Evolutionary divergence of mitochondrial genomes in two *Tetranychus* species distributed across different climates

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Abstract

There is increasing evidence that mitochondrial genomes (mitogenomes) can be under selection, whereas the selective regimes shaping mitogenome evolution remain largely unclear. To test for mitogenome evolution in relation to the climate adaptation, we explored mtDNA variation in two spider mite (*Tetranychus*) species that distribute across different climates. We sequenced 26 complete mitogenomes of *Tetranychus truncatus*, which occurs in both warm and cold regions, and nine complete mitogenomes of *Tetranychus pueraricola*, which is restricted to warm regions. Patterns of evolution in the two species' mitogenomes were compared through a series of d_N/d_S methods and physicochemical profiles of amino acid replacements. We found that: (1) the mitogenomes of both species were under widespread purifying selection; (2) elevated directional adaptive selection was observed in the *T. truncatus* mitogenome, perhaps linked to the cold climates adaptation of *T. truncatus*; and (3) the strength of selection varied across genes, and diversifying positive selection detected on *ND4* and *ATP6* pointed to their crucial roles during adaptation to different climatic conditions. This study gained insight into the mitogenome evolution in relation to the climate adaptation.

Keywords: mitochondrial DNA, d_N/d_S , positive selection, purifying selection, climate.

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Introduction

Mitochondria are the site for the citric acid cycle and for oxidative phosphorylation (OXPHOS), the final steps of adenosine triphosphate (ATP) synthesis via cellular respiration (Chong & Mueller, [2013](#)). In addition to their crucial role in ATP production, mitochondria are also associated with many other processes essential for cell survival and function, including cellular Ca^{2+} signalling, apoptosis, cell transport, thermoregulation and immunity (Brand, [2000](#); Detmer & Chan, [2007](#); Chong & Mueller, [2013](#); Ballard & Pichaud, [2014](#)). The typical animal mitochondrial genome (mitogenome) is circular and 15–20 kb in length, with 37 genes (Boore, [1999](#)). Of these, 13 genes encode subunits of protein complexes directly involved in the OXPHOS pathway responsible for most of the energy produced in the cell (Ballard & Pichaud, [2014](#)).

In the past 30 years, mtDNA has been extensively used in evolutionary and ecological studies because of several advantages, eg a large number of copies of mtDNA, high mutation rate and maternal inheritance (Awise *et al.*, [1987](#)). Given the important roles of all 13 mtDNA-encoded peptides in cellular ATP production and other functions, mtDNA variation may result in significant metabolic and fitness consequences (Ballard & Pichaud, [2014](#)). Genetic diversity of mtDNA may therefore be shaped not only by random genetic drift, but also by natural selection. Consistent with this, some empirical studies have found evidence for positive selection on the mitogenome (Fontanillas *et al.*, [2005](#); Dalziel *et al.*, [2006](#); da Fonseca *et al.*, [2008](#); Foote *et al.*, [2011](#); Garvin *et al.*, [2011](#); Gagnaire *et al.*, [2012](#); Bélanger-Deschênes *et al.*, [2013](#); Jacobsen *et al.*, [2014](#); Jacobsen *et al.*, [2015](#)). This process of positive selection may be related to thermal adaption and/or aerobic requirements linked to migration, swimming speed, size and diet [see review by Garvin *et al.* ([2015](#))]. The way selection shapes mitogenome evolution has now become a focus of mitochondrial research. Several recent studies have begun to explore how trait and environmental variation link to variation in the mitogenome (Mishmar *et al.*, [2003](#); da Fonseca *et al.*, [2008](#); Balloux *et al.*, [2009](#); Sun *et al.*, [2011](#); Chong & Mueller, [2013](#); Melo-Ferreira *et al.*, [2014](#); Silva *et al.*, [2014](#); Morales *et al.*, [2015](#); Harrison *et al.*, [2016](#)). However, selection processes shaping mitogenome evolution generally remain unclear.

An obvious potential selective regime shaping mitogenome evolution is climate. Climate affects bioenergetic demand and leads to metabolic adaptation, particularly in ectotherms (Franks & Hoffmann, [2012](#)). Mitochondria play an important role in thermoregulation (Brand, [2000](#)), and when populations/species from different climates are compared they often differ for physiological processes connected to mitochondria, such as oxygen consumption, mitochondrial densities and mitochondrial respiration rates (Sommer & Pörtner, [1999](#),

2012; Peck, 2002; Martinez-Cruz *et al.*, 2012). Temperature adaptation may involve variation in mtDNA, as recently confirmed or inferred in fish (Silva *et al.*, 2014), planthoppers (Sun *et al.*, 2015), *Drosophila* (Ballard *et al.*, 2007) and humans (Elson *et al.*, 2004; Ruiz-Pesini *et al.*, 2004). Most of these inferences were based on analyses of the geographic distributions of mtDNA variation of single genes or a limited number of mitochondrial protein-coding genes (PCGs) within a population genetic framework, and studies at the whole mitogenome level have been rare (eg Melo-Ferreira *et al.*, 2014; Morales *et al.*, 2015; Harrisson *et al.*, 2016; Jacobsen *et al.*, 2016).

