Experimental and Applied Acarology (2005) **35:** 29–46
DOI: 10.1007/s10493-004-1953-0

Identification of astigmatid mites using the second internal transcribed spacer (ITS2) region and its application for phylogenetic study★

KOJI NOGE[1,*], NAOKI MORI[1], CHIHIRO TANAKA[2], RITSUO NISHIDA[2], MITSUYA TSUDA[2] and YASUMASA KUWAHARA[1]
*[1]Laboratory of Chemical Ecology, Division of Applied Life Sciences, Graduate School of Agriculture, Kyoto University, Sakyo-ku, Kyoto 606-8502, Japan; [2]Laboratory of Environmental Mycoscience, Division of Environmental Science and Technology, Graduate School of Agriculture, Kyoto University, Sakyo-ku, Kyoto 606-8502, Japan; *Author for correspondence (E-mail: noge@kais.kyoto-u.ac.jp; phone: +81-75-753-6307; fax: +81-75-753-6312)*

Received 20 August 2003; accepted in revised form 19 August 2004

Key words: Astigmata, Astigmatid mites, Identification of species, ITS2, Molecular phylogeny, Taxonomy

Abstract. The second internal transcribed spacer (ITS2) of nuclear ribosomal DNA from 73 specimens of Astigmata was analyzed by PCR amplification and DNA sequencing. The length of the ITS2 region varied from 282 to 592 bp. The interspecific variation based on consensus sequences was more than 4.1%, while the intraspecific or intra-individual variation was from 0 to 5.7%. The variation between geographically separated populations (0–3.2%) was almost the same as the variation within strains. The sequences of the ITS2 region of Astigmata were concluded to be species-specific. The phylogenetic tree inferred from the ITS2 region supported Zachvatkin's morphological classification in the subfamily Rhizoglyphinae. The species-specific ITS2 sequence is useful for the species identification of astigmatid mites and for studying low-level phylogenetic relationships.

Introduction

Astigmata, a suborder of Acari, consists of two cohorts, Psoroptidia and Acaridia. A total of 65 families are included in Astigmata (OConnor 1982). Most of them function as decomposers of organic residues and may cause no harm to humans and human environments directly, but some are known as noxious pests to agriculture and public health (Sasa 1965; Nakao and Kurosa 1988; Kuwahara et al. 1991).

Species identification of these mites is difficult because of their restricted morphological characters. The higher category of classification is still in a state of flux. Moreover, the following factors have made the taxonomy confusing: numerous synonymical names, few type specimens preserved, no figures available for descriptions, morphological polymorphism in adults, and

★Chemical Ecology of Astigmatid Mites LXXV

descriptions of only a hypopus available without the relationship to the corresponding adult being given.

These species secrete from their opisthonotal glands a mixture of the following classes of volatile compounds: normal-chain hydrocarbons ($C_{11} - C_{21}$), monoterpenes, aromatic compounds and other miscellaneous compounds. Parts of these compounds function as alarm, aggregation or sex pheromones (Kuwahara et al. 1975, 1982; Leal et al. 1989a). The profile of secretion has been found to be characteristic for each species, and provides a chemical key which can be used to confirm the identification of a given species based on conventional morphological characters (Leal et al. 1989b; Kuwahara 1999). However, these profiles are not always consistent in terms of quantity and quality due to variations among conspecific populations and analytical conditions (Sakata et al. 2003).

Recent developments in molecular biology enable us to understand the systematics and phylogenetic relationships of various organisms, by comparing nucleotide sequences of a certain ribosomal DNA (rDNA) region (Kuperus and Chapco 1994; Wada and Satoh 1994). In eucaryotes, the rDNA region consists of three highly conserved regions (18S rDNA, 5.8S rDNA and 28S rDNA) and two rapidly evolving regions, named the internal transcribed spacer regions (ITS1 and ITS2) (Hillis and Dixon 1991 and references therein). The latter regions have been used to study closely related species and to analyze low-level phylogenetic relationships (Porter and Collins 1991; Gotoh et al. 1998).

In order to obtain a new taxonomical key based on molecular biology, the sequence data of the ITS2 region of Astigmata were collected, and the feasibility of using the data for species identification was discussed referring to morphological taxonomy. Taxonomical relationships among groups of mites were also discussed, based on molecular phylogeny using the ITS2 region.

