

Molecular-Based Identification and Phylogeny of *Oligonychus* Species (Acari: Tetranychidae)

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ABSTRACT The genus *Oligonychus* has been morphologically divided into two groups based on the direction of curvature of the aedeagus and includes some morphologically similar species that are difficult to distinguish. To develop DNA-based methods for identifying *Oligonychus* species and to determine the phylogenetic relationships among them, we examined the cytochrome *c* oxidase subunit I gene of mitochondrial DNA and the internal transcribed spacer and 28S regions of nuclear ribosomal RNA gene for 17 species. Based on the genetic distances (*p*-distances) of the three DNA regions, the range of intraspecific divergence was found to be below (and not overlap) the range of interspecific divergence, which allowed the 17 species to be discriminated correctly, consistent with their classification based on morphology. Phylogenetic trees constructed by neighbor-joining and Bayesian methods clearly showed two clades, consisting of species whose aedeagi curve ventrally and dorsally, respectively. Three *Oligonychus* species inhabiting gramineous plants formed clearly defined subclades.

KEY WORDS phylogenetic relationship, *Oligonychus*, morphology, mitochondrial DNA, rDNA

The family Tetranychidae, including ≈1,200 species, contains the most injurious plant-feeding mites and represents one of the most cosmopolitan and economically important groups of terrestrial arthropods (Jeppson et al. 1975, Bolland et al. 1998, Migeon and Dorkel 2006, Gotoh et al. 2009). *Oligonychus*, one of the genera in this family, has received little attention as an agricultural pest compared with the genera *Panonychus* and *Tetranychus*. *Oligonychus* includes several worldwide pest species such as the polyphagous *Oligonychus coffeae* (Nietner) and *Oligonychus biharensis* (Hirst). *O. coffeae* has a host range of >85 plant genera, including mango (*Mangifera* spp.), coffee (*Coffea* spp.), tea (*Camellia* spp.), cotton (*Gossypium* spp.), and jute (*Corchorus* spp.), and it is the most serious pest in tea-growing areas of tropical and subtropical regions (Bolland et al. 1998, Gotoh and Nagata 2001). The genus also includes mono-, oligophagous species, such as *Oligonychus orthius* Rimando, *Oligonychus modestus* (Banks), and *Oligonychus rubicundus* Ehara that inhabit only gramineous plants.

The genus *Oligonychus* can be divided morphologically into two groups depending on whether the aedeagi curve ventrally or dorsally (cf. Table 1). Closely related species are difficult to distinguish by morphological characters alone because of intraspecific vari-

ations of the key traits and the limited number of potential diagnostic characters (Wauthy et al. 1998, Zhang and Jacobson 2000, Gotoh et al. 2009). For example, the number of setae on the legs varies intraspecifically in *Oligonychus castaneae* Ehara & Gotoh and *O. coffeae* (Ehara and Gotoh 2007), so that unskilled persons have some difficulty in identifying these species.

Methods based on short DNA sequences (DNA barcoding) have proved to be an effective tool for species identification of many animals (Hebert et al. 2003a,b) and have been recently used to identify spider mite species (Ben-David et al. 2007, Hinomoto et al. 2007, Ros and Breeuwer 2007). DNA-based identification can improve the control of spider mites and can help to resolve international trade barriers related to plant quarantine (Gotoh et al. 2007, Osakabe et al. 2008) as well as discover new invasive species (Wang and Qiao 2009) and cryptic species (Hebert et al. 2004, Carew et al. 2011). Navajas et al. (1996) and Ros and Breeuwer (2007) carried out phylogenetic analyses of Tetranychidae including three *Oligonychus* species [*Oligonychus ununguis* (Jacobi), *Oligonychus platani* (McGregor), and *Oligonychus gossypii* (Zacher)] by using the cytochrome *c* oxidase subunit I (COI) gene of mitochondrial DNA (mtDNA). 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 a phylogenetic tree based on the COI gene, *O. gossypii* also placed closer to *Tetranychus* species whose aedeagi curve dorsally, whereas *O. ununguis* and *O. platani* formed a separate group with a 98% bootstrap value. Polyphyly in the

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Table 1. Collection records of 17 species of the genus *Oligonychus* and three species of the genus *Panonychus* (outgroup) used in this study