Based on our long-term (2008–2014) survey ranging from north-eastern China to south-western China (data not published), we found seven species of *Tetranychus*, namely *Tetranychus urticae*, *Tetranychus pueraricola*, *Tetranychus kanzawai*, *Tetranychus truncatus*, *Tetranychus phaselus*, *Tetranychus ludeni* and *Tetranychus malaysiensis*. Among them, *T. pueraricola* and *T. truncatus* are predominant and distributed in different climate zones, providing an opportunity to investigate the potential effect of climate on the evolution of their mitogenome. Both these *Tetranychus* species are agricultural pests (Ehara & Gotoh, 1996; Seeman & Beard, 2011; Xue *et al.*, 2016). Based on surveys, *T. pueraricola* is mainly distributed in the southern subtropical zone (in southern China), and *T. truncatus* is mainly distributed in the moderate temperate zone and warm temperate zone (in northern China). The genetic distance between the complete mitogenome sequence of these species is 12.7% (Chen *et al.*, 2014, 2016). Different climatic distributions may be associated with different metabolic rates and thermoregulation ability of the two species' mitochondria, leading to varied selection pressure acting on the two species' mitogenomes.

In order to explore the potential effect of climate on the evolution of mitogenomes, we sequenced 26 *T. truncatus* mitogenomes and nine *T. pueraricola* mitogenomes that belonged to different mitochondrial haplotypes based on a partial 768 bp sequence of the mitochondrial cytochrome *c* oxidase subunit 1 (*COXI*) gene. We compared evolution patterns of the two species' mitogenomes through a series of d_N/d_S methods and physicochemical profiles of amino acid replacements [where d_N is the non-synonymous (Nsyn) mutation substitution rate and d_S is the synonymous (Syn) mutation substitution rate]. Our results provide new insights on the evolution of mitogenomes in these mites.

Results

Mitogenome haplotype characterization and sequencing

In total, 684 *T. truncatus* individuals from 13 localities and 314 *T. pueraricola* individuals from nine localities were sampled (Fig. 1A). In order to capture as many genetic variations as possible with low expense, we first identified unique mitogenomes before sequencing. By analysing a 768 bp *COX1* gene sequence, we characterized 29 mtDNA haplotypes for *T. truncatus* and 12 for *T. pueraricola*. The relationship among different haplotypes in each species is shown in Fig. 1B, C. Then one individual representing a unique mitogenome was selected for subsequent mitogenome sequencing. Nine complete mitogenomes for *T. pueraricola* and 26 complete mitogenomes for *T. truncatus* were successfully amplified and sequenced. All of the mitogenome sequences have been deposited in GenBank (accessions MG518326–MG518360).

Mitogenome genetic diversity

The proportions of Syn and Nsyn substitutions for each of the 13 mitochondrial PCGs were in the ranges 1–3.6% and 0.3–3.1% respectively in *T. pueraricola* mitogenomes; and the equivalent ranges for *T. truncatus* mitogenomes were 0.0–2.7% and 0.0–1.3% respectively (Fig. 2). Wilcoxon signed-rank tests indicated that both the proportions of Syn and Nsyn substitutions were significantly higher in the *T. pueraricola* mitogenomes than in the *T. truncatus* mitogenomes globally (for Syn, $P = 0.001$; for Nsyn, $P = 0.006$). However, the Nsyn substitutions in *ND3*, *COX1* and *COX2* were lower or similar in *T. pueraricola* mitogenomes when compared with those of *T. truncatus* (Fig. 2). In addition, *T. truncatus* and *T. pueraricola* consistently had fewer Nsyn substitutions for the *COX1* and *COX2* genes than for the other genes.

