*581/586	*645/649
T. kanzawai	568	650
Amphitetranychus viennensis	535	623
Panonychus ulmi	624	692
P. citri	604	708
Petrobia harti	545	538

* *ITS1* length of *T. cinnabarinus* from Yunnan and Henan was 581, while that of *T. cinnabarinus* from other places was 586; *ITS2* length of *T. cinnabarinus* from Yunnan and Henan was 645, while that of *T. cinnabarinus* from other places was 649.

Nuclear rDNA ITS1 and ITS2

The *ITS1* of 23 populations and *ITS2* of 15 populations representing nine species were sequenced. The length of the *ITS* region (*ITS1* and *ITS2*) varied widely across different tetranychid species (Table 5). Due to the length variation in the *ITS* region and the uncertain homology of its position with the positions of *ITS* sequences from other taxa, there were no suitable non-Tetranychidae sequences to use as outgroups. The number of parsimony informative sites was much higher than in the mitochondrial markers examined (Table 2). The Kimura-2-parameter distances were obtained from the *ITS1* and *ITS2* sequences between nine species (Table 6). The genetic distances within genus and between genera are given in Table 7. In order to assess intraspecific nucleotide polymorphism, the *ITS* region was amplified from seven *T. cinnabarinus*, eight *T. urticae* and five *P. harti* specimens collected from different geographical populations (Table 1). The lengths of the *ITS* PCR products of *T. truncatus*, *T. turkestani* and *T. urticae* were the same (582 bp for *ITS1* and 645 bp for *ITS2)* (Table 5). The level of intraindividual variation revealed by ambiguous positions was low: the mean genetic distances (calculated by the function 'mean distances within group' of MEGA) within *T. urticae* and *P. harti* for *ITS1* were 0.018 and 0, respectively, and for *ITS2* they were 0 and 0.0008, respectively. However, the distances between geographical populations in *T. cinnabarinus* were higher than 0.02 (see Table 2), which is very similar to the result based on the *COI* sequence data. We also assessed saturation of *ITS* gene by

plotting the number of transitions and transversions against TN93 distance (Fig. 2), and obtained the similar result compared with *COI* sequence.