Species	Date	Locality, country	Host plant	Direction of curvature of the aedeagus	Voucher no. ^a	Accession no.		
						COI	ITS	28S
<i>Oligonychus</i>								
<i>O. clavatus</i> (Ehara)	5 July 2009	Okinawa, Japan	<i>Pinus</i> sp.	Ventral	0340	AB683653	AB683685	AB683717
<i>O. clavatus</i>	28 July 2009	Kanagawa, Japan	<i>Pinus thunbergii</i>	Ventral	0360	AB683654	AB683686	AB683718
<i>O. pustulosus</i> Ehara	22 Aug. 2009	Aomori, Japan	<i>Cryptomeria japonica</i>	Ventral	0363	AB683655	AB683687	AB683719
<i>O. karamatus</i> (Ehara)	27 Aug. 2009	Hokkaido, Japan	<i>Larix kaempferi</i>	Ventral	0358	AB683656	AB683688	AB683720
<i>O. hondoensis</i> (Ehara)	27 June 2008	Ibaraki, Japan	<i>Cryptomeria japonica</i>	Ventral	0084	AB683657	AB683689	AB683721
<i>O. hondoensis</i>	22 Aug. 2009	Aomori, Japan	<i>Cryptomeria japonica</i>	Ventral	0376	AB683658	AB683690	AB683722
<i>O. tsudomei</i> Ehara	9 July 2009	Okinawa, Japan	<i>Pinus</i> sp.	Ventral	0341	AB683659	AB683691	AB683723
<i>O. ilicis</i> (McGregor)	30 Oct. 2000	Kagoshima, Japan	<i>Camellia sinensis</i>	Ventral	0081	AB683660	AB683692	AB683724
<i>O. ilicis</i>	30 Sept. 2008	Ibaraki, Japan	<i>Rhododendron</i> sp.	Ventral	0241	AB683661	AB683693	AB683725
<i>O. camelliae</i> Ehara & Gotoh	13 May 2000	Fukushima, Japan	<i>Camellia japonica</i>	Ventral	0082	AB683662	AB683694	AB683726
<i>O. camelliae</i>	31 Mar. 2002	Tokyo, Japan	<i>Camellia japonica</i>	Ventral	0083	AB683663	AB683695	AB683727
<i>O. ununguis</i> (Jacobi)	27 July 2008	Hokkaido, Japan	<i>Cryptomeria japonica</i>	Ventral	0088	AB683664	AB683696	AB683728
<i>O. perditus</i> Pritchard & Baker	17 Sept. 2009	Kanagawa, Japan	<i>Juniperus</i> sp.	Ventral	0364	AB683665	AB683697	AB683729
<i>O. castaneae</i> Ehara & Gotoh	4 May 2009	Ibaraki, Japan	<i>Castanea crenata</i>	Ventral	0296	AB683666	AB683698	AB683730
<i>O. castaneae</i>	5 May 2009	Ibaraki, Japan	<i>Castanea crenata</i>	Ventral	0297	AB683667	AB683699	AB683731
<i>O. gotohi</i> Ehara	1 July 2007	Ibaraki, Japan	<i>Lithocarpus edulis</i>	Ventral	0076	AB683668	AB683700	AB683732
<i>O. gotohi</i>	8 Mar. 2008	Okinawa, Japan	<i>Lithocarpus edulis</i>	Ventral	0019	AB683669	AB683701	AB683733
<i>O. coffeae</i> (Nietner)	30 May 2005	Okinawa, Japan	<i>Mangifera indica</i>	Ventral	0078	AB683670	AB683702	AB683734
<i>O. coffeae</i>	13 June 2008	Okinawa, Japan	<i>Litchi chinensis</i>	Ventral	0025	AB683671	AB683703	AB683735
<i>O. amiensis</i> Ehara & Gotoh	13 July 2005	Ibaraki, Japan	<i>Lithocarpus edulis</i>	Ventral	0116	AB683672	AB683704	AB683736
<i>O. amiensis</i>	29 Mar. 2007	Hiroshima, Japan	<i>Lithocarpus edulis</i>	Ventral	0118	AB683673	AB683705	AB683737
<i>O. orthius</i> Rimando	21 Oct. 2008	Hanoi, Vietnam	<i>Saccharum officinarum</i>	Dorsal	0289	AB683674	AB683706	AB683738
<i>O. orthius</i>	9 July 2009	Okinawa, Japan	<i>Saccharum officinarum</i>	Dorsal	0378	AB683675	AB683707	AB683739
<i>O. modestus</i> (Banks)	1 July 2008	Yunlin, Taiwan	<i>Saccharum officinarum</i>	Dorsal	0066	AB683676	AB683708	AB683740
<i>O. modestus</i>	9 Sept. 2008	Okinawa, Japan	<i>Digitaria ciliaris</i>	Dorsal	0092	AB683677	AB683709	AB683741
<i>O. biharensis</i> (Hirst)	21 Dec. 2007	Okinawa, Japan	<i>Mangifera indica</i>	Dorsal	0012	AB683678	AB683710	AB683742
<i>O. biharensis</i>	30 June 2008	Taipei, Taiwan	<i>Dimocarpus longan</i>	Dorsal	0064	AB683679	AB683711	AB683743
<i>O. rubicundus</i> Ehara	1 July 2008	Chiayi, Taiwan	<i>Saccharum officinarum</i>	Dorsal	0022	AB683680	AB683712	AB683744
<i>O. rubicundus</i>	17 Oct. 2008	Kochi, Japan	<i>Miscanthus sinensis</i>	Dorsal	0290	AB683681	AB683713	AB683745
<i>Panonychus</i>								
<i>P. mori</i> Yokoyama	22 April 2007	Hokkaido, Japan	<i>Morus australis</i>		0239	AB683682	AB683714	AB683746
<i>P. citri</i> (McGregor)	6 May 1993	Ibaraki, Japan	<i>Ilex crenata</i>		0226	AB683683	AB683715	AB683747
<i>P. osmanthi</i> Ehara & Gotoh	16 Nov. 2001	Guilin, China	<i>Osmanthus fragrans</i>		0229	AB683684	AB683716	AB683748