Materials and methods

Mites

The 73 species and/or strains used in this study are listed and encoded in Tables 1 and 2. The genus *Caloglyphus* was tentatively synonymized as the genus *Sancassania*. Several unidentified species were also examined in this study. From the morphological characters, each unidentified species could be identified at the genus level. For example, an unidentified species *Rhizoglyphus* sp. 1 (RH6) was characterized as belonging to the genus *Rhizoglyphus* based on e.g. the shape of seta *ba* (stout conical spine) on legs I and II. However, compared with the described species, morphological characters were somewhat different. Thus, these species were tentatively characterized as unidentified in the present study. The origins of unidentified species were appended in Tables 1 and 2. Strains from different localities were treated as distinct samples

Table 1. Sampled mites of the suborder Astigmata

Taxonomic status of species	Locality	Origin of unidentified species	Code	Length (bp)	GenBank Accession No.	Genetic distance
Superfamily Pyroglyphoidea						
Family Pyroglyphidae						
Genus *Dermatophagoides*						
D. farinae	Tokyo Pref., Japan		DM59	335	AB105001	0.000–0.003
D. pteronyssinus	Tokyo Pref., Japan		DM63	324	AB105008	0.032–0.041
				321	AB105009	
Superfamily Psoroptoidea						
Family Psoroptidae						
Genus *Psoroptes*						
P. caniculi	Tokyo Pref., Japan		PS74	330	AB105019	0.028–0.045
				329	AB105020	
				326	AB105021	
Superfamily Histiostomatoidea						
Family Histiostomatidae						
Genus *Histiostoma*						
H. laboratorium	Ibaraki Pref., Japan		HT57	302	AB104999	0.007–0.017
Superfamily Hemisarcoptoidea						
Family Carpoglyphidae						
Genus *Carpoglyphus*						
C. lactis	Tokyo Pref., Japan		CP65	592	AB105012	0.000
Family Chaetodactylidae						
Genus *Chaetodactylus*						
C. nipponicus	Tokyo Pref., Japan		CH69	514	AB105016	0.000–0.006
Genus *Sennertia*						
Sennertia sp.	Kyoto Pref., Japan	*Xylocopa appendiculata circumvolans**	SN61	427	AB105004	0.005–0.012
				426	AB105005	

Table 1. Continued

Taxonomic status of species	Locality	Origin of unidentified species	Code	Length (bp)	GenBank Accession No.	Genetic distance
Family Winterschmidtiidae						
Genus *Oulenzia*						
Oulenzia sp.	Philippines	Pesticide-free banana	OL50	289	AB104992	0.004–0.040
				282	AB104993	
Superfamily Glycyphagoidea						
Family Echimyopoidae						
Genus *Blomia*						
B. tropicalis	Okinawa Pref., Japan		BL60	326	AB105002	0.000–0.019
				324	AB105003	
Superfamily Acaroidea						
Family Suidasiidae						
Genus *Suidasia*						
S. medanensis	Ibaraki Pref., Japan		SD40	350	AB104974	0.009–0.024
				347	AB104975	
				346	AB104976	
Genus *Tortonia*						
Tortonia sp. 1	Tokyo Pref., Japan	Megachilid bee hive	TT48	402	AB104987	0.018–0.031
				405	AB104988	
Tortonia sp. 2	Aomori Pref., Japan	Megachilid bee hive	TT49	410	AB104989	0.002–0.022
				412	AB104990	
				411	AB104991	
Family Lardoglyphidae						
Genus *Lardoglyphus*						
L. kanoi	Tokyo Pref., Japan		LD64	437	AB105010	0.000–0.005
				436	AB105011	
Family Acaridae	See Table 2					

*Collected as a deutonymph.

Table 2. Sampled mites of the family Acaridae (Suborder Astigmata)