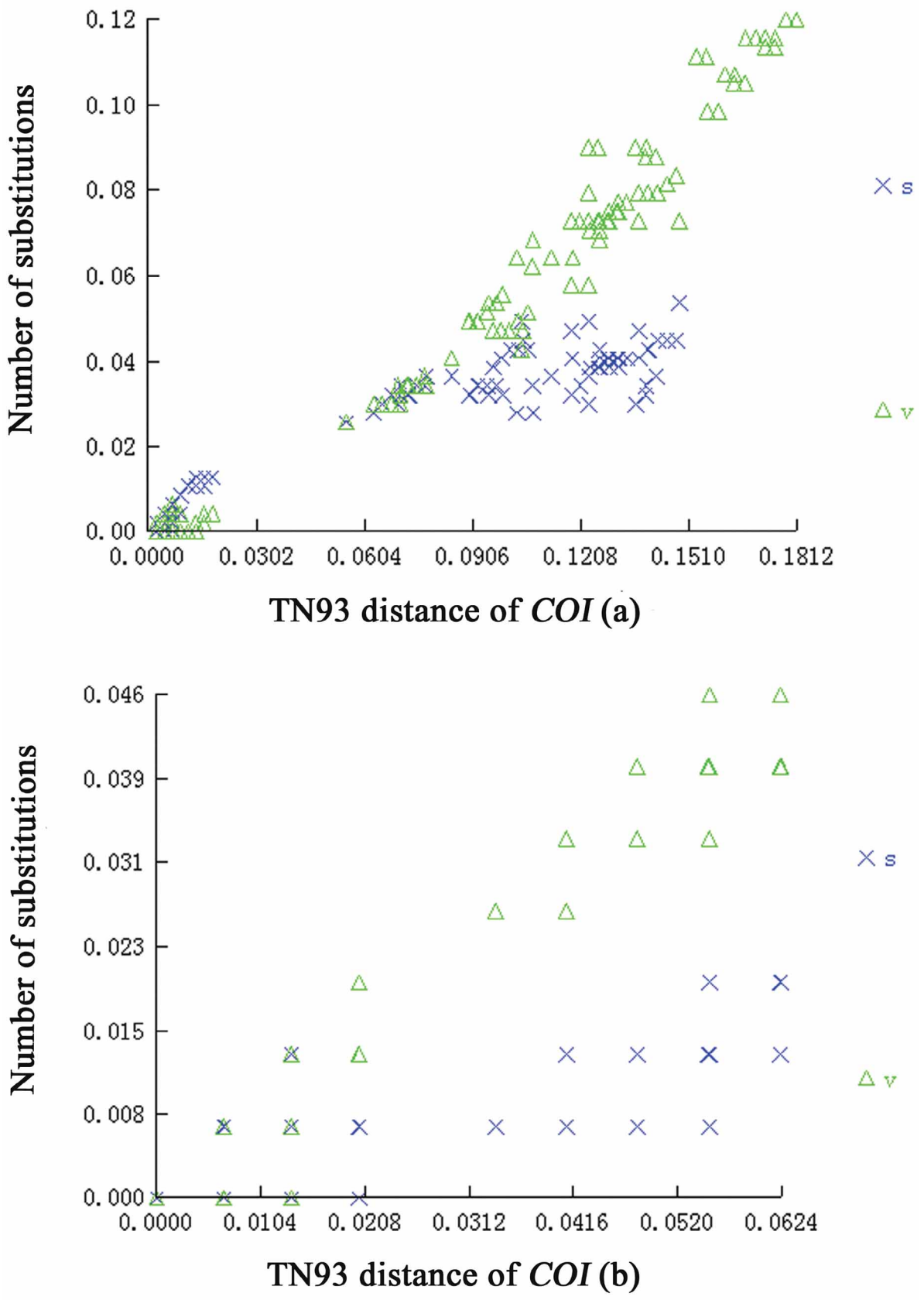

FIGURE 1. Scatter plot for the number of transitions (s) and transversions (v) versus TN distance of *COI* gene in pairwise comparisons between spider mites, (a) all codon positions; (b) third codon position.