^a Voucher specimens are preserved at the Laboratory of Applied Entomology and Zoology (Faculty of Agriculture, Ibaraki University) under the serial voucher specimen numbers.

genus *Oligonychus* also was reported in the internal transcribed spacer (ITS) region of the nuclear ribosomal RNA gene (rDNA) (Ben-David et al. 2007). However, the phylogenetic relationships among *Oligonychus* species are poorly understood because only limited DNA sequence data are available.

DNA sequences of a part of the COI gene of mtDNA and the ITS and 28S regions of rDNA have been used to identify and infer phylogenetic relationships among species of a taxon (Morse and Normark 2006, Wagener et al. 2006, Wang and Qiao 2009). Here, we collected 17 *Oligonychus* species, representing most of the known *Oligonychus* species in Japan, and we examined

whether they could be distinguished by these sequences. We also examined whether the phylogenies based on these sequences coincide with the morphology of the aedeagus and whether they can elucidate the evolution of host plant specificity.

Materials and Methods

Mites. Seventeen *Oligonychus* species (29 strains) were used in this study together with three *Panonychus* species (three strains) as outgroups (Table 1). Among the mite samples, those that could be reared in the laboratory were maintained on leaf discs of the

Table 2. Description of the primers used in PCR amplification and sequencing of the mtDNA (COI gene) and rDNA (ITS and 28S regions)

Primer name		Sequence (5' to 3')	Reference
5' half of COI (≈700 bp)			
CI-J-1718	F primer	5'-GGAGGATTTGAAAATTGATTAGTTCC-3'	Simon et al. (1994)
COI REVA	R primer	5'-GATAAAACGTAATGAAAATGAGCTAC-3'	Gotoh et al. (2009)
3' half of COI (≈300 bp)			
mt03nf	F primer	5'-TTYGAYCCWAGAGGAGGAGG-3'	This study
mt01nr	R primer	5'-AAACCTARAAATGTTGWGG-3'	This study
ITS region			
rD02	F primer	5'-GTCGTAACAAGGTTTCCGTAGG-3'	Hinamoto and Takafuji (2001)
HC2	R primer	5'-ATATGCTTAAAGTTACGCGGG-3'	Navajas et al. (1994)
28S region			
28v-5'	F primer	5'-AAGGTAGCCAAATGCCTCATC-3'	Hillis and Dixon (1991), Palumbi (1996)
28jj-3'	R primer	5'-AGTAGCGTAAAACTAACCT-3'	Hillis and Dixon (1991), Palumbi (1996)

