Phylogenetic Analysis of the Spider Mite Sub-Family Tetranychinae (Acari: Tetranychidae) Based on the Mitochondrial COI Gene and the 18S and the 5' End of the 28S rRNA Genes Indicates That Several Genera Are Polyphyletic

Tomoko Matsuda¹, Maiko Morishita¹, Norihide Hinomoto², Tetsuo Gotoh¹*

¹ Laboratory of Applied Entomology and Zoology, Faculty of Agriculture, Ibaraki University, Ibaraki, Japan, ² NARO Agricultural Research Center, National Agriculture and Food Research Organization, Ibaraki, Japan

Abstract

The spider mite sub-family Tetranychinae includes many agricultural pests. The internal transcribed spacer (ITS) region of nuclear ribosomal RNA genes and the cytochrome c oxidase subunit I (COI) gene of mitochondrial DNA have been used for species identification and phylogenetic reconstruction within the sub-family Tetranychinae, although they have not always been successful. The 18S and 28S rRNA genes should be more suitable for resolving higher levels of phylogeny, such as tribes or genera of Tetranychinae because these genes evolve more slowly and are made up of conserved regions and divergent domains. Therefore, we used both the 18S (1,825–1,901 bp) and 28S (the 5’ end of 646–743 bp) rRNA genes to infer phylogenetic relationships within the sub-family Tetranychinae with a focus on the tribe Tetranychini. Then, we compared the phylogenetic tree of the 18S and 28S genes with that of the mitochondrial COI gene (618 bp). As observed in previous studies, our phylogeny based on the COI gene was not resolved because of the low bootstrap values for most nodes of the tree. On the other hand, our phylogenetic tree of the 18S and 28S genes revealed several well-supported clades within the sub-family Tetranychinae. The 18S and 28S phylogenetic trees suggest that the tribes Bryobiini, Petrobiini and Eurytetranychini are monophyletic and that the tribe Tetranychini is polyphyletic. At the genus level, six genera for which more than two species were sampled appear to be monophyletic, while four genera (Oligonychus, Tetranychus, Schizotetranychus and Eotetranychus) appear to be polyphyletic. The topology presented here does not fully agree with the current morphology-based taxonomy, so that the diagnostic morphological characters of Tetranychinae need to be reconsidered.

Introduction

The spider mite sub-family Tetranychinae includes some pests that cause serious economic losses throughout the world [1], [2], [3]. The family consists of more than 1,200 species, some of which have a wide host range, whereas others are highly host-specific [4], [5]. For example, Tetranychus urticae Koch, Panonychus citri (McGregor) and Oligonychus coffeee (Nietner), have an especially strong effect on agricultural and horticultural crops, and they are polyphagous. However, these genera also include mono-oligophagous species, such as Tetranychus bambusae Wang & Ma, Panonychus bambusicola Ehara & Gotoh, Oligonychus orthius Rimando, Oligonychus modestus (Banks) and Oligonychus rubi-cundus Ehara which inhabit only gramineous plants.