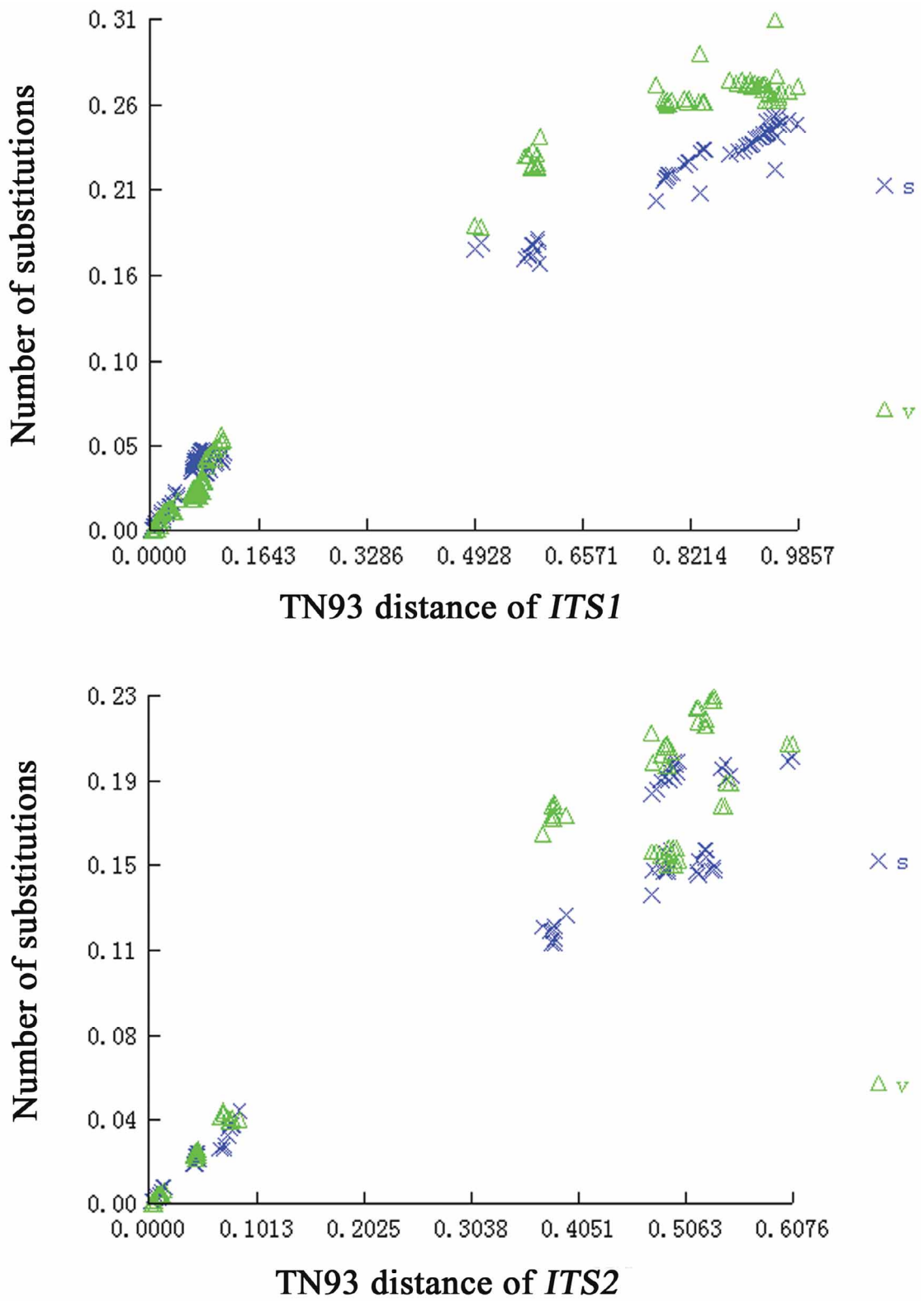

FIGURE 2. Scatter plot for the number of transitions (s) and transversions (v) versus TN distance of *ITS1* and *ITS2* in pairwise comparisons between spider mites.

Overall, the ML trees based on *ITS1*, *ITS2* and *ITS1+ITS2* agreed well with the taxonomic positions based on morphological criteria. Four clades delegating four genera emerge clearly, although the exact branching order remains unresolved. The topology of the *ITS1* tree (Fig. 4) is similar to that of the *ITS1+ITS2* tree (not shown), although bootstrap support values were slightly lower in the *ITS1+ITS2* tree. However, the positions

of *Amphitetranychus viennensis* (Zacher) and *T. urticae* from Yunnan in the *ITS1* tree and *ITS2* tree (Fig. 5) were different. In the clade of the genus *Tetranychus*, the positions of the Henan and Yunnan populations from the *T. cinnabarinus* remain ambiguous. However, the bootstrap analysis based on *ITS2* strongly supported the monophyly of the species in *Tetranychus* (Fig.5), agreeing well with the study of Ben-David *et al.* (2007).

TABLE 6. Kimura-2-parameter distances between nine species based on *ITS1* (above the diagonal) and *ITS2* (below the diagonal) sequences.

	1	2	3	4	5	6	7	8	9
[1]*T. cinnabarinus*	–	0.0465	0.0434	0.0943	0.0545	0.7907	0.7502	0.5358	0.7940
[2]*T. truncatus*	0.0318	–	0.0243	0.0888	0.0293	0.7850	0.7600	0.5272	0.7927
[3]*T. urticae*	0.0317	0.0158	–	0.0848	0.0230	0.7796	0.7457	0.5322	0.7861
[4]*T. kanzawai*	0.0535	0.0546	0.0462	–	0.0989	0.7611	0.7412	0.5435	0.8243
[5]*T. turkestani*	0.0329	0.0158	0.0098	0.0504	–	0.7700	0.7455	0.5272	0.7817
[6]*P. ulmi*	0.4513	0.4503	0.4429	0.4567	0.4389	–	0.0815	0.6807	0.4608
[7]*P. citri*	0.4350	0.4366	0.4256	0.4319	0.4285	0.0674	–	0.6873	0.4817
[8]*A. viennensis*	0.3561	0.3587	0.3584	0.3554	0.3489	0.4738	0.4500	–	0.7957
[9]*Petrobia harti*	0.4718	0.4672	0.4551	0.4523	0.4666	0.5392	0.5530	0.6046	–

TABLE 7. Kimura-2-parameter distances within genera (first number) and between genera (above the diagonal) based on differences of the *ITS1*, and the distances within genera (second number) and between genera (below the diagonal) based on *ITS2* nucleotide sequences respectively. NA, not applicable because the genus had only one species.