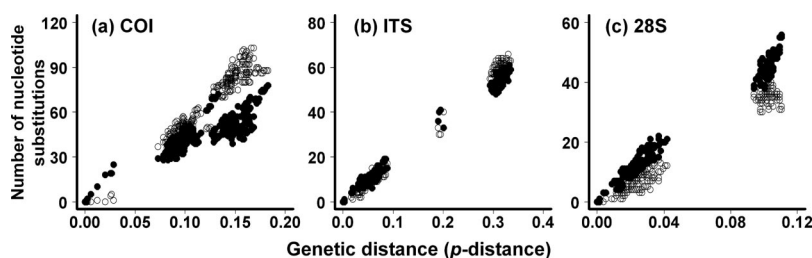


Fig. 1. Relationships between genetic distances (p -distance) and the number of nucleotide substitutions from pairwise comparisons of COI gene of mtDNA sequences (a), ITS region of rDNA (b), and 28S region of rDNA (c). Closed and open symbols represent transitional and transversional substitutions, respectively.

original host plants placed on a water-saturated polyurethane mat in a plastic dish (90 mm in diameter, 20 mm in depth) at 25°C under a photoperiod of 16:8 (L:D) h 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 DNA analyses and 70% 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 Agri-

culture, Ibaraki University, Ibaraki, Japan) under the serial voucher specimen numbers.

DNA Extraction, Amplification, Cloning, and Sequencing. One or two strains of each species were examined in the experiments (Table 1). Initially we analyzed five individual females of each strain, but in all cases the range of divergence among individuals within a strain did not exceed the range of intra-specific divergence (data not shown). Therefore, to keep data calculations simple, a single adult female randomly chosen from each strain was put into a 0.5-ml microcentrifuge tube containing 50 μ l of PrepMan Ultra Reagent (Applied Biosystems, Foster City, CA) and then crushed with the tip of pipette. Each tube was then heated at 100°C for 10 min, and this solution was used for further analyses. The polymerase chain reaction (PCR) primers are given in Table 2. 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 40–45°C, 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 QIAquick PCR Purification kits (QIAGEN, Valencia, CA) and sequenced directly or after cloning into the pGEM-T vector (Promega, Madison, WI). Sequencing was carried out in both directions by using the amplifying primers with a BigDye Terminator Cycle Sequencing kit v.3.1 (Applied Biosystems) and on an ABI 3130xl automated sequencer.

Data Analysis. All sequence data obtained were deposited in DDBJ/EMBL/GenBank International Nucleotide Sequence Databases under the accessions AB683653–AB683748 (Table 1). Sequences obtained were aligned using ClustalW (Thompson et al. 1994), and numbers of parsimony informative sites were calculated using MEGA4 software (Tamura et al. 2007). Gaps included in the ITS and 28S sequences were treated using the complete deletion option in MEGA4 that removes sites containing missing data or alignment gaps before the analyses. Genetic distances (p -distances; intraspecific and interspecific divergences) of *Oligonychus* species were calculated by MEGA4. Twelve of the species were collected in two locations. Intraspecific divergences were calculated for these 12 pairs.

Before constructing the phylogenetic tree, we estimated parameter values of nucleotide substitutions