Although exact species identification is the first step in any biological study, spider mites are difficult to distinguish by morphological characters alone because of their small size (< 0.5 mm) and limited number of diagnostic characters [6], [7], [8]. Therefore, the use of DNA-based methods to identify species has increasingly been used for some genera of the Tetranychinae. For example, Navajas and Boursot [9] showed that T. urticae and Tetranychus turkestanii Ugarov & Nikolskii, which are very closely related species, can be identified by using the internal transcribed spacer 2 (ITS2) region of nuclear ribosomal RNA (rRNA) genes. More recently, Matsuda et al. [10], [11] revealed that almost all species of Japanese Oligonychus (17 of 18 species) and all species of Tetranychus (13 species) can be identified by using the cytochrome c oxidase subunit I (COI) gene of mitochondrial DNA.
| Sub-family | Tribe | Genus | Species | Date       | Locality      | Host plant          | Voucher specimen no. | Accession no. | COI    | 18S    | 28S    |
|------------|-------|-------|---------|------------|---------------|---------------------|---------------------|--------------|--------|--------|--------|
| Bryobiinae | Bryobiini | Bryobia | *B. eharai* Pritchard & Keifer | Sept. 11, 2012 | Ibaraki, Japan | Chrysanthemum morifolium | 0612 | –          | AB926227 | AB926318 |
|            |       |       | *B. praeftosa* Koch | July 27, 2008 | Hokkaido, Japan | Trifolium repens | 0609 | AB981203 | AB926228 | AB926319 |
|            | Petrobiini | Petrobia | *P. latens* (Müller) | Mar. 30, 2012 | Tokushima, Japan | Daucus carota | 0482 | AB981204 | AB926229 | AB926320 |
|            | Tetranychina | T. hartii (Eving) | | June 11, 2012 | Ibaraki, Japan | Oxalis corniculata | 0602 | –          | AB926230 | AB926321 |
|            | Eurytetranychina | Eurytetranychoides | *E. japonicus* (Ehara) | Sept. 22, 2010 | Tokyo, Japan | Lithocarpus edulis | 0493 | AB981205 | AB926231 | AB926322 |
|            | Eutetranychus | *A. firmianae* (Ma & Yuan) | | Aug. 7, 2010 | Ibaraki, Japan | Firmiana simplex | 0405 | –          | AB926234 | AB926325 |
|            | Panonychus | *P. bambusicola* Ehara & Gotoh | | June 4, 1989 | Hokkaido, Japan | Sasa senanensis | 0606 | AB981207 | AB926235 | AB926326 |
|            | Sasanychus | *P. citri* (McGregor) | | May 6, 1993 | Ibaraki, Japan | Ilex crenata | 0226 | AB981208 | AB926237 | AB926328 |
|            | Sasanychus | *P. elongatus* Manson | | July 27, 2010 | Hangzhou, China | Broussonetia papyrifera | 0398 | –          | AB926238 | AB926329 |
|            | Sasanychus | *P. mori* Yokoyama | | Apr. 22, 2007 | Hokkaido, Japan | Moussiaustralis | 0239 | AB981209 | AB926239 | AB926330 |
|            | Sasanychus | *P. osmanthi* Ehara & Gotoh | | Nov. 16, 2001 | Guilin, China | Osmanthus fragrans | 0229 | AB981210 | AB926240 | AB926331 |
|            | Sasanychus | *P. ulmi* (Koch) | | Aug. 4, 2010 | Hokkaido, Japan | Ulmus davidiana | 0407 | AB981211 | AB926241 | AB926332 |
| Schizotetranychus | S. aktnus (Ehara) | | | June 23, 1986 | Hokkaido, Japan | Sasa senanensis | 0605 | AB981212 | AB926242 | AB926333 |
|            | Schizotetranychus | *S. pusullus* Ehara & Gotoh | | July 31, 2012 | Hokkaido, Japan | Sasa chartacea | 0575 | AB981213 | AB926243 | AB926334 |
| Schizotetranychus | *S. bambusicola* Reck | | | Aug. 27, 2011 | Chiba, Japan | Phyllostachys edulis | 0503 | AB981214 | AB926244 | AB926335 |
|            | Schizotetranychus | *S. brevisetosus* Ehara | | Oct. 13, 2011 | Kochi, Japan | Quercus glauca | 0527 | AB981215 | AB926245 | AB926336 |
|            | Schizotetranychus | *S. cercidiphylli* Ehara | | Aug. 3, 2010 | Hokkaido, Japan | Cercidiphyllum japonicum | 0411 | AB981216 | AB926246 | AB926337 |
|            | Schizotetranychus | *S. vitus* Ehara & Ohashi | | May 22, 2012 | Nara, Japan | Quercus glauca | 0549 | AB981217 | AB926247 | AB926338 |
|            | Stigmaeopsis | *S. lepedezae* Beglijarov & Mitrofanov | | Aug. 26, 2011 | Ibaraki, Japan | Pueraria montana | 0515 | AB981218 | AB926248 | AB926339 |
|            | Stigmaeopsis | *S. reki* Ehara | | Aug. 4, 2010 | Hokkaido, Japan | Sasa senanensis | 0408 | AB981219 | AB926249 | AB926340 |
|            | Stigmaeopsis | *S. schizopus* (Zacher) | | June 14, 2010 | Tokyo, Japan | Salix integra | 0532 | AB981220 | AB926250 | AB926341 |
|            | Stigmaeopsis | *S. shii* (Ehara) | | June 14, 2010 | Tokyo, Japan | Castanea sieboldii | 0533 | AB981221 | AB926251 | AB926342 |
|            | Stigmaeopsis | *S. eurialis* Banks | | Aug. 7, 2011 | Ibaraki, Japan | Pleioblastus chino | 0506 | AB981222 | AB926252 | AB926343 |
|            | Stigmaeopsis | *S. longus* (Saito) | | June 4, 1989 | Hokkaido, Japan | Sasa senanensis | 0542 | AB981223 | AB926253 | AB926344 |
|            | Stigmaeopsis | *S. miscanthi* (Saito) | | Feb. 16, 2009 | Nagasaki, Japan | Miscanthus sinensis | 0404 | AB981224 | AB926254 | AB926345 |
Table 1. Cont.