	Panonychus	*Tetranychus*	*Amphitetranychus*	*Petrobia*	Intra-genus
[1]*Panonychus*	–	0.7035	0.6433	0.4306	*0.0815/0.0676
[2]*Tetranychus*	0.4362	–	0.5128	0.7706	*0.0414/0.0263
[3]*Amphitetranychus*	0.5155	0.4196	–	0.7957	NA/NA
[4]*Petrobia*	0.5816	0.4461	0.3762	–	NA/NA

*First number is Kimura-2-parameter distances within genus based on *ITS1*, second number is distance based on *ITS2*.

Discussion

Although recent studies have identified spider mites based on *COI* and *ITS* sequences (Ben-David *et al.* 2007; Hinomoto *et al.* 2007; Ros & Breeuwer 2007; Osakabe *et al.* 2008), the present study is the first to use three DNA sequences simultaneously for identifying tetranychid species. The sequence divergences of three markers were generally much greater between species than within them, and the genetic distances between genera were much higher. Although errors in the course of DNA replication in PCR and sequencing might happen, we believe they did not affect our estimates of nucleotide variation, because the results we obtained based on the three DNA sequences were similar and resembled the previous study (Ben-David *et al.* 2007; Ros & Breeuwer 2007; Osakabe *et al.* 2008). Intraspecific sequence variation in *T. urticae* and *P. harti* collected from geographically distinct populations were lower than the 2% threshold proposed by Hebert *et al.* (2003b), but this was not the case for the complex species *T. cinnabarinus*. Although intraspecific variation was not detected in the other species, its occurrence in these species cannot be ruled out. For this reason and limited sample size in the study, intraspecific sequence variation should be investigated further, particularly for those species that are also found outside China.

The substitution saturation was analyzed by plotting the number of transitions and transversions versus TN distance of mitochondrial and nuclear DNA sequences in pairwise comparisons between spider mites. We can conclude that all the molecular markers used in this study are suitable for phylogenetic analysis because of unsaturated substitution of them. And the best choice of DNA region for tetranychid mite phylogeny analysis should depend on the analytic results.