Taxonomic status of species	Locality	Origin of unidentified species	Code	Length (bp)	GenBank Accession No.	Genetic distance
Subfamily Rhizoglyphinae						
Genus *Cosmoglyphus*						
C. hughesi	Kyoto Pref., Japan		CS23	493	AB104958	0.000–0.002
	Mie Pref., Japan		CS39	493	AB104973	0.000–0.002
Cosmoglyphus sp. 1	Okinawa Pref., Japan	Decaying sweet potato	CS45	500	AB104983	0.000–0.004
Genus *Sancassania* (= *Caloglyphus*)						
S. rodriguezi	Ibaraki Pref., Japan		SS1	488	AB104923	0.000–0.006
				489	AB104924	
Sancassania sp. 1	Wakayama Pref., Japan	*Melolontha frater**	SS2	447	AB104925	0.005–0.009
				450	AB104926	
	Tokyo Pref., Japan	*Melolontha japonica**	SS24	449	AB104959	0.000–0.005
Sancassania sp. 2	Saitama Pref., Japan	*Heptophylla picea**	SS18	437	AB104949	0.000–0.005
				434	AB104950	
	Kouchi Pref., Japan	Organic soil	SS20	437	AB104952	0.000–0.007
				434	AB104953	
	Shiga Pref., Japan	Onion	SS27	434	AB104961	0.000–0.005
	Ibaraki Pref., Japan	Organic soil	SS35	434	AB104970	0.005–0.012
	Kyoto Pref., Japan	Organic soil	SS36	434	AB104971	0.000–0.005
	Hokkaido Pref., Japan	Organic soil	SS37	434	AB104972	0.000–0.005
Sancassania sp. 3	Okinawa Pref., Japan	Organic soil	SS21	490	AB104954	0.000–0.004
				500	AB104955	
				498	AB104956	
	Ibaraki Pref., Japan	Culture bed of beetle	SS28	498	AB104962	0.000–0.004
				496	AB104963	
				492	AB104964	
S. polyphillae	Ehima Pref., Japan		SS22	448	AB104957	0.000
Sancassania sp. 4	Kouchi Pref., Japan	Organic soil	SS31	440	AB104966	0.000–0.002
				442	AB104967	

Table 2. Continued

Taxonomic status of species	Locality	Origin of unidentified species	Code	Length (bp)	GenBank Accession No.	Genetic distance
S. aff. shanghaiensis	Hokkaido Pref., Japan		SS33	426	AB104968	0.000
	Kyoto Pref., Japan-A		SS67	433	AB105013	0.000
	Kagoshima Pref., Japan		SS76	433	AB105025	0.000
	Kyoto Pref., Japan-B		SS83	433	AB105028	0.000
S. shanghaiensis	Kyoto Pref., Japan		SS56	426	AB104998	0.000
S. spinitarsus	Fukushima Pref., Japan-A		SS43	441	AB104979	0.000
				447	AB104980	
	Fukushima Pref., Japan-B		SS68	447	AB105014	0.000
				441	AB105015	
	Yamanashi Pref., Japan		SS88	441	AB105032	0.000
				447	AB105033	
	Kyoto Pref., Japan		SS89	441	AB105034	0.000–0.002
Sancassania sp. 6	Kagoshima Pref., Japan	Organic soil	SS4	446	AB104929	0.007–0.009
				467	AB104930	
Genus *Histiogaster*						
Histiogaster sp. 1	Oita Pref., Japan	*Trichoderma harzianum*	HS9	416	AB104935	0.000–0.005
				415	AB104936	
	Nara Pref., Japan	Culture bed of beetle	HS10	421	AB104937	0.002–0.010
				417	AB104938	
Histiogaster sp. 2	Ibaraki Pref., Japan	Sap	HS11	469	AB104939	0.000
				474	AB104940	
H. rotundus	Akita Pref., Japan		HS72	436	AB105017	0.002–0.007
Subfamily *Rhizoglyphinae*						
Genus *Schwiebea*						
S. elongata	Ibaraki Pref., Japan		SC7	433	AB104933	0.000–0.009
				439	AB104934	
	Kyoto Pref., Japan		SC29	431	AB104965	0.000
Schwiebea sp. 1	Chiba Pref., Japan	A contaminant of RH3	SCl3	461	AB104942	0.000–0.002
Schwiebea sp. 2	Unknown	Unknown	SC15	450	AB104946	0.000