| Sub-family     | Tribe                | Genus          | Species          | Date       | Locality       | Host plant          | Voucher specimen no. | Accession no. |
|----------------|----------------------|----------------|------------------|------------|----------------|---------------------|----------------------|---------------|
|                |                      | S. saharai Saito & Mori |                | Aug. 5, 2011 | Chiba, Japan | Heteroblastus chino | 0501                | AB981226, AB926256, AB926347 |
|                |                      | S. trilobata Saito & Mori |                | Oct. 27, 1997 | Hokkaido, Japan | Sasa senanensis     | 0541                | AB981227, AB926257, AB926348 |
| Yeonymys       |                      | Y. sappoensis Ebara |                | Aug. 4, 2010 | Hakkoaido, Japan | Sasa senanensis     | 0406                | AB981228, AB926258, AB926349 |
| Eotetanychus   |                      | E. asiaticus Ebara | Mar. 19, 2007 | Nagasaki, Japan | Citrus reticulata | 0546                | AB981229, AB926259, AB926350 |
|                |                      | E. boreus Ebara | June 3, 2010 | Chiba, Japan | Armenisca mume | 0415 –              | AB981226, AB926260, AB926351 |
|                |                      | E. celtis Ebara | Aug. 27, 2011 | Chiba, Japan | Aphananthe aspera | 0406                | AB981227, AB926256, AB926351 |
|                |                      | E. cinnamomea Ebara |                | Aug. 5, 2011 | Chiba, Japan | Cornus controversa | 0406                | AB981228, AB926259, AB926351 |
|                |                      | E. dissectus Ebara |                | Aug. 3, 2010 | Hokkaido, Japan | Acer pictum          | 0412                | AB981229, AB926260, AB926351 |
|                |                      | E. nomurai Ebara | Aug. 20, 2011 | Ibaraki, Japan | Celtis sinensis | 0541                | AB981226, AB926259, AB926351 |
|                |                      | E. prun if (Oudemans) | Sept. 1, 2012 | Ibaraki, Japan | Castanea crenata | 0546                | AB981229, AB926256, AB926351 |
|                |                      | E. quercifoliace Ebara & Goto | | July 6, 2011 | Ibaraki, Japan | Quercus serrata | 0507                | AB981234, AB926267, AB926351 |
|                |                      | E. rubricans Ebara | Sept. 1, 2012 | Ibaraki, Japan | Carpinus tschonoskii | 0559 –        | AB981228, AB926256, AB926351 |
|                |                      | E. smithii Pritchard & Baker | Aug. 14, 2007 | Nagasaki, Japan | Rosa multiflora | 0545                | AB981229, AB926256, AB926351 |
|                |                      | E. spectabilis Ebara | Sept. 7, 2001 | Hokkaido, Japan | Acer pictum | 0524 –              | AB981226, AB926256, AB926351 |
|                |                      | E. sugianisensis (Yokoyama) | Aug. 26, 2011 | Ibaraki, Japan | Monus australis | 0517                | AB981226, AB926256, AB926351 |
|                |                      | E. Harium (Herrmann) | Aug. 3, 2010 | Hokkaido, Japan | Alnus hirsuta | 0409 –              | AB981226, AB926256, AB926351 |
|                |                      | E. yoshimai Ebara & Goto | Aug. 29, 2011 | Ibaraki, Japan | Magnolia obovata | 0519 –              | AB981226, AB926256, AB926351 |
|                |                      | E. yoshii Ebara | Aug. 15, 2011 | Hokkaido, Japan | Ulmus davidiana | 0528                | AB981226, AB926256, AB926351 |
|                |                      | E. uncus Garman | Aug. 3, 2010 | Hokkaido, Japan | Betula platyphylla | 0413 –        | AB981226, AB926256, AB926351 |
| Oligonychus    |                      | O. amiresis Ebara & Goto | July 13, 2005 | Ibaraki, Japan | Lithocarpus edulis | 0116              | AB981226, AB926256, AB926351 |
|                |                      | O. bhuiensis (Hirst) | Dec. 3, 2005 | Okinawa, Japan | Magnifera indica | 0012               | AB981226, AB926256, AB926351 |
|                |                      | O. camelliace Ebara & Goto | May 13, 2000 | Fukushima, Japan | Camellia japonica | 0008               | AB981226, AB926256, AB926351 |
|                |                      | O. castaneae Ebara & Goto | May 5, 2009 | Ibaraki, Japan | Castanea crenata | 0297               | AB981226, AB926256, AB926351 |
|                |                      | O. clavatus (Ebara) | July 28, 2005 | Kanagawa, Japan | Pinus thunbergii | 0360               | AB981226, AB926256, AB926351 |
|                |                      | O. coffeae (Nieter) | May 30, 2005 | Okinawa, Japan | Magnifera indica | 0078               | AB981226, AB926256, AB926351 |
|                |                      | O. gotohi Ebara | July 1, 2007 | Ibaraki, Japan | Lithocarpus edulis | 0076               | AB981226, AB926256, AB926351 |
|                |                      | O. hondensis (Ebara) | Aug. 22, 2009 | Aomori, Japan | Cryptomeria japonica | 0376           | AB981226, AB926256, AB926351 |
| Sub-family | Tribe | Genus | Species | Date       | Locality          | Host plant       | Voucher specimen no. | Accession no. | COI  | 18S  | 28S  |
|------------|-------|-------|---------|------------|-------------------|------------------|--------------------|---------------|------|------|------|
| O. /lisc (McGregor) |       |       |         | Oct. 30, 2000 | Kagoshima, Japan | Camellia sinensis | 0081               | AB8683640     | AB826284 | AB826375 |
| O. karonatus (Ehara) |       |       |         | Aug. 27, 2009 | Hokkaido, Japan  | Larix kaempferi   | 0358               | AB8683656     | AB826285 | AB826376 |
| O. modestus (Banks) |       |       |         | Sept. 9, 2008 | Okinawa, Japan   | Digiataris ciliaris | 0092             | AB8683677     | AB826286 | AB826377 |
| O. orthus Rimando |       |       |         | July 9, 2009  | Okinawa, Japan   | Saccharum officinarum | 0378             | AB8683675     | AB826287 | AB826378 |
| O. perditus Pritchard & Baker |       |       |         | Sept. 17, 2008 | Kanagawa, Japan | Juniperus sp.       | 0364             | AB8683656     | AB826288 | AB826379 |
| O. pustulosus Ehara |       |       |         | Aug. 22, 2009 | Aomori, Japan    | Cryptomeria japonica | 0363             | AB8683655     | AB826289 | AB826380 |
| O. rubicundus Ehara |       |       |         | Oct. 17, 2008 | Kochi, Japan     | Miscanthus sinensis | 0290             | AB8683681     | AB826290 | AB826381 |
| O. ununguis (Jacobi) |       |       |         | July 27, 2008 | Hokkaido, Japan  | Cryptomeria japonica | 0088             | AB8683664     | AB826291 | AB826382 |
| Amphitetranychus A. quercivorus (Ehara & Gotoh) |       |       |         | July 9, 2003  | Ibaraki, Japan   | Quercus crispula   | 0610             | AB981238      | AB826292 | AB826383 |
| Amphitetranychus T. bambusea Wang & Ma |       |       |         | July 5, 2009  | Okinawa, Japan   | Phyllostachys edulis | 0343             | AB826294      | AB926384 |
| Tetanychus T. evansi Baker & Pritchard |       |       |         | Nov. 3, 2006  | Tokyo, Japan     | Salarium nigrum    | 0210             | AB736039      | AB826295 | AB826386 |
| Tetanychus T. ezoensis Ehara |       |       |         | Sept. 3, 2008 | Ibaraki, Japan   | Taxus cuspidata     | 0281             | AB736042      | AB826296 | AB826387 |
| Tetanychus T. hulhotensis Ehara, Gotoh & Hong |       |       |         | July 26, 2007 | Inner Mongolia Autonomous Region, Mongolia | Zea mays | 0201 | -- | AB826297 | AB826388 |
| T. karazawai Kishida |       |       |         | May 19, 1993 | Shizuoka, Japan | Thea sinensis      | 0158             | AB736043      | AB826298 | AB826389 |
| T. lombardini Baker & Pritchard |       |       |         | July 10, 2008 | Durban, South Africa | Erthrina variegata | 0381 | -- | AB826299 | AB826390 |
| T. ludeni Zacher |       |       |         | Oct. 17, 1995 | Ibaraki, Japan   | Solidago virgaurea | 0189             | AB736051      | AB826300 | AB826391 |
| T. mactarlani Baker & Pritchard |       |       |         | Sept. 30, 2008 | Mymensingh, Bangladesh | Dolichos lablab | 0398 | -- | AB826301 | AB826392 |
| T. mergansier Boudreaux |       |       |         | Apr. 6, 2007  | El Talo, Sonora, Mexico | Cucurbita maxima | 0225 | -- | AB826302 | AB826393 |
| T. misumaeensis Ehara & Gotoh |       |       |         | Aug. 23, 2005 | Hokkaido, Japan   | Apios sp.         | 0218             | AB736054      | AB826303 | AB826394 |
| T. neocaledonicus Andre |       |       |         | May 27, 1998  | Tokyo, Japan     | Morus australis     | 0192             | AB736055      | AB826304 | AB826395 |
| T. okinawanus Ehara |       |       |         | June 19, 2003 | Okinawa, Japan   | Pueraria montana    | 0208             | AB736058      | AB826305 | AB826396 |
| T. parakashawae Ehara |       |       |         | June 5, 1993 | Ibaraki, Japan   | Pueraria montana    | 0155             | AB736060      | AB826306 | AB826397 |
| T. phaselus Ehara |       |       |         | June 29, 2000 | Ibaraki, Japan   | Glycine max         | 0191             | AB736066      | AB826307 | AB826398 |
| T. piersci McGregor |       |       |         | Dec. 20, 2007 | Okinawa, Japan   | Cucumis melo        | 0014             | AB736068      | AB826308 | AB826399 |
| T. puerarica Ehara & Gotoh |       |       |         | Oct. 23, 1993 | Ibaraki, Japan   | Pueraria montana    | 0203             | AB736071      | AB826309 | AB826400 |
| T. truncatus Ehara |       |       |         | May 8, 2004  | Kyoto, Japan     | Solaranum nigrum   | 0195             | AB736075      | AB826310 | AB826401 |
Despite recent advances in DNA-based methods for identifying spider mites, most phylogenetic relationships of sub-families, tribes and genera of the Tetranychinae remain poorly understood, as is reflected by the low support values for most nodes of the phylogenetic trees. However, phylogenetic trees clearly show that the genus *Oligonychus* is polyphyletic. Navajas et al. [12] and Ros and Breeuwer [13] analyzed the phylogeny of Tetranychinae including three *Oligonychus* species (*Oligonychus ununguis* (Jacobi), *Oligonychus platani* (McGregor) and *Oligonychus gossypii* (Zacher)) using the COI gene. Although these three species have the same empodium shape, *O. gossypii*, whose aedeagus curves dorsally, can be easily distinguished from *O. ununguis* and *O. platani* whose aedeagi curve ventrally. In the phylogenetic trees of these two studies, *O. gossypii* clustered more closely with *Tetranychus* species whose aedeagi also curve dorsally, while *O. ununguis* and *O. platani* formed a separate group. Polyphyly in the genus *Oligonychus* was also reported in the ITS2 region [14].