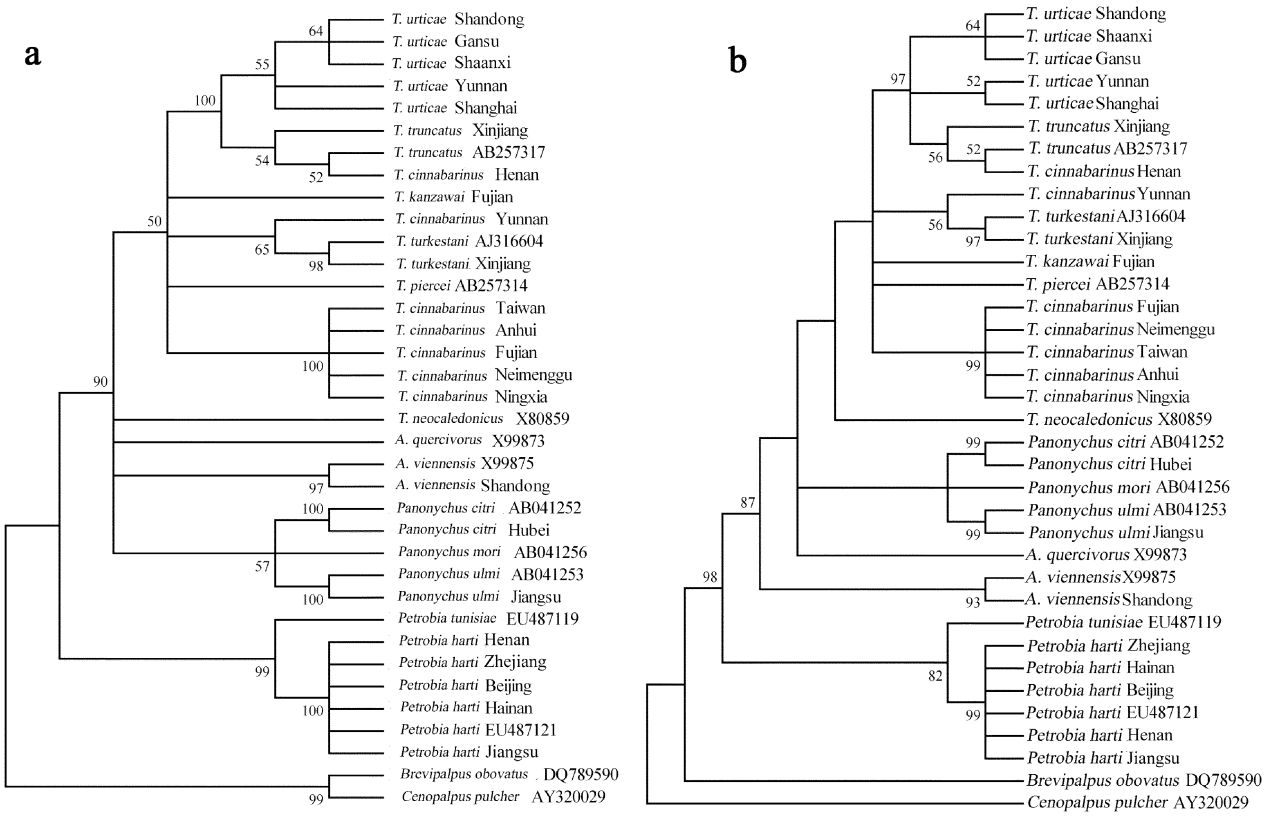

FIGURE 3. Neighbor-joining (NJ) tree (a) and maximum likelihood (ML) tree (b) based on *COI* sequences. Twenty-three of the *COI* sequences were obtained from the nine Chinese tetranychid species analyzed in this study. In addition, thirteen acarine *COI* sequences were obtained from the GenBank: the *COI* sequence (GenBank nos. DQ789590 and AY320029) from *Brevipalpus obovatus* and *Cenopalpus pulcher* were used as outgroups; the other *COI* sequences *Tetranychus truncatus, T. turkestani, T. piercei, T. neocaledonicus, Panonychus citri, Pa. ulmi, Pa. mori, Amphitetranychus viennensis, A. quercivorus, Petrobia harti* and *P. tunisiae* (GenBank nos. AB257317, AJ316604, AB257314, X80859, AB041252, AB041253, AB041256, X99875, X99873, EU487121 and EU487119 respectively) from GenBank also included into our phylogenetic analysis. Numbers adjacent to branches show the bootstrap values (> 50%) of 1000 replicates.

The analysis of mitochondrial and nuclear DNA sequences presented in this paper supported the previously proposed *Tetranychus*, *Amphitetranychus*, *Panonychus* and *Petrobia* genera within the Tetranychidae. A similar phylogeny was obtained using both mitochondrial and nuclear DNA sequences within the *Tetranychus* group. The phylogeny of *T. cinnabarinus*, and *T. urticae* has not been fully resolved, and the positions of *T. truncatus*, and *T. turkestani* within *Tetranychus* remain ambiguous based on the markers used here and in previous studies (Ros & Breeuwer 2007; Ben-David *et al.* 2007). Inclusion of more taxa of *Tetranychus* as well as the use of multiple markers should help to resolve the phylogeny of *Tetranychus* in the future.

The divergence between *T. urticae* and *T. truncatus* was only 0.0143 and 0.0158 in the *COI* and *ITS2* sequences, respectively, but 0.0243 in the *ITS1* sequences. In addition, low divergences based on *ITS2* were observed between *T. urticae* and *T. truncatus* (0.0158), *T. truncatus* and *T. turkestani* (0.0158) and *T. urticae* and *T. turkestani* (0.0098), in agreement with previous studies (Navajas & Boursot 2003; Ros & Breeuwer 2007; Ben-David *et al*. 2007). However, such cases of divergence between species under 2% should be rare (Barrett & Hebert 2005). The genetic distances for *ITS1* between all pairs of species studied ranged from 2.3% to 82.43%, which are well over the threshold of 2% divergence for interspecies comparisons. We recommend that nucleotide divergences between 1% and 2% should be treated with caution and that, in agreement with Ben-David *et al.* (2007), species identity should be confirmed by morphological criteria.