Selection pressure comparison in gene level

d_N/d_S for pairwise sequences was used to infer the selective constraints on each gene of each species. Because there were no Syn mutations between some pairwise sequences, we calculated the ratio of $d_N/(d_S + \text{constant})$ as an alternative to d_N/d_S to avoid dividing by zero, following the method of Mishmar *et al.* (2003). The constant was of the d_S value for one Syn mutation. The ratios of $d_N/(d_S + \text{constant})$ were consistently <1 , suggesting purifying selection predominated the evolution of the mitogenomes (Fig. 3). Wilcoxon rank-sum test revealed that six out of the 13 PCGs possessed significantly lower $d_N/(d_S + \text{constant})$ ratios in *T. truncatus* than in *T. pueraricola*. In contrast, relatively higher ratios in *T. truncatus* were only found on *ND4*, *ND5*, *Cytb* and *ATP6*, but only with significance detected in *ND4* and *ND5*. Additionally, relatively lower d_N/d_S ratios were also observed in three COX genes in both species, particularly in *COX1* and *COX2*. the McDonald–Kreitman (MK) test (McDonald & Kreitman, 1991) detected significant positive

selection for the *ND4* (NI = 0.379, $P = 0.013$) and *ATP6* (NI = 0.380, $P = 0.039$, Table 1) genes, involving an excess of divergent Nsyn mutations between the two species.

Positive selection sites

A Bayesian phylogeny tree with high posterior probability on each node was constructed based on the concatenated sequences of the 13 PCGs and used for the subsequent selection analyses (Fig. 4). No substitution saturation was detected for any codon positions in either the symmetrical or asymmetrical topology tests ($I_{ss} < I_{ss,c}$, $P < 0.001$). Saturation plots showing the relationship between general time reversible (GTR) distance and the simple p -distance are in Supporting Information Fig. S1. The phylogenetic relationship of the 10 species was congruent with accepted relationships based on nuclear genes (18S and 28S) or mitochondrial genes in previous studies (Matsuda *et al.*, 2014; Xue *et al.*, 2017). To compare selection pressures acting on the mitogenomes of two species globally, we first applied the branch-model method implemented by CodeML in PAML4 (Yang, 2007) to estimate the d_N/d_S values of branches of the two species. In the branch-model test, a comparison was made between the three- ω branch model (ω_0 : background; ω_1 : *T. pueraricola* branches; ω_2 : *T. truncatus* branches) and the M0 (one-ratio) model; M0 – -57373.57 was rejected in favour of the three- ω branch model (log-likelihood $\ln L = -57352.56$, $P < 0.001$), suggesting that d_N/d_S varies among the three defined branch groups. The d_N/d_S ratios for the branches of *T. pueraricola*, *T. truncatus* and the background were 0.031, 0.036 and 0.0190 respectively.

Both the branch-site model method implemented by the CodeML program and the mixed effects model of evolution (MEME) method (Murrell *et al.*, 2012) detected positive selection sites. Only amino acids that diverged from the ancestral state for the two species and fixed within *T. pueraricola* or *T. truncatus* were considered as positive selection sites. The ancestral sequence was inferred by TreeSAAP 3.2 under the REV model with other parameters set as default (Woolley *et al.*, 2003). We used this stringent criterion because inferred positive selection sites in the terminal branch of each species that remain polymorphic could represent cases in which the purifying selection site has not yet purged deleterious forms (Rand *et al.*, 2004). The branch-site model method implemented by CodeML identified two codons in the *T. truncatus* mitogenome under positive selection (64I on *Cytb* and 111K on *ATP6*), when the branch leading to *T. truncatus* was assigned as the ‘foreground’. However, no positively selected codon was detected in the *T. pueraricola* mitogenome when the branch leading to *T. pueraricola* was assigned as the ‘foreground’. The MEME test (Murrell *et al.*, 2012) found evidence of episodic positive selection at four codons in the *T. pueraricola* mitogenome and three codons in the *T. truncatus* mitogenome (Table 2). To further

validate whether the inferred sites are associated with changes of physicochemical properties and to determine the selection direction, the changes in physicochemical properties of amino acids caused by replacements across the phylogeny were estimated using TreeSAAP 3.2 software. TreeSAAP results revealed that only the five amino acid changes to the *T. truncatus* direction were associated with significant radical changes in physicochemical properties (Table 2).