The unresolved phylogeny among the taxa of the sub-family Tetranychinae based on the COI sequences is probably due to the strongly biased nucleotide composition and the saturation at the third codon positions [13]. Because both the 18S and 28S rRNA genes evolve more slowly and are made up of conserved regions and divergent domains [15], these genes have been used for phylogenetic analyses of higher taxonomic relationships (from “phyla” to “classes” within Ecdysozoa) [16], [17]. In resolving tick genera (Acarina: Ixodida), combining the 18S and 28S rRNA genes provided more detailed relationships than did the 18S gene alone [18], [19]. Therefore, we used both the 18S (1,825–1,901 bp) and 28S (the 5’ end of 646–743 bp) rRNA genes to infer phylogenetic relationships within the sub-family Tetranychinae. Then, we compared the trees based on the 18S and 28S genes with the tree based on the mitochondrial COI gene (618 bp). Another problem in previous studies [12], [13], [14] was that only 16 to 25 species were used for the phylogenetic analyses. Limited taxon sampling can seriously influence the resulting phylogenetic inferences (for reviews, see [20], [21], [22]). Therefore, to assess the phylogenetic relationships among tribes and genera of the sub-family Tetranychinae, we examined a total of 88 strains (15 genera and 4 tribes) most of which were from Japan.