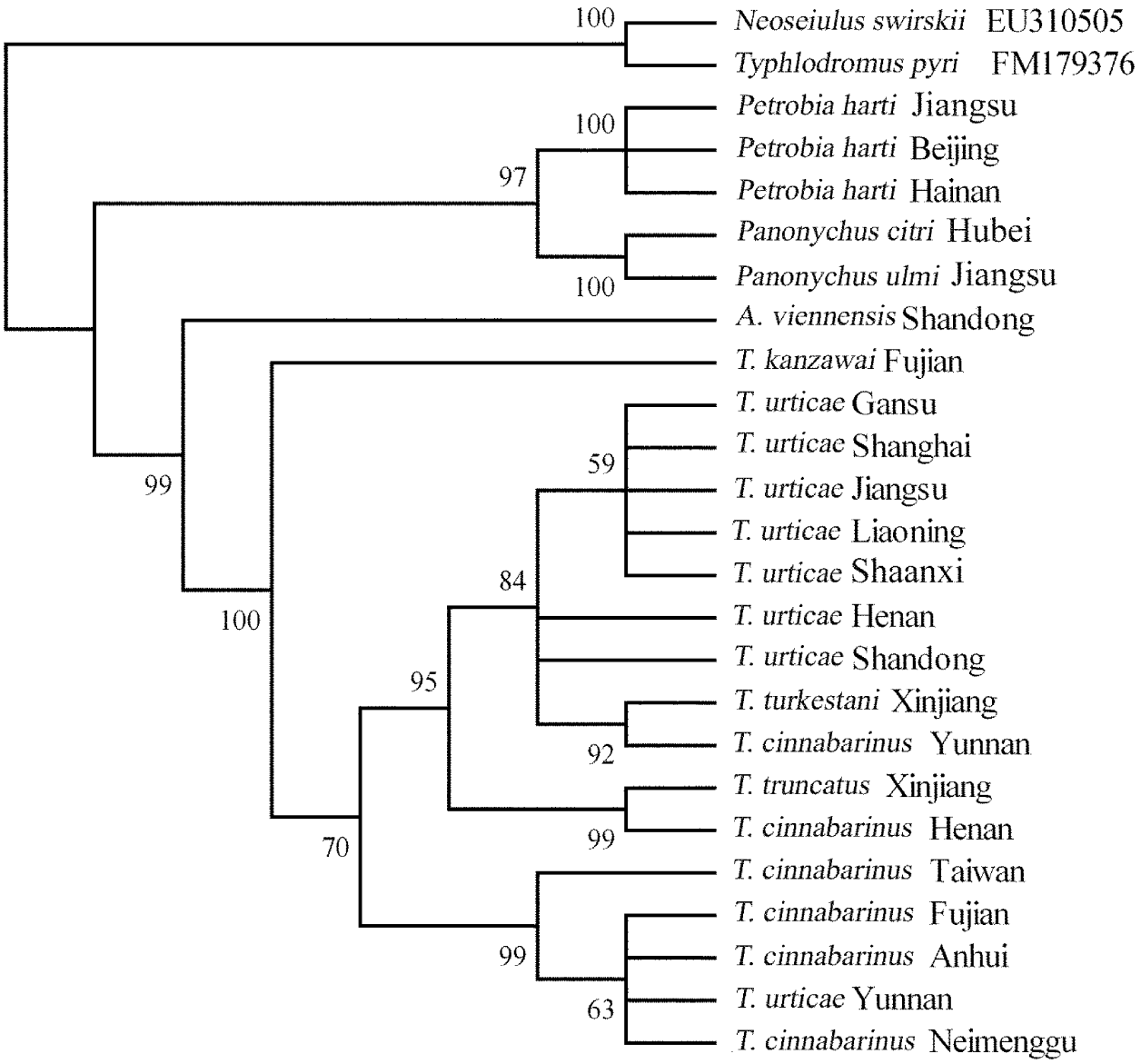

FIGURE 4. ML tree based on *ITS1* sequences. Sequence data for the *ITS1* was aligned from a total of 23 individuals from nine species. The outgroups *Neoseiulus swirskii* and *Typhlodromus pyri* (GenBank nos. EU310505 and FM179376, respectively) were used to root the *ITS1* tree. Numbers on the branches indicate the percentage bootstrap values (>50) based on NJ bootstrapping with ML settings (1,000 replicates).