S. araujoae	Kyoto Pref., Japan		SCI6	524	AB104947	0.002–0.004
	Okinawa Pref., Japan		SCI7	524	AB104948	0.000
	Kouchi Pref., Japan		SC25	524	AB104960	0.000
S. similis	Hokkaido Pref., Japan		SC19	450	AB104951	0.000
Schwiebea sp. 4	Kyoto Pref., Japan	Organic soil	SC52	439	AB104995	0.000–0.002
				440	AB104996	
Schwiebea sp. 5	Kyoto Pref., Japan	A contaminant of SS83	SC84	441	AB105029	0.000
Genus *Rhizoglyphus*						
R. setosus	Thailand		RH3	448	AB104927	0.000–0.007
				449	AB105028	
Rhizoglyphus sp. 1	Okinawa Pref., Japan	Organic soil	RH6	460	AB104931	0.002–0.007
				461	AB104932	
	Kyoto Pref., Japan	Organic soil	RH73	460	AB105018	0.000
	Kyoto Pref., Japan	Organic soil	RH87	453	AB105031	0.000
R. robini	Mie Pref., Japan		RH12	467	AB104941	0.000–0.002
	Chiba Pref., Japan		RH51	467	AB104994	0.000–0.004
	Kyoto Pref., Japan		RH86	467	AB105030	0.000–0.004
Rhizoglyphus sp. 2	Hokkaido Pref., Japan	Organic soil	RH34	447	AB104969	0.000
Rhizoglyphus sp. 3	Okinawa Pref., Japan	Organic soil	RH79	406	AB105026	0.012–0.025
				407	AB105027	
Genus *Thyreophagus*						
Thyreophagus sp. 1	Kyoto Pref., Japan	Humus	TH14	334	AB104943	0.012–0.025
				338	AB104944	
				335	AB104945	
Thyreophagus sp. 2	China	Dried flower products	TH44	337	AB104981	0.009–0.037
				339	AB104982	
Subfamily Tyrophaginae						
Genus *Aleuroglyphus*						
A. ovatus	Tokyo Pref., Japan		AL47	422	AB104984	0.049–0.176
				432	AB104985	
				426	AB104986	

Table 2. Continued

Taxonomic status of species	Locality	Origin of unidentified species	Code	Length (bp)	GenBank Accession No.	Genetic distance
Genus *Tyrophagus*						
T. similis	Kyoto Pref., Japan		TY42	458	AB104978	0.000–0.002
T. longior	Taiwan		TY55	460	AB104997	0.000
T. putrescentiae	Kyoto Pref., Japan		TY41	488	AB104977	0.002–0.008
	Tokyo Pref., Japan–A		TY58	488	AB105000	0.004–0.006
	Tokyo Pref., Japan–B		TY107	488	AB105037	0.004–0.006
T. neiswanderi	Chiba Pref., Japan		TY90	486	AB105035	0.000–0.002
				490	AB105036	
Genus *Tyroborus*						
T. lini	Kagoshima Pref., Japan		TY75	422	AB105022	0.000–0.002
				422	AB105023	
				424	AB105024	
Subfamily Acarinae						
Genus *Acarus*						
A. immobilis	Tokyo Pref., Japan		AC62	514	AB105006	0.039–0.078
				515	AB105007	

*Collected as a deutonymph.

for discussing variation of the ITS2 region between strains of different geographic origins.

All species examined are maintained as propagative forms at the Laboratory of Chemical Ecology, Kyoto University. *Dermatophagoides* mites (DM59 and DM63) and *Lardoglyphus konoi* Sasa et Asanuma (LD64) were reared at around 70% RH on a mixture of dry yeast and dried fish meat (1:1). *Carpoglyphus lactis* Linnaeus (CP65) were maintained on a mixture of dry yeast and sugar (1:1). The following species were reared on dry yeast at around 70% RH: *Suidasia medanensis* Oudemans (SD40), *Aleuroglyphus ovatus* (Troupeau) (AL47) and genus *Tyrophagus* mites (TY41, 42, 55, 58, 90 and 107). *Histiostoma laboratorium* R. Hughes (HT57) were kept submerged. All of the other mites were reared on an agar medium composed of dry yeast and corn powder in a Petri dish (85 mm i.d., 20 mm ht.) (Kuwahara, unpublished).

DNA extraction, PCR and DNA sequencing

Genomic DNA was extracted from 1 to 20 fresh mite(s), depending upon their body size, according to the protocol described in Kuwahara et al. (1998). PCR was performed in a reaction mixture (50 μl) containing 1.25 units of KOD Dash (TOYOBO), 1 × KOD Dash buffer, 0.2 mM of dNTP and 10 pmol each of two oligonucleotide primers. The primers for amplification were designed in the well conserved 5.8S and 28S rRNA coding regions, based on the nucleotide sequence of mosquitoes (Diptera: Culicidae, Wesson et al. 1992) and modified for astigmatid mites. The sequence of the forward primer was 5′-CGACTTTC GAACGCATATTGC-3′, and the reverse was 5′-GCTTAAATTCAGG-GGGTAATCTCG-3′. Amplification was carried out with an initial denaturation step at 94 °C, 2 min followed by 25 cycles of (1) denaturation at 94 °C, 30 s (2) annealing at 56 °C, 30 s and (3) extension at 74 °C, 2 min. DNA sequencing of the ITS2 region was performed as previously described (Kuwahara et al. 1998). Three clones of the ITS2 region of all mites examined were sequenced.