### Results

**Mitochondrial COI gene**

We obtained the COI sequences of 38 strains determined in this study (Table 1) and 30 strains from previously published data [10], [11]. The COI sequences contained no insertions or deletions. After alignment, the COI fragment had 618 nucleotides, of which 282 were parsimony-informative sites (File S1). The AT contents of the COI sequences of the tetranychid mites were very high (75.5%), especially at the 3rd codon position (93.0%). Chi-square tests revealed no significant heterogeneity in the first and second codon positions of the COI sequences, but significant heterogeneity at third codon positions (Figure 1). Similar high AT contents have been observed in previous studies of tetranychid mites [10], [11], [12], [13].

A phylogenetic tree of the sub-family Tetranychinae based on the COI gene is shown in Figure 2. Among the eight genera for which more than two strains were sampled, four genera (*Panonychus*, *Sasanychus*, *Stigmaeopsis* and *Amphitetranychus*) appear to be monophyletic with >80 bootstrap values, while the other four (*Oligonychus*, *Tetranychus*, *Schizotetranychus* and *Eoetetranychus*) are polyphyletic. The four monophyletic genera are in clades 8, 3, 5 and 2, respectively (Figure 2). As was observed in previous studies, *Oligonychus* species whose aedeagus curves...
ventrally (clade 7) can be easily distinguished from *Oligonychus biharensis* (Hirst), *O*. modestus, *O*. orthius and *O*. rubinclus whose aedeagi curve dorsally. Although *Schizotetranychus* and *Eotetranychus* are scattered across the tree, some species formed well-supported clades. *Schizotetranychus bambusae* Reck & *Schizotetranychus recki* Ehara clustered with *Sasanychus* and *Yezonychus* species (clade 4). The clade including *Schizotetranychus cercidiphylli* Ehara, *Eotetranychus asiaticus* Ehara and *Eotetranychus cornicola* Ehara are supported with high bootstrap value (clade 6: bootstrap value (BP) = 88). The COI tree also shows monophyly of closely related species that morphologically and molecularly resemble each other, such as *P*. citri and *Panonychus osmanthus* Ehara & Gotoh [23], [24] (clade 9) and *T*. urticae and *T*. turkestani [9] (clade 1). These results are consistent with the 18S and 28S topologies described below. However, the COI phylogeny was not resolved and the deep-level relationships were especially unresolved, as shown by the low bootstrap values (Figure 2), as was observed in previous studies [12], [13]. The deep-level phylogeny of the sub-family Tetranychinae was also not resolved in the Bayesian tree (data not shown).

18S and 28S rRNA genes

We determined the 18S and the 5′ end of the 28S rRNA sequences of all 68 strains used in this study (Table 1). The lengths of the 18S sequences obtained were 1,825–1,901 bp. The 18S and 28S sequences contained a number of gaps (insertions and deletions). After alignment and deletion of the ambiguous part of the aligned data, the final length was 1,863 bp, containing 495 parsimony-informative sites. The lengths of the 28S sequences were 646–743 bp, with a final length of 671 bp, containing 201 parsimony-informative sites. The aligned sequences before and after deleting the ambiguous parts are shown in Supporting Information (Files S2–S4). Chi-square tests revealed no significant heterogeneity in the nucleotide composition of the 18S and 28S sequences (Figure 3).