Using either *COI* or *ITS* data, the four genera were separated with strong bootstrap support. However, *A. viennensis* and *P. harti* are sister groups to the other taxa in the *ITS2* tree constructed with the ML method (bootstrap support 62%), but in both the *ITS1* and *ITS1+ITS2* trees, *A. viennensis* was a sister taxon to the *Tetranychus* genera with bootstrap support values of 99%. Although the phylogeny of *T. cinnabarinus*, *T. truncatus* and *T. urticae* has not been fully resolved, analysis based on *COI* separated *T. turkestani* from the *T. urticae* group at least. However, it is very likely that by including more taxa of *Tetranychus* in the tree, the phylogeny of *Tetranychus* genera could be well resolved based on *ITS* region (*ITS1* and *ITS2*). The *ITS1* tree supported the positions of samples defined by morphological criteria with exceptions of *T. urticae* from Yunnan and *T. cinnabarinus* from Yunnan and Henan, generally in agreement with Osakabe *et al.* (2008). The *ITS2* tree solved the phylogeny of *Tetranychus* species well with an exception of *T. cinnabarinus*. *ITS2* was also found to be useful in several other insects (Di Muccio *et al.* 2000; Hackett *et al.* 2000). Therefore, we can tentatively conclude that nuclear ribosomal *ITS* region is more suitable than *COI* for identification and

phylogenetic analysis of tetranychid species. We can also speculate that the evolutionary rate of *COI* in spider mites is lower than that of *ITS1*, but higher than that of *ITS2*.