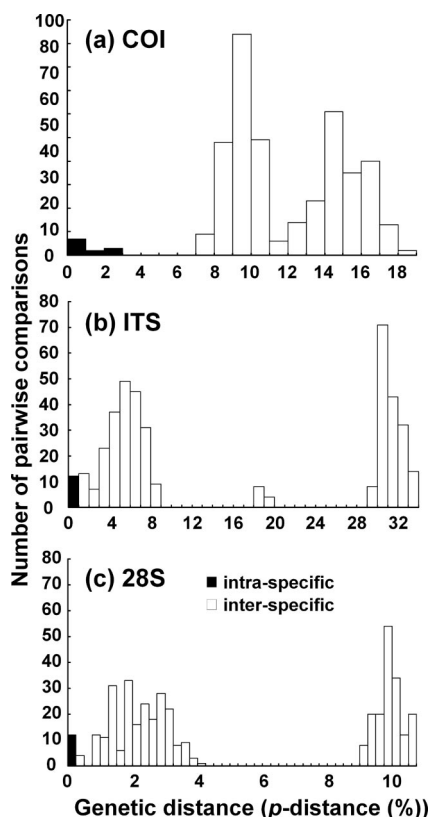


Fig. 2. Comparison of intra- and interspecific genetic distances (p -distances) in COI gene of mtDNA (a), ITS region of rDNA (b), 28S region of rDNA (c) among 17 species of the genus *Oligonychus*.

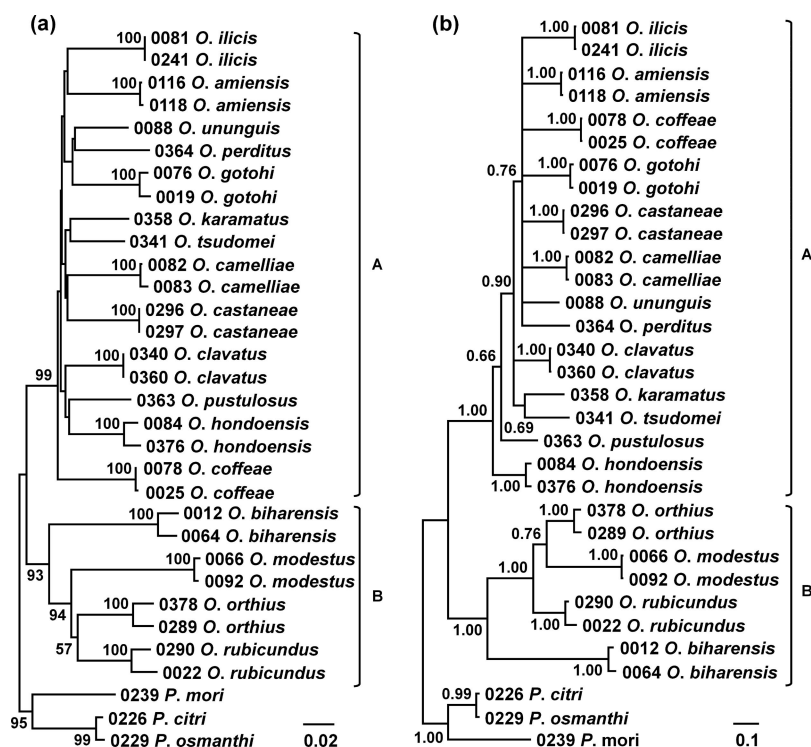


Fig. 3. NJ and Bayesian phylogenetic trees of the genus *Oligonychus* based on the mitochondrial COI gene. (a) NJ tree by using the Tamura and Nei (1993) model. (b) Bayesian tree using the GTR+I+G model. Bootstrap values based on 1,000 replications, and Bayesian posterior probabilities are indicated above nodes. Only bootstrap values >50% and Bayesian posterior probabilities >0.50 are shown. Each operational taxonomic unit is indicated by a voucher specimen number and species name. A, species whose aedeagi curve ventrally; and B, species whose aedeagi curve dorsally.

(transitional substitutions and transversional substitutions) by using PAUP* version 4.0b10 software (Swofford 2002). The genetic distance (p -distance) is obtained by dividing the number of nucleotide differences by the total number of nucleotides compared. The transversion/transition ratio was plotted against genetic distance (p -distances) using R software version 2.11.1 (R Development Core Team 2008) to assess substitution saturation. The transition/transversion ratio approaches one when the divergence between the two sequences is large and when substitution saturation occurs (Moritz et al. 1987, Purvis and Bromham 1997, Yang and Yoder 1999, Hinomoto and Takafuji 2004).