Phylogenetic trees based on a single gene were not as well resolved as phylogenetic trees based on the combined 18S and 28S data sets. Therefore, only the combined data set was used for the ML and Bayesian analyses. The 18S and 28S trees suggest that the tribes Bryobiini and Petrobiini of the sub-family Bryobiinae, which were used as outgroups, are both monophyletic (Figures 4A and 5A, clades 22 and 23). Within the Tetranychinae, Clade 15 is composed of species of Eurytetranychini, and clades 12, 17 and 20 are composed of species of Tetranychini (Figures 4A and 5A). Among the 10 genera for which more than two strains were sampled, six genera (*Bryobia*, *Aponychus*, *Panonychus*, *Sasanychus*, *Stigmaeopsis* and *Amphitetranychus*), appear to be monophyletic with >95 bootstrap values and 1.00 posterior probabilities, while four genera (*Oligonychus*, *Tetranychus*, *Schizotetranychus* and *Eotetranychus*) are polyphyletic. The monophyletic genera are in clades 22, 14, 5, 7, 17 and 21, respectively (Figures 4A–4D and 5A–5D). Species of the genus *Oligonychus* are separated into 2 clades (clades 1 and 19), with the *Tetranychus* species included in clade 19 (Figures 4B, 4D, 5B and 5D). *Schizotetranychus* species, with the exception of *S*. *cercidiphylli*, are separated into 3 clades (clades 3, 4 and 9), with the *Sasanychus* and *Yezonychus* species included in clade 9 (Figures 4B and 5B). In the ML tree (Figures 4B–4C), *S*. *cercidiphylli* and *Eotetranychus* species, with the exception of *Eotetranychus uchidai* Ehara, are paraphyletic with respect to clade 10. *E. uchidai* forms a sister group with *Panonychus*, *Sasanychus*, *Schizotetranychus* and *Yezonychus* species (Figure 4B, clade 8). In the Bayesian tree (Figures 5B–5C), a well-supported clade consisting of *S*. *cercidiphylli* and *Eotetranychus* species, with the exception of *E. uchidai*

![Figure 1. Base compositions of the codons of the mitochondrial COI gene. (A) All codon positions, (B) 1st codon position, (C) 2nd codon position, (D) 3rd codon position, averaged over all 68 mite strains used in this study. Error bars depict range. Results of the homogeneity test are given for each codon position. doi:10.1371/journal.pone.0108672.g001](Fig1.png)
clade 10: Bayesian posterior probabilities (BPP) = 0.96) clustered with clade 8.  

As was observed in the COI tree, the 18S and 28S trees also show the monophyly of \textit{P. citri} and \textit{P. osmanthi} which are closely related species (Figures 4B and 5B, clade 6). \textit{S. cercidiphylli} forms a well-supported clade with four \textit{Eotetranychus} species (\textit{E. asiaticus}, \textit{Eotetranychus borus} Ehara, \textit{E. cornicola} and \textit{Eotetranychus toyoshimai} Ehara & Gotoh) in both ML and Bayesian trees (Figures 4C and 5C, clade 11; BP/BPP = 93/1.00). On the other hand, closely related \textit{Eotetranychus} species (\textit{E. pruni} Oudemans, \textit{E. querci} Reeves and \textit{E. uncatus} Garman), which have long, flagellate and undulate aedeagi [25], did not cluster together in either tree (Figures 4C and 5C).
Phylogeny of Tetranychidae Based on rDNA

Discussion

Only a few studies have examined the molecular phylogeny of the sub-family Tetranychinae, and they often used genes or regions that had limited discriminating ability. As observed in previous studies, our tree based on the COI gene did not resolve deep-level phylogeny because of the low bootstrap values for deep nodes of tree (Figure 2). Therefore, we used the 18S and 28S rRNA genes for phylogenetic analyses because of their better discriminating ability. Indeed, our phylogenetic tree of the 18S and 28S sequences revealed several well-supported clades, allowing us to consider the phylogenetic relationships among the sub-family Tetranychinae.

Our phylogenetic trees based on the 18S and 28S rRNA genes suggest that the tribes Bryobiini and Petrobiini of the sub-family Bryobiinae are both monophyletic, but the tribe Tetranychini is polyphyletic because the monophyletic clade of Eurytetranychini is placed inside Tetranychini (Figures 4A and 5A). At the generic level, 4 genera (Oligonychus, Tetranychus, Schizotetranychus and Eotetranychus) are polyphyletic. The phylogenetic tree separates the Oligonychus species into two clades (Figures 4B, 4D, 5B and 5D, clades 1 and 19). That is, the two clades comprising the genus Oligonychus coincide with their morphology based on the direction of curvature of the aedeagus. These results are in agreement with our COI phylogeny (Figure 2) and previous phylogenies based on the COI gene and ITS2 region [10], [12], [13], [14]. Although phylogenies based on the COI gene and ITS2 region could not establish the exact phylogenetic positions of the two clades of Oligonychus, our tree suggests that species whose aedeagi curve ventrally form a sister group with some of the Schizotetranychus species (Figures 4B and 5B, clade 2) and species whose aedeagi curve dorsally are more closely related to Tetranychus species whose aedeagi also curve dorsally (Figures 4D and 5D, clade 19). Though Oligonychus and Tetranychus can be distinguished by their empodium shape, our phylogenetic trees reveal that the shape of the aedeagi can help to discriminate these two genera.

Species of the genus Schizotetranychus and Eotetranychus appear to be polyphyletic within clade 12 (Figures 4B–4C and 5B–5C). Puzzlingly, S. cerdiciphylly and E. uchidai are separated from other congeneric species in the tree. The placement of Eotetranychus species is different between the ML and Bayesian trees. In the ML tree (Figures 4B–4C), we could not establish the exact phylogenetic position of the species of Eotetranychus which are paraphyletic respect to clade 10 because bootstrap values are relatively low. On the other hand, in the Bayesian tree (Figure 5C), S. cerdiciphylly and the Eotetranychus species, with the exception of E. uchidai, clustered into a well-supported clade (clade 10: BPP = 0.96). Similarly, the phylogenetic position of the genus Stigmaeopsis is resolved in the Bayesian analysis but not in the ML analysis. In the ML tree (Figure 4C), Stigmaeopsis species (clade 17) clustered with clade 13, which includes the Eurytetranychini species and some of the Tetranychini species, but the topology is not well supported (clade 16: BP = 50). In the Bayesian tree (Figure 5C), Stigmaeopsis species (clade 17) clustered with clade 13 with high Bayesian posterior probabilities (clade 16: BPP = 0.91). Although our data suggests that the Bayesian tree (Figures 4A–4D) is better supported than the ML tree (Figures 4A–4D), it is common knowledge that posterior probabilities are generally higher than bootstrap values [26].