Discussion

The identification of positive Darwinian selection at the molecular level has been a central task in evolutionary biology (Vitti *et al.*, 2013). In this study, we explored the evolution of mtDNA in relation to climatic adaptation by using two *Tetranychus* species that distribute across different climates. Owing to the fact that natural selection and random genetic drift always interplay mutually in shaping genetic diversity, it has been challenging to distinguish them from each other. Less sensitivity to population demography makes d_N/d_S a powerful measure of selection pressure, and a variety of d_N/d_S -based methods under different theoretical models have been developed to explore the footprints of natural selection (Nielsen, 2005). Although these methods have been successfully used in some studies at the population level (Mishmar *et al.*, 2003; Silva *et al.*, 2014; Morales *et al.*, 2015; Jacobsen *et al.*, 2016), it remains risky to use them in some instances, because some of the variations in populations are just transient segregating polymorphisms rather than actual substitutions, particularly the Nsyn segregates, which would not fix in the populations but may lead to inflated d_N/d_S ratios or even false-positive results. In this study, to circumvent the problem caused by transient Nsyn segregates, the MK test was also used to explore the genes subjected to adaptive evolution. By comparing the d_N/d_S ratios for the sites that are polymorphic within species (P_n/P_s) to the d_N/d_S ratios for the sites that are divergent between species (D_n/D_s), the MK test [$NI = (P_n/P_s)/(D_n/D_s)$] can detect adaptive selection, with $NI < 1$ indicating positive selection. It is suitable for closely related species because of the limited number of multiple mutations at single nucleotide sites (McDonald & Kreitman, 1991; Egea *et al.*, 2008). So, the Nsyn segregates just lead to inflated P_n/P_s , making the MK test result more conservative instead of false positive. In addition, we also identified diversifying positive selection sites both by codon-based d_N/d_S methods and changes in physicochemical properties of amino acids under a phylogenetic framework. Although the results of different approaches were not always consistent, several patterns emerged for the evolution of the mitogenome of these species.

The d_N/d_S ratio for the branches of each species estimated by CODEML under the branch model were all < 1 . This indicates that patterns of mtDNA variation are not consistent with strictly neutral molecular evolution, instead suggesting that purifying selection was the predominant

force shaping mitogenome evolution. Purifying selection may act to conserve functionality of OXPHOS proteins (Meiklejohn *et al.*, [2007](#)). This process may not be acting with equal intensity on the two species, since the ω rate on the *T. truncatus* branches (0.036) was higher than on the *T. pueraricola* branches (0.031), which could reflect directional evolution at some sites in the face of mostly purifying selection, or by relaxed purifying selection (Bazin *et al.*, [2006](#); Meiklejohn *et al.*, [2007](#)). Considering that widespread lower intraspecific $d_N/(d_S + \text{constant})$ ratios across the 13 PCGs in *T. truncatus* than in *T. pueraricola* and that more positive selection sites were identified in the *T. truncatus* mitogenome, we propose that elevated directional selection in the *T. truncatus* mitogenome may be involved. Our results are consistent with analyses performed in hares (Melo-Ferreira *et al.*, [2014](#)), which pointed to the mitogenomes of species living in cold climates having higher ω rates than those in warm climates.

Although the mitogenome is inherited as a linked unit, variable levels of genetic diversity and $d_N/(d_S + \text{constant})$ ratios were observed across the mitochondrial PCGs of the two species, suggesting the strength of selection acting on each PCG was different. Notably, both species were consistently possessed of relatively lower $d_N/(d_S + \text{constant})$ ratios on the *COX1* and *COX2* genes than on other genes. Obvious lack of Nsyn substitutions pointed to stronger purifying selection (functional constraints). Consistently, a previous study on vertebrates suggested that Nsyn mutations in these two genes may have disproportionate effects on fitness, making them typically the most highly conserved in the mitogenome (Zhang & Broughton, [2013](#)).

Multiple lines of evidence, including the MK test, $d_N/(d_S + \text{constant})$ ratios, positive selection sites and changes in physicochemical properties, point to positively diversifying selection on the *ND4* and *ATP6* genes. The peptides encoded by *ND4* and *ATP6* constitute mitochondrial complexes I and V respectively. Mitochondrial complex I is the first and largest enzyme in the chain of complexes that participate in OXPHOS. It catalyses the transfer of two electrons from NADH to ubiquinone, coupled to the translocation of four protons across the inner mitochondrial membrane. A recent study on the atomic structure of mammalian mitochondrial complex I revealed that a proton translocation pathway existed in *ND4*-encoded peptides (Fiedorczuk *et al.*, [2016](#)). By the translocation pathway, protons are able to transfer across the inner mitochondrial membrane electrochemically to generate the proton gradient required by ATP synthase. *ATP6* is a subunit of the F_0 proton channel in ATP synthase, the final complex of the OXPHOS chain catalysing synthesis of ATP from adenosine diphosphate (da Fonseca *et al.*, [2008](#)). Changes within *ATP6* have been associated with differences in metabolism and selection linked to energetic requirements. In agreement with our results, previous studies in humans (Mishmar *et al.*, [2003](#); Balloux *et al.*, [2009](#))