Phylogenetic trees were constructed by the neighbor-joining (NJ) method by using MEGA4 and Bayesian analysis by using MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003). For the NJ analysis, the Tamura and Nei (1993) model was used, and the robustness of the branches was tested by bootstrap analysis (Felsenstein 1985) with 1,000 replications. For the Bayesian analysis, we used the best-fit model (GTR+I+G model for COI, GTR+G model for ITS and GTR+I+G model for 28S) chosen by a hierarchical likelihood ratio test by using MrModeltest 2.1 (Nylander 2004). Markov chain Monte Carlo iterations were run under the selected model for 1,500,000 generations for COI; 6,000,000 generations for ITS; and 2,000,000 genera-

tions for 28S, keeping one tree every 100 generations. The first 25% of the trees were discarded as burn-in, and phylogenetic relationships were based on the remaining 75% of the trees. The consensus tree with posterior probabilities was constructed based on the trees sampled after the burn-in.

Results

The lengths of the COI, ITS, and 28S sequences that we obtained were 909, 393–422, and 794–834 bp, respectively. The COI sequences of the *Oligonychus* species contained no insertions or deletions, whereas the ITS and 28S sequences contained a number of insertions and deletions. After alignment, the COI fragment had 909 nucleotide sites, of which 329 were parsimony informative sites. Similarly, after alignment, the ITS fragment had 355 nucleotide sites (when gaps were treated using the complete deletion option in MEGA4), of which 168 were parsimony informative sites. The 28S fragment had 784 nucleotide sites, of which 128 were parsimony informative sites. The COI sequences of the *Oligonychus* species were extremely rich in A+T (76.0%), especially at the third codon position (92.7%). Similar high AT contents have been observed in previous studies of tetranychid mites (Navajas et al. 1996, Hinomoto and Takafuji 2001, Hinomoto et al. 2001, Ros and Breeuwer



Fig. 4. NJ tree of the genus *Oligonychus* based on the ITS region by using Tamura and Nei (1993) model. Bootstrap values based on 1,000 replications are indicated above nodes, whereas Bayesian posterior probabilities are given below the nodes. Only bootstrap values >50% and Bayesian posterior probabilities >0.50 are shown. Each operational taxonomic unit is indicated by a voucher specimen number and species name. A, species whose aedeagi curve ventrally; and B, species whose aedeagi curve dorsally.

2007, Ito and Fukuda 2009) and insects (Lunt et al. 1996, Lin and Danforth 2004). The AT contents of the ITS and 28S sequences (58.7 and 52.0%, respectively) showed little or no bias. The substitutions analyses showed that the COI gene and ITS region were saturated (Fig. 1a and b), especially at higher genetic distance. In contrast, the 28S region was not saturated even at higher genetic distance (Fig. 1c).

Comparison of the genetic distances of the COI gene used for species identification showed that intraspecific divergence (0–2.9%) of the genus *Oligonychus* (black bars in Fig. 2a) was lower than interspecific divergence (7.3–18.3%; white bars in Fig. 2a). Furthermore, the intraspecific divergence did not overlap with interspecific divergence, i.e., interspecific divergence was at least 4.3% higher than intraspecific divergence. Similarly, the genetic distances of the ITS region showed that intraspecific divergence (0–0.3%) was lower than interspecific divergence (1.6–33.5%; Fig. 2b), and the genetic distances of the 28S region showed that intraspecific divergence (0–0.1%) was lower than interspecific divergence (0.4–10.7%; Fig. 2c).

The trees obtained from the NJ and Bayesian analyses of the COI gene clearly show two clades (A and B) with high bootstrap values, comprised of species whose aedeagi curve ventrally and dorsally, respec-

tively (Fig. 3; bootstrap values >93%, posterior probabilities = 1.00). The NJ and Bayesian trees based on COI sequences showed the same topology with two differences: 1) *Oligonychus hondoensis* (Ehara) and *Oligonychus pustulosus* Ehara were positioned at the base of clade A in the Bayesian tree, but not in the NJ tree; and 2) *O. orthius* and *O. rubicundus* formed a sister group in the NJ tree, whereas *O. orthius* and *O. modestus* formed a sister group and *O. rubicundus* was positioned at the base of these two species in the Bayesian tree. However, the phylogenetic relationships among these three species were ambiguous, because the bootstrap value (57%) and the posterior probability (0.76) were relatively low. The topologies of the ITS and 28S trees obtained by the NJ and Bayesian analyses were almost the same, so only the NJ tree is shown for ITS (Fig. 4) and 28S (Fig. 5). In these trees (ITS and 28S), 17 *Oligonychus* species clearly separated into clades A and B with high bootstrap values, and were comprised of species whose aedeagi curved ventrally (A) and dorsally (B), respectively, as was observed in the COI trees (Fig. 3).