Phylogenetic trees can be used to assess associations between spider mites and their host plants [13]. In the ML and Bayesian trees (Figures 4D and 5D), Olgonychus and Tetranychus species inhabiting graminaceous plants (O. orthius, O. modestus, O. rubicundus and T. bambusae) clustered separately from other species and formed a monophyletic clade (Figures 4D and 5D, clade 18). Clade 4 includes Schizoetetranychus brevisetosus Ehara, Schizoetetranychus gilicus Ehara & Ohashi and Schizoetetranychus shii (Ehara) which inhabit fagaceous plants (Figures 4B and 5B). Clade 9 include species irrespective of genus, which inhabit bamboo plants, Sasanychus akaitasus (Ehara), Sasanychus pusillus Ehara & Gotoh, S. bambusae, S. recki and Yezonychus sapporoensis Ehara (Figures 4B and 5B). All Stigmaeopsis species inhabiting graminaceous plants are separated from other Tetranychini species and appear to be monophyletic (Figures 4C and 5C, clade 17). These results indicate that the phylogenetic relationships of some species of spider mites are closely linked with their host plant, as reported in other phytophagous arthropods [27], [28], [29].

We consider the phylogenies of the Tetranychinae based on the 18S and 28S rRNA genes to be a major improvement over previous phylogenies because they reveal several well-supported clades that were not distinguished by phylogenetic relationships based on the COI gene and ITS2 region. Our finding that the tribe Tetranychini and four genera (Oligonychus, Tetranychus, Schizotetranychus and Eotetranychus) are polyphyletic indicates that the diagnostic morphological characters of tribes and genera of Tetranychidae need to be reconsidered. Although we examined a large number of species in this study, most of them were collected in Japan. Analyzing a number of undescribed genera remaining throughout the world may help achieve a deeper understanding of the phylogenetic relationships among the family.
Tetranychinae. In addition, a large number of nuclear genes need to be examined to resolve poorly understood relationships in the ML tree (Figures 4A–4D), such as the phylogenetic positions of the genera *Eotetranychus* and *Stigmaeopsis*.

**Materials and Methods**

**Mites**

Eighty-four strains representing 12 genera and two tribes in Tetranychinae, were used in this study and four strains of the tribes *Bryobiini* and *Petriobiini* of the sub-family *Bryoibiinae* (Acari: Tetranychidae) were used as outgroups (Table 1). Mite samples that could be reared in the laboratory were maintained on leaf discs of common bean leaves (*Phaseolus vulgaris* L.), mulberry leaves (*Morus bombycis* Koidz.) or the original host plants placed on a water-saturated polyurethane mat in a plastic dish (90 mm diameter, 20 mm depth) at 25°C under a 16L:8D photoperiod until analysis. Samples that could not be maintained in the laboratory and samples that were imported from abroad were preserved in 99.5% ethanol for molecular analyses and 70% ethanol.

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**Figure 4. Maximum likelihood (ML) phylogenetic tree of the sub-family Tetranychinae based on the 18S and 28S rRNA genes using the GTR Gamma model.** Bootstrap values (>50%) based on 1,000 replications are indicated at nodes. Each operational taxonomic unit is indicated by the voucher specimen no. and scientific name. Black circles with numbers indicate the clade no. which corresponds with the article. The tree is divided into three sections: (A) The entire tree, (B) Tetranychini-1, (C) Tetranychini-1, Eurytetranychini and Tetranychini-2 and (D) Tetranychini-3. doi:10.1371/journal.pone.0108672.g004
ethanol for morphological identification. Specimens were mounted in Hoyer’s medium and identified under phase-contrast and differential interference-contrast microscopes. Voucher specimens are preserved at the Laboratory of Applied Entomology and Zoology, Faculty of Agriculture, Ibaraki University under the serial voucher specimen numbers (Table 1).

DNA extraction, amplification, cloning and sequencing

Total DNA was extracted from the whole body of each female individual by using a Wizard Genomic DNA Purification Kit (Promega). Live female individuals for DNA samples and female individuals for voucher specimen were obtained from the same leaf discs. A few of the strains could not be maintained in the laboratory. For these strains, DNA samples were obtained from ethanol-preserved female individuals. The PCR primers are given in Table 2. The mitochondrial COI fragments were amplified using primer sets C1-J-1718 \[30\] and COI REVA \[8\] for species of 12 genera (Bryobia, Petrobia, Eurytetranychoides, Aponychus, Panonychus, Sasanychus, Schizotetranychus, Yezonychus, Eotetra-nychus, Oligonychus, Amphitetranychus and Tetranychus) and primer sets C1-J-1718-stig and COI REVA-stig for species of the genus Stigmaeopsis. COI sequences for Oligonychus and Tetra-

Figure 5. Bayesian phylogenetic tree of the sub-family Tetranychinae based on the 18S and 28S rRNA genes using the GTR Gamma model. Bayesian posterior probabilities (\(>0.50\)) are indicated at nodes. Each operational taxonomic unit is indicated by the voucher specimen no. and scientific name. Black circles with numbers indicate the clad no. which corresponds with the article. The tree is divided into three sections: (A) The entire tree, (B) Tetranychini-1, (C) Tetranychini-1, Eurytetranychini and Tetranychini-2 and (D) Tetranychini-3.