and birds (Morales *et al.*, 2015) also found that variations on the two genes differ across climate regions, probably associated with mitochondrial climate adaptation. The physiological mechanism underlying mitochondrial climate adaptation is that mitochondrial OXPHOS not only generates ATP, but also generates heat to maintain body temperature under cold conditions (Wallace, 2005; Das, 2006). During the process of OXPHOS, mitochondrial complexes I, III and IV use the energy from electron transfer to pump protons from the mitochondrial inner membrane into the intermembrane space, creating an electrochemical gradient. The energy stored in this gradient is coupled to ATP synthesis by a complex (ATPase) as the protons flow back into the mitochondrial matrix through a proton channel in complex V. However, protons also can bypass ATPase via uncoupling mechanisms (proton leak), which generate heat instead of ATP. The balance between the two antergic functions of OXPHOS is determined by the efficiency of coupling efficiency of OXPHOS (Brand, 2000; Wallace, 2005; Das, 2006).

In consideration of the foregoing, we propose that amino acid changes within *ND4* and *ATP6* genes may affect the efficiency of proton translocation, and thus the coupling efficiency, coordinating the ratio of ATP synthesis efficiency to thermogenesis efficiency, enabling the two species to adapt to different habitats. We assume that the positively selected sites identified in the *T. truncatus* mitogenome probably lead to elevated efficiency for OXPHOS uncoupling, facilitating *T. truncatus* mitochondria to generate more heat in resistance to a cold climate. In contrast, with the lack of cold constraint for *T. pueraricola* under a subtropical climate, the positive sites in *T. pueraricola* mitogenome are likely associated with increased coupling efficiency, producing more ATP. Further study is necessary to compare the ATP synthesis efficiency and proton leakage efficiency between the two species.

In addition to the mitochondrial uncoupling mechanism of cold adaptation, quantitative regulation of mitochondrial proteins can occur in response to cold temperature through changes in DNA copy number, transcription, translation, messenger RNA stability or protein stability (Das, 2006). Recently, Camus *et al.* (2015) found that sequence variation within mtDNA affects both the copy number of mitogenomes and patterns of gene expression across key mitochondrial PCGs. Whether the mutations on the positively selected sites lead to varied copy number of mitogenomes or patterns of gene expression, enabling these two *Tetranychus* species to adapt to different climates, needs to be studied in the future.

Conclusion

Two species' mitogenomes appear to be under widespread purifying selection, rather than evolving as neutral entities. Elevated directional adaptive selection was observed in the *T. truncatus* mitogenome. The diversifying positive selection on *ND4* and *ATP6* was probably associated with the different climate adaptations.

Experimental procedures

Sampling and DNA extraction

From 2013 to 2014, 314 adult females of *T. pueraricola* and 684 of *T. truncatus* were collected from nine locations and 13 locations respectively over a large area of China (Fig. 1). Briefly, the *T. pueraricola* populations were mainly sampled from the middle subtropical zone (MS) and the plateau climate zone (PC), and the *T. truncatus* populations were mainly sampled from the moderate temperate zone (MT) and the warm temperate zone (WT). Details of sampling information are presented in Supporting Information Table S1. At each locality, individuals were haphazardly collected in a 5 m × 5 m square. All of the samples were immediately preserved in 100% ethanol and returned to the laboratory for species identification and DNA extraction. Genomic DNA was extracted from the entire body with a DNeasy Blood and Tissue kit (Qiagen, Valencia, CA, USA) following the manufacturer's protocol.

Mitochondrial haplotype characterization

Unique mitogenomes were first identified by amplifying and sequencing a partial sequence of the mitochondrial *COX1* gene (768 bp) for each individual, and then individuals classified according to haplotype. We found 12 haplotypes in the *T. pueraricola* populations and 29 haplotypes in the *T. truncatus* populations. The relatedness between haplotypes in each species was represented through a median-joining network constructed with Network 4.6 (Bandelt *et al.*, 1999). We randomly selected one individual representing each haplotype for subsequent mitogenome sequencing.

Mitogenome sequencing and annotations

The complete mitogenome sequence of each selected individual was produced from five (for *T. pueraricola*) and seven (for *T. truncatus*) overlapping regions, which were amplified by PCR using primers designed in our previously reported mitogenome sequence of the two species (Chen *et al.*, 2014). Each PCR amplification was performed in a total volume of 50 µl PCR mix

containing 1× Gflex PCR Buffer, 1.25 U TKS Gflex DNA polymerase (TaKaRa, Beijing, China), 0.3 µM of each primer and approximately 10 ng of genomic DNA. The thermal profile used an initial denaturation step of 94 °C for 2 min followed by 30 cycles of denaturing at 98 °C for 10 s, annealing at 60 °C for 15 s, and extension at 68 °C for 1–1.5 min (depending on the PCR product length; Supporting Information Tables S2 and S3).