Phylogenetic analysis

DNA sequences were aligned using the CLUSTAL W (v. 1.81) multiple alignment program (Thompson et al. 1994). The setting for this run was as follows: Fast pairwise alignment parameters (Gap penalty = 5, K-tuple size = 2, No. of top diagonals = 4, Window size = 4); Multiple alignment parameters (Gap open penalty = 15, Gap extension penalty = 6.66). The aligned sequences were checked manually. Phylogenetic trees were inferred using by neighbor-joining (NJ), maximum parsimony (MP) and maximum likelihood (ML) methods. The NJ method was performed using NEIGHBOR

in the PHYLIP ver 3.573c package (Felsenstein 1995). The distance matrix was calculated using DNADIST with Kimura's two-parameter method and the topology was tested with 1000 bootstrap trials (Felsenstein 1985) with the programs SEQBOOT and CONSENSE. MP analysis was done with the PAUP* program version 4.0bl0, written by David L. Swofford (2002). Gaps were treated as fifth base. Support for phylogeny derived from MP algorithm was measured by bootstrapping over 1000 replicates. ML analysis was also done with the PAUP* (version 4.0bl0). Heuristic likelihood searches under the K2P model were performed with all of the characters included. Bootstrap support values were obtained from 100 replicate re-sampled data sets for MP analysis. For MP and ML analyses, one of the sequences from three clones was chosen randomly as a representative sequence.

Results

Sequence analysis

PCR products containing the complete ITS2 region and portions of the flanking 3′ end of the 5.8S and the 5′ end of the 28S rRNA coding regions were successfully obtained from all species of Astigmata, and their DNA sequences were determined completely. The PCR amplification of some molds that were presumably originating from the surface of mites as contaminants was successfully excluded with our designed primers. All sequences exhibited homology to the published sequences containing partial sequences of the 5.8S and 28S rRNA genes and complete ITS2 region obtained from *Sarcoptes scabiei* (Acari: Sarcoptidae) (GenBank Accession No. AF387730) and *Psoroptes* sp. (Acari: Psoroptidae) (GenBank Accession No. AF123080) by BLAST Search on the web page of the DNA Data Bank of Japan (DDBJ, http://www.ddbj.nig.ac. jp/Welcome-j.html). The boundaries between the ITS2 region and the rRNA coding regions were estimated by comparing with the 5.8S and 28S rRNA genes of *Psoroptes* sp. (GenBank Accession No. AF123080). In Astigmata, both ends of the rRNA (5.8S and 28S rRNA) coding regions were well conserved, while the ITS2 region showed significant diversification among the species examined. The length of the ITS2 region containing partial sequences of 5.8S and 28S rRNA genes also varied from 282 to 592 bp as summarized in Tables 1 and 2. Three DNA sequences were collected for each species and/or strain of mite. No variation among these sequences was observed in the following 17 specimens: *Carpoglyphus lactis* (CP65), *Sancassania polyphyllae* (SS22), *S. aff. shanghaiensis* (SS33, SS67, SS76 and SS83), *S. shanghaiensis* (SS56), *Schwiebea elongata* (SC29), *Schwiebea* sp. 2 (SC15), *S. araujoae* (SC17 and SC25), *S. similis* (SC19), *Schwiebea* sp. 5 (SC84), *Rhizoglyphus* sp. 1 (RH73 and RH87), *Rhizoglyphus* sp. 2 (RH34) and *T. longior* (TY55). However, a few variations between clones were observed among 55 specimens. The differences of the length of the ITS2 region were