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Data analysis

PCR amplification was performed with the following profile: 3 min at 94°C, followed by 35 cycles of 1 min at 94°C, 1 min at 45°C for COI, 60°C for 28S and 65°C for 18S and 1.5 min at 72°C. An additional 10 min at 72°C was allowed for last strand elongation. The resultant DNA solutions were purified by using MinElute PCR Purification Kit (Qiagen) and sequenced directly. Sequencing was carried out using the sequencing primers (Table 2) with a BigDye Terminator Cycle Sequencing Kit v.3.1 (Applied Biosystems) and on an ABI 3130xl automated sequencer.

| Primer name | Sequence | Application | References |
|-------------|----------|-------------|------------|
| COI         | Forward  | 5’-GGAGGATTTTGGAAATTTGAGTATTGTTCC-3’ | PCR amplification & sequencing | Simon et al. [30] |
| COI REVA    | Reverse   | 5’-GATAAACGTAATGAAGATGCTAC-3’ | PCR amplification & sequencing | Gotoh et al. [8] |
| C1-J-1718   | Forward   | 5’-GGAGGTTTTTGGATTGGTTATGCC-3’ | PCR amplification & sequencing | This study |
| COI REVA-stig| Reverse | 5’-GAAGAATCAATAGAAATGACCAC-3’ | PCR amplification & sequencing | This study |

**18S**

| Primer name | Sequence | Application | References |
|-------------|----------|-------------|------------|
| 18S-1F      | Forward   | 5’-ACCGCGATTTTGGACATCAATACATTT-3’ | PCR amplification & sequencing | This study |
| 18S-2F      | Forward   | 5’-TTGCTCTGAGCGGACGAT-3’ | Sequencing | This study |
| 18S-2R      | Reverse   | 5’-ACCCCAAGTTGCGACTAAATC-3’ | Sequencing | This study |
| 18S-3R      | Reverse   | 5’-TCCAATGAATCTGTGAATGAT-3’ | Sequencing | This study |
| 18S-8R      | Reverse   | 5’-TCTGTGTTATCGGAATATCA-3’ | Sequencing | This study |
| 18S-9F      | Forward   | 5’-AGCTCGGAAAACACAGTTT-3’ | Sequencing | This study |
| 18S-9R      | Reverse   | 5’-AGGCCATACACACCTGTATT-3’ | Sequencing | This study |
| 18S-10F     | Forward   | 5’-AGTTGGTGAGTGGATGCTGTGTT-3’ | Sequencing | This study |
| 18S-10R     | Reverse   | 5’-ACAAGGGCCAGGACGTATCAA-3’ | PCR amplification & sequencing | This study |

**28S**

| Primer name | Sequence | Application | References |
|-------------|----------|-------------|------------|
| 28v-5’      | Forward   | 5’-AAGGTAAGCCAAATGCCTC-3’ | PCR amplification & sequencing | Hillis and Dixon [31], Palumbi [32] |
| 28v-3’      | Reverse   | 5’-AGTAAGGAAACTAACC-3’ | PCR amplification & sequencing | Hillis and Dixon [31], Palumbi [32] |

Supporting Information

- **Table S2.** Primers used in polymerase chain reaction amplification and sequencing of the mitochondrial COI gene and the 18S and 28S rRNA genes.

Information Criterion (AIC) using the program Kakusan4 [38]. The RAxML search was executed for the best-scoring ML tree in one single program run (the ‘-i a’ option) instead of the default maximum parsimony-starting tree. Statistical support was evaluated with 1,000 rapid bootstrap inferences. The MrBayes5D analyses were implemented with two parallel runs of 10 million generations each and using one cold and two incrementally heated Markov chains and sampling every 100 steps. Tracer v.1.6 [39] was used to assess if the search had reached stationarity and to check whether the sample sizes for each parameter (ESS>100) were adequate. The first 10% of the trees were discarded as burn-in and the consensus tree with Bayesian posterior probabilities was constructed based on the trees sampled after the burn-in.

**Supporting Information**

- **File S1** Aligned COI sequences in FASTA format. (ZIP)
- **File S2** Aligned 18S sequences in FASTA format. (ZIP)
- **File S3** Aligned 28S sequences in FASTA format. (ZIP)
- **File S4** Aligned 18S sequences after deleting the ambiguous parts in FASTA format. (ZIP)
- **File S5** Aligned 28S sequences after deleting the ambiguous parts in FASTA format. (ZIP)
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Author Contributions

Conceived and designed the experiments: TM TG. Performed the experiments: TM MM. Analyzed the data: TM NH. Contributed to the writing of the manuscript: TM TG.