Amplicons were purified separately with a SanPrep Column PCR Product Purification Kit (Sangon Biotech, Shanghai, China), quantified by NANO and then pooled in approximately equimolar quantities. Pooled amplicons for each individual were sent to BGI Company (Beijing, China) for sequencing with the Illumina Hiseq 4000 platform. We sequenced 1 Gbp of data for each mitogenome. Clean sequence reads obtained from the mitogenome amplicons of each individual were assembled and annotated in Geneious 6.1.6 (Biomatters Ltd, Auckland, New Zealand) by using published mitogenome sequences of the two species (GenBank: KM111296 and KJ729021) as references. The Highest Quality option under the Consensus threshold option was selected for mitogenome assembly, which led to mapping of the highest quality base at each column onto the reference sequence. The minimum coverage for each based was 500×. The sequence of the 13 PCGs was extracted from the assembled integer mitogenome of each individual. The sequences of the 13 PCGs were aligned individually by codons using the MUSCLE method in Mega 6.06 software (Tamura *et al.*, 2013), and concatenated for subsequent analyses.

Mitogenome genetic diversity comparison

We calculated the proportions of Syn and Nsyn substitutions for each PCG within each of the two *Tetranychus* species using DNASP v5.10 (Librado & Rozas, 2009). Subsequently, we used Wilcoxon signed-rank tests to determine whether the proportions of Syn and Nsyn substitutions differed significantly between the two species.

Phylogenetic tree construction

In order to infer the selection in the phylogeny framework, a Bayesian phylogeny tree was constructed using MrBayes 3.2.2 (Ronquist *et al.*, 2012) for the sequenced mitogenomes here coupled with the mitogenomes of another nine species of Tetranychidae (Supporting Information Table S4). We only used the concatenated alignment of the 13 PCGs' sequences with length of 10 242 bp for phylogenetic analyses. The optimal partitioning schemes and models of molecular evolution were identified by PartitionFinder (Lanfear *et al.*, 2012). The GTR+I+G model with five partitioning schemes were identified and used for the subsequent analyses (Supporting

Information Table S5). We ran MrBayes 3.2.2 for 10 million generations with a sampling frequency of 1000 generations after 1 million generations of burn-in. *P. citri* was used as an outgroup. To evaluate the occurrence of substitution saturation, we estimated the index of substitution saturation in DAMBE 6 (Xia, 2017) using the method of Xia *et al.* (2003). Each codon position was performed both in the symmetrical and asymmetrical topology test. In order to infer the direction of amino acid changes, we reconstructed the sequence at the ancestral node of *T. truncatus* and *T. pueraricola* with TreeSAAP 3.2 under the REV model with other parameters set as default (Woolley *et al.*, 2003).

d_N/d_S analysis

To compare selective constraints acting on each mitochondrial PCG in each species, we calculated the d_N/d_S ratio. d_N and d_S were calculated by using DnaSP 5.10 software (Librado & Rozas, 2009). Because Syn mutation was lacking between some pairwise sequences, we calculated the ratio of $d_N/(d_S + \text{constant})$ as an alternative to d_N/d_S , where the constant was of the d_S for one Syn mutation to avoid dividing by zero following the method of Mishmar *et al.* (2003). The Wilcoxon rank-sum test implemented in R 3.3.1 software (R Development Core Team, 2005) was used to determine whether these values for each gene differed significantly between the species.

McDonald–Kreitman test analysis

In order to infer which genes had undergone positive selection, the MK test (McDonald & Kreitman, 1991) was performed on each PCG. This test compares the proportion of Nsyn to Syn mutations within species with the divergence between species to infer selection, assuming Syn sites as effectively neutral. We conducted the MK test using web-based standard and generalized MK-test software (Egea *et al.*, 2008) with Jukes and Cantor corrections (Jukes & Cantor, 1969). *P* values were corrected for multiple comparisons through false discovery rates using the p.adjust function in R 3.3.1.