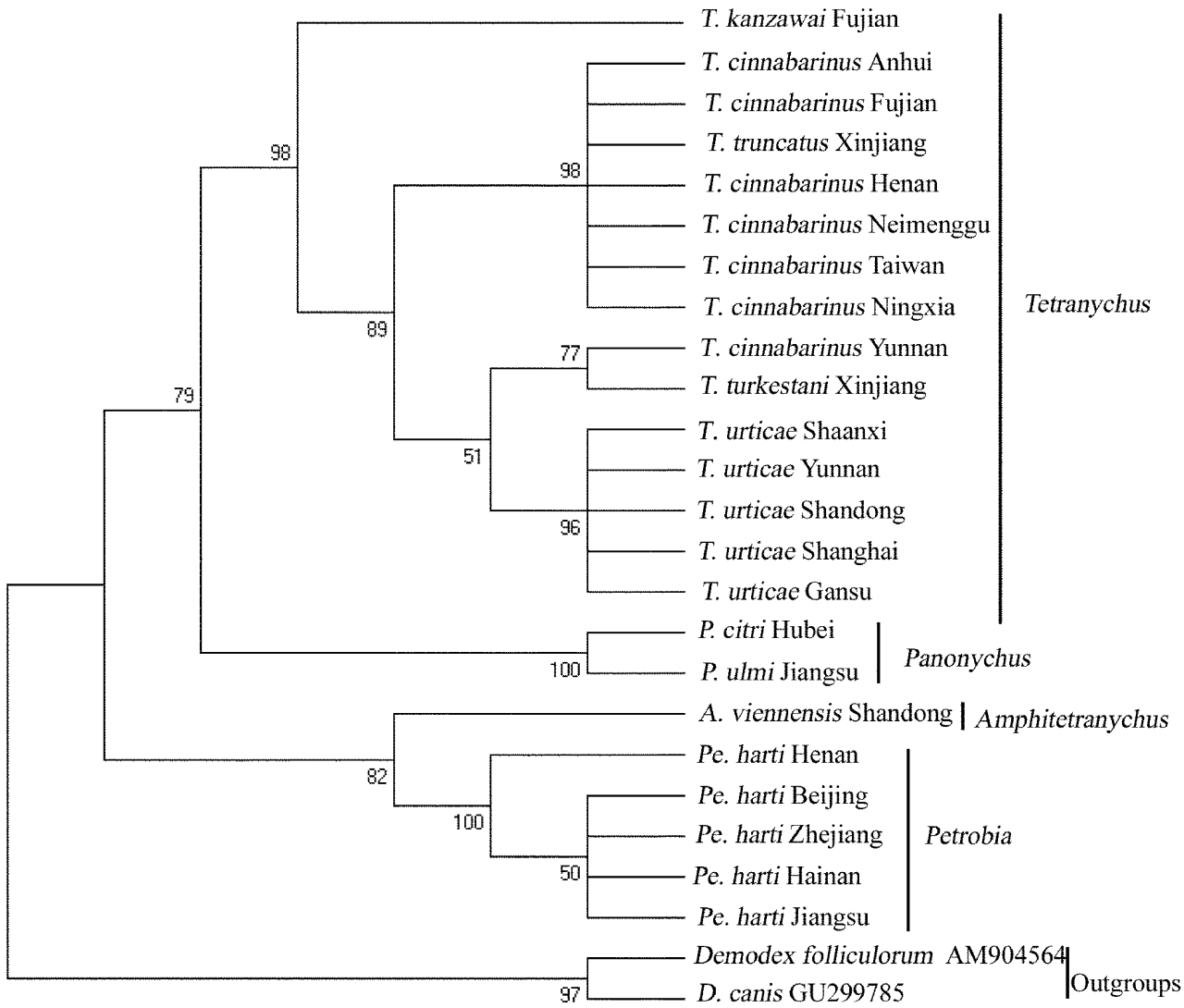

FIGURE 5. ML tree based on *ITS2* sequences. Sequence data for the *ITS2* was aligned from a total of 23 individuals from nine species. *Demodex folliculorum* and *D. canis* (GenBank nos. AM904564 and GU299785, respectively) were selected as the outgroups of *ITS2* tree. Numbers on the branches indicate the percentage bootstrap values (>50) based on NJ bootstrapping with ML settings (1,000 replicates).

The question whether *T. cinnabarinus* mites should be considered as a separate species has not been clearly answered. When considering the present data together with that of Xie *et al.* (2006a), the distances based on the three markers used suggest that *T. cinnabarinus* is the most divergent. The diversity within *T. cinnabarinus* was especially high with a maximum of 10.18%. Based on all the molecular markers examined in this study, the Henan and Yunnan populations of *T. cinnabarinus* were far from the *T. cinnabarinus* group. Although *T. cinnabarinus* and *T. urticae* were separated by ML analysis based on *ITS2* with strong bootstrap support values of 99% and 92% respectively, the phylogenetic analysis based on other markers in this study could not solve their positions well.

A restriction fragment length polymorphism (RFLP) approach, based on the variation between species, may be a quicker and cheaper method for distinguishing mites. This method has been used for *Tetranychus spp*. in Japan (Osakabe *et al.* 2002, 2008) and for *T. urticae* and *T. turkestani* in France (Navajas & Boursot 2003).

To resolve with greater confidence the phylogeny of the tetranychid species, especially for the genus *Tetranychus*, there is an urgent need for better nuclear markers. Mitochondrial DNA is useful, but could be misleading due to possible uniparental inheritance and introgression events. The marker of choice should be neutral, have a higher mutation rate than the *ITS2* region, and unlike *ITS1*, it should not have a high length polymorphism. The challenge is to find suitable nuclear markers and sufficient geographic sampling designs allowing for the assessment of intra- and interspecific variation. Navajas & Fenton (2000) and Cruickshank (2002) investigated the suitability of various molecular markers, but there is still a need for nuclear markers suitable for distinguishing closely related species. Recently, Sonnenberg *et al.* (2007) suggested the *D1-D2* region of the nuclear *28S* rDNA gene as a taxonomic marker. It could complement DNA barcoding studies based on mitochondrial DNA and *ITS* region sequences. In addition, molecular testing for reproductive parasites and crossing experiments should be standard procedures for delineating biological species (Ros & Breeuwer, 2007). With the arrival of genomic sequence data, we are currently developing new nuclear DNA markers for identification of spider mites.

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