Among the clade B species (whose aedeagi curve dorsally), mono- or oligophagous species that inhabit gramineous plants (*O. orthius*, *O. modestus*, and *O. rubicundus*) and polyphagous species that inhabit trees (*O. biharensis*) were clearly separated into two

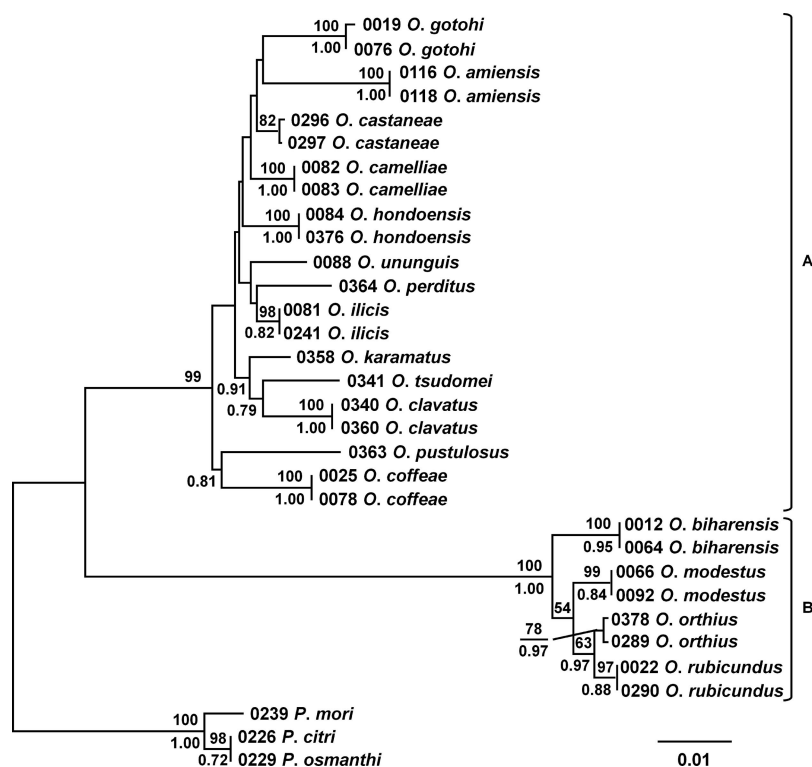


Fig. 5. NJ tree of the genus *Oligonychus* based on the 28S region by using Tamura and Nei (1993) model. Bootstrap values based on 1,000 replications are indicated above nodes, whereas Bayesian posterior probabilities are given below the nodes. Only bootstrap values >50% and Bayesian posterior probabilities >0.50 are shown. Each operational taxonomic unit is indicated by a voucher specimen number and species name. A, species whose aedeagi curve ventrally; and B, species whose aedeagi curve dorsally.

distinct subclades, which were supported by high bootstrap values and posterior probabilities in the COI, ITS, and 28S trees (Figs. 3, 4, and 5, respectively). The subclades consisting of polyphagous species were also well resolved in the COI and ITS trees, but not in the 28S tree, where the bootstrap value (54%) and posterior probability (<0.5) were low.

Discussion

Although DNA-based identification methods and phylogenetic analyses have been used for some agricultural pests, so far they have been used for the genus *Oligonychus* only as part of phylogenetic analyses of the family Tetranychidae (Navajas et al. 1996, Ben-David et al. 2007, Ros and Breeuwer 2007). Therefore, we examined the efficiency of DNA-based identification as well as their phylogenetic relationships.