Branch-model-based selection analyses

In order to compare selection pressures acting on the mitogenomes of two species globally, we applied the branch-model method implemented by CodeML in PAML4 (Yang, 2007) to estimate the d_N/d_S ratios of branches of the two species. A likelihood ratio test for the log likelihood values of the maximum likelihood method was used to determine whether the three- d_N/d_S branch model (ω_0 , background; ω_1 , *T. pueraricola* branches; ω_2 , *T. truncatus* branches) was preferred to the one-ratio (M0) model.

Positive selection sites identification

Strong purifying selection acts to preserve mitochondrial function and can mask positive selection acting on individual sites, and positive selection on PCGs is also frequently transient or episodic, occurring at a few time points and affecting a few codons. We therefore used two branch-site model-based methods within a phylogenetic framework to identify positive selection sites. First, the branch-site model method implemented by the CodeML program in PAML4 software (Yang, 2007) was used to identify positively selected sites along each branch leading to each of the two species. The likelihood of a model (A) in which site classes in a background lineage are restricted to values ≤ 1 , while site classes of the foreground lineage are allowed to take values > 1 , is compared with the likelihood of a null model that fixes ω at 1 in the foreground lineage, using a likelihood ratio test. Second, MEME was also used to detect positive selection. MEME can identify both episodic and pervasive positive selection (Murrell *et al.*, 2012). The best codon-based substitution model for the data was selected within HyPhy (Delpont *et al.*, 2010).

Detecting radical changes in physicochemical properties of amino acids

The changes in physicochemical properties of amino acids caused by replacements at the inferred positively selected codons across the phylogeny were estimated using TreeSAAP 3.2 software (Woolley *et al.*, 2003). TreeSAAP applies a sliding window approach to compare the observed pattern of amino acid replacements and changes in physiochemical properties with those expected under neutrality. We ran the analysis with sliding-window size 15 codons in one-codon increments. To minimize the false-positive detection rate, only amino acid changes with magnitude categories ≥ 6 and z-scores above 3.09 or below -3.09 ($P < 0.001$) were considered as potentially under positive selection. Furthermore, we only employed 23 amino acid properties with reported accuracies $> 75\%$ in the analysis (McClellan & Ellison, 2010).

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Figure 1. Sampling locations (A), haplotype network for *Tetranychus truncatus* (B) and for *Tetranychus pueraricola* (C) based on a partial sequence of the mitochondrial *COX1* gene (768 bp). (A) *T. pueraricola* (Used), the *T. pueraricola* populations used in this study; *T. tuncatus* (Used), the *T. tuncatus* populations used in this study;

T. pueraricola (Recorded), the recorded occurrence of *T. pueraricola*; *T. truncatus* (Recorded), the recorded occurrence of *T. pueraricola*. The records are based on cumulative surveys conducted by our group during 2008–2014. PC, plateau climate zone; CT, cold temperate zone; MT, moderate temperate zone; WT, warm temperate zone; NS, northern subtropical zone; MS, middle subtropical zone; SS, southern subtropical zone; MTr, middle tropical zone (the map was made using ArcGIS 10.2 software, <http://www.arcgis.com/features/>). The regionalization of climatic zones is according to Zheng *et al.* (2010). In (B) and (C), the haplotypes labelled by red characters indicate that the mitogenomes corresponding to these haplotypes were not sequenced.

Figure 2. Proportion of (A) synonymous (Syn) and (B) non-synonymous (Nsyn) substitutions in each mitochondrial protein-coding gene of the two *Tetranychus* species. Wilcoxon signed-rank tests indicated that both the proportions of Syn and Nsyn substitutions were significantly higher in the *Tetranychus pueraricola* mitogenomes than in the *Tetranychus truncatus* mitogenomes globally (for Syn, $P = 0.001$; for Nsyn, $P = 0.006$). *ND1–4L*: NADH dehydrogenase complex genes; *Cytb*: cytochrome *b* gene; *COX1–3*: cytochrome *c* oxidase subunit genes; ATP6, -8: ATP synthase genes.

Figure 3. Comparison of relative constraints [$d_N/(d_S + \text{constant})$] for each mitochondrial protein coding gene of the two *Tetranychus* species. The constant was of the d_S for one synonymous mutation following the method of Mishmar *et al.* (2003). For each gene, the bottom and top of the line indicates the minimum and maximum values respectively. The bottom, intermediate and top horizontal lines in the boxes represent the 25th, 50th (median) and 75th percentile values respectively. Numbers above plots are the P values (Wilcoxon rank-sum test) for the comparison.

Figure 4. Bayesian phylogeny tree based on 13 mitochondrial protein coding gene sequences. *Panonychus citri* was used as outgroup. The posterior probability is given at each node. Branch length was omitted. The d_N/d_S ratios for the branches of each of

the species were estimated by the branch model using CodeML in PAML4 software.

A. viennensis, *Amphitetranychus viennensis*; *P. ulmi*, *Panonychus ulmi*.

Table 1. McDonald–Kreitman test for each of the 13 protein-coding genes

Gene	Length (bp)	Syn divergence	Syn polymorphism	Nsyn divergence	Nsyn polymorphism	NI	<i>P</i> values
<i>ND1</i>	855	60.42	31	31.97	14	0.853	0.808
<i>ND2</i>	888	33.49	16	38.29	12	0.655	0.500
<i>ND3</i>	333	53.10	10	21.04	7	1.766	0.500
<i>ND4</i>	1152	96.78	45	96.26	17	0.379	0.013
<i>ND4L</i>	243	21.66	12	13.60	4	0.530	0.500
<i>ND5</i>	1563	129.62	61	152.49	44	0.613	0.137
<i>ND6</i>	399	39.73	14	25.24	10	1.124	0.876
<i>Cytb</i>	1062	83.70	39	55.33	17	0.659	0.468
<i>COX1</i>	1536	111.18	83	26.37	15	0.761	0.573
<i>COX2</i>	636	52.10	36	18.43	4	0.314	0.137
<i>COX3</i>	786	50.80	32	36.35	12	0.524	0.273
<i>ATP6</i>	618	54.69	37	58.32	15	0.380	0.039
<i>ATP8</i>	129	9.58	2	18.04	4	1.061	0.949

Syn, synonymous; Nsyn, non-synonymous. NI, neutrality index; NI > 1 indicates negative selection, NI = 1 indicates neutral selection; NI < 1 indicates positive selection. *P*-values have been corrected by the false discovery rate method; values <0.05 are highlighted in bold.

Table 2. Codons with evidence of positive selection

Gene	C	Pos	Probabilit	An	Amino acid identity		Significant properties
	x	y		c	<i>Tetranychus truncatus</i>	<i>Tetranychus pueraricola</i>	
<i>ND4</i>	1	321	0.009a	M	M	F	—
<i>ND5</i>	1	79	0.002a	M	M	F	—
<i>ND5</i>	1	157	0.037a	M	M	L	—
<i>ND4</i>	1	329	0.049a	K	M	K	M : $B_r, R_F, h, F, P, P_r, E_t$
<i>ND5</i>	1	385	0.034a	K	I	S/M	I : $N_s, B_r, R_F, pK', h, F, H_{nc}, P, R_a, H_p, E_t$
<i>Cytb</i>	3	64	0.971b	E	I	E	I : $N_s, B_r, pK', h, P_r, P, a_C, H_t, E_t$
<i>ATP6</i>	5	31	0.013a	F	H	F	H : R_F, h, P

ATP6	5	111	0.971b	S	K	S	K: E_t
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Cx, oxidative phosphorylation system complex. Pos, amino acid position number in the gene.

Probability: a, *P*-value of MEME analysis; b, posterior probability of positive selection inferred by Bayes empirical Bayes approach in CodeML. Anc, amino acid identity for the ancestral sequence of the two *Tetranychus* species. Amino acid identity in *T. truncatus* and *T. pueraricola*, bold font indicates amino acid identified to be evolving under positive selection by TreeSAAP. Significant properties, physicochemical properties with evidence of positive selection: *N_s*, average number of surrounding residues; *B_r*, buriedness; *R_F*, chromatographic index; *pK'*, equilibrium constant (ionization of COOH); *h*, hydropathy; *F*, mean root-mean-square fluctuation displacement; *H_{nc}*, normalized consensus hydrophobicity; *E_t*, total non-bonded energy; *P_r*, polar requirement; *P*, polarity; *α_C*, power to be at the C-terminal; *R_a*, solvent accessible reduction ratio; *H_p*, surrounding hydrophobicity; *H_t*, thermodynamic transfer hydrophobicity; *P_t*, turn tendencies; *P_c*, coil tendencies.

Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1. Sampling information for *T. pueraricola* and *T. truncatus* in China.

Table S2. Information on the primer sequences used for mitochondrial genome amplification in *T. truncatus*.

Table S3. Information on the primer sequences used for mitochondrial genome amplification in *T. pueraricola*.

Table S4. The mitogenome sequences of the other nine species of Tetranychidae used for phylogenetic reconstruction.

Table S5. Partition strategies used in this study.

Figure S1. Substitution saturation plots for each codon position. (A) the first codon position, (B) the second codon position, (C) the third codon position, green X marks represent the observed data for transitions, blue triangular symbols for transversions.

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