Our DNA-based identification was effective for discriminating *Oligonychus* species because the ranges of intra- and interspecific divergences do not overlap (Fig. 2). Furthermore, the taxonomic status of each species was robustly supported because all 17 species analyzed in the current study were independently clustered in all three trees (Figs. 3–5), implying that DNA-based identification was valid in *Oligonychus* species, even for species that are difficult to distin-

guish by morphological traits such as *O. castaneae* and *O. coffeae* (Ehara and Gotoh 2007). In contrast, DNA-based methods for identifying species in other tetranychid genera have encountered some difficulties. For example, *Tetranychus urticae* Koch and *Tetranychus turkestanii* (Ugarov & Nikolskii) formed separate clades in an ITS tree, but the two species were not distinguishable in a COI tree (Navajas and Boursot 2003, Ben-David et al. 2007, Ros and Breeuwer 2007). Osakabe et al. (2008) were able to discriminate eight of 11 *Tetranychus* species by using restriction fragment length polymorphism after PCR of the ITS region but were unable to separate the other three species (*Tetranychus kanzawai* Kishida, *Tetranychus parakanzawai* Ehara, and *Tetranychus ezoensis* Ehara), which are morphologically close. In the genus *Panonychus*, the degree of intraspecific divergence of the COI gene among Japanese *P. mori* populations was higher than the degree of interspecific divergence between *P. citri* and *P. osmanthi* (Toda et al. 2000), i.e., the ranges of intra- and interspecific divergences overlapped. Thus, the COI sequences were unable to separate these *Panonychus* species, and DNA-based identification may be more effective for *Oligonychus* species than for other genera of Tetranychidae.

Our phylogenetic analyses (Figs. 3–5) well resolved the monophyly of *Oligonychus* species whose aedeagi

curve ventrally (clade A) and dorsally (clade B) with high support values (bootstrap values >93%, posterior probabilities = 1.00) except for the Bayesian trees based on the ITS and 28S regions (posterior probabilities were 0.61 and <0.50, respectively). Thus, the phylogenetic trees of the *Oligonychus* species coincided with their morphology based on the direction of curvature of the aedeagus. These results are in agreement with previous phylogenies based on COI (Navajas et al. 1996, Ros and Breeuwer 2007) and ITS (Ben-David et al. 2007) that separated *Oligonychus* species into two clades that coincided with the direction of curvature of the aedeagus.

The bootstrap values and posterior probabilities within clade A (Figs. 3–5) were too low to resolve the species in clade A. This could be caused by saturated sequence data owing to the application of an inappropriate gene region because transitional substitution in the COI gene (Fig. 1a) and ITS region (Fig. 1b) was saturated. Despite a lack of substitutional saturation in the 28S region (Fig. 1c), the 28S tree also poorly resolved the clade A species (Fig. 5), possibly because the divergence of the 28S sequences was lower than the divergences of the COI and ITS sequences. Thus, the low resolution of the phylogenetic relationships among species within the clade A could be caused by saturated sequence data (COI and ITS) or low sequence divergence (28S).

Another reason for the low resolution of the phylogenetic relationships in clade A may be a rapid speciation event within the clade. If this happened, the trees would show short internal branches and long terminal branches leading to each species, because the time between the respective speciation events was too short to accumulate substitutions (Barth et al. 2008). Such a scenario has been proposed to explain poorly resolved phylogenies in many groups of organisms, including aphids, black flies, bees, birds, turtles, mammals and higher plants (Whitfield and Lockhart 2007). Our phylogenetic trees (Figs. 3–5) showed short and conflicting internal branches and long terminal branches within clade A. The low resolution of clade A suggests that it experienced a rapid speciation event, but more sequence data are needed to test this idea. The finding that mono- or oligophagous species inhabiting gramineous plants (*O. orthius*, *O. modestus*, and *O. rubicundus*) were clearly separated from polyphagous species that inhabits various trees (*O. biharensis*) in all three trees (Figs. 3–5) suggests that the phylogenetic relationships of the species inhabiting gramineous plants could be affected by their close host association, as was shown to be the case for *Bryobia* mites (Ros et al. 2008).

The present results show that the COI, ITS, and 28S sequences are able to identify essentially 17 species of the genus *Oligonychus* and to determine their phylogenetic relationships. Several undescribed *Oligonychus* species remain throughout the world. Analyzing these species may help achieve a deeper understanding of the phylogenetic relationships among *Oligonychus* species.

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