mainly due to insertions/deletions of single nucleotides at several sites. Microsatellite-like repeats also attributed to the length variation with two patterns observed, one consisted of short repeats, e.g., in the sequences of *Schwiebea similes* (SC7), $(TC)_n$ ($n = 2$ or 4) and $(GT)_{n'}$ ($n' = 1$ or 2) were observed (see GenBank Accession Nos. AB104933 and AB104934). In the ITS2 sequences of *Tyrophagus neiswanderi* (TY90), $(GCCT)_n$ ($n = 1$ or 2) was repeated in tandem (see GenBank Accession No. AB105035 and AB105036). These short repeats were observed in nine specimens [*Psoroptes caniculi* (PS74), *Suidasia medanensis* (SD40), *Sancassania* sp. 3 (SS21 and SS28), *Histiogaster* sp. 1 (HSIO), *H. rotundus* (HS72), *S. similis* (SC7), *Aleuroglyphus ovatus* (AL47) and *T. neiswanderi* (TY90)]. Half the number (five specimens) of these repeats was composed of TG or GT repeats. Another pattern was single nucleotide repetition (2–8). For example, in the sequences of *Sennertia* sp. (SN40), T was repeated seven or eight times in tandem. These repeats were observed in nine specimens [*Sennertia* sp. (SN61), *Tortonia* sp. 1 (TT48), *Tortonia* sp. 2 (TT49), *Sancassania rodriguezi* (SSl), *Sancassania* sp. 1 (SS2), *Sancassania* sp. 4 (SS31), *Sancassania* sp. 6 (SS4), *Histiogaster* sp. 1 (HS9) and *Schwiebea* sp. 4 (SC52)].

Intraspecific and interspecific variations

The variation within strain remained within the range of 0.2 to 3.7% except for AL47 (4.5 to 5.7%). Slight differences were also observed among strains from geographically separated locations, such as *Cosmoglyphus hughesi* (0–0.4%, CS23 and CS39), *Sancassania* sp.1 (0.7–1.8%, SS2 and SS24), *Sancassania* sp. 2 (0–0.7%, SS18, SS20, SS27, SS35, SS36 and SS37), *Sancassania* sp. 3 (0.2–2%, SS21 and SS28), *S. aff. shanghaiensis* (0–3.2%, SS33, SS67, SS76 and SS83), *S. spinitarsus* (0–1.6%, SS43, SS68, SS88, and SS89), *Histiogaster* sp. 1 (2.6–2.9%, HS9 and HS10), *Rhizoglyphus* sp. 1 (0–2.6%, RH6, RH73 and RH87), *R. robini* (0–0.6%, RH12, RH51 and RH86), *Schwiebea elongata* (1.8–2.7%, SC7 and SC29), *S. araujoae* (0–0.6%, SC16, SC17 and SC25) and *Tyrophagus putrescentiae* (0–0.8%, TY41, TY58 and TY107). The variation among strains was largely similar to those within strains. The minimum interspecific variation of ITS2 sequences among astigmatid mites was 4.1% [between *T. similis* (TY42) and *T. longior* (TY55)].

Genetic distances

Because of length variation of the ITS2 region among astigmatid mites, the percentage homology is not sufficient for the comparison between species. Thus, the genetic distances within and between strains and between species were considered to be more informative. The genetic distances (from minimum to maximum size) within strains are shown in Tables 1 and 2. The comparison of genetic distances within strains, between strains and between species in

Table 3. The comparison of genetic distances within strain, between strains, and between species of genus *Rhizoglyphus*

Species	Genetic distance		
	Within strain	Between strains	Between species[†]
RH3	0.000–0.007		0.187 (vs. RH6)
			0.135 (vs. RH12)
			0.139 (vs. RH34)
			0.177 (vs. RH79)
RH6	0.002–0.007	0.000–0.004 (vs. RH73)	0.140 (vs. RH12)
		0.011–0.016 (vs. RH87)	0.136 (vs. RH34)
		0.016 (RH73 vs. RH87)	0.155 (vs. RH79)
RH12	0.000–0.002	0.000–0.007 (vs. RH51)	0.023 (vs. RH34)
		0.000–0.007 (vs. RH86)	0.125 (vs. RH79)
		0.000–0.002 (RH51 vs. RH86)	
RH34	0.000		0.120 (vs. RH79)
RH79	0.012–0.015		

[†]Mean distances are shown between species.

Table 4. Mean of the genetic distances between congeneric species and among genera in the subfamily Rhizoglyphinae

Species	Mean of the genetic distance	
	Between congeneric species	Between genera
RH3	0.135 (vs. RH12)	0.391 (vs. HS11)
		0.294 (vs. SC7)
		0.393 (vs. CS23)
		0.348 (vs. SS1)
HS11	0.118 (vs. HS72)	0.459 (vs. SC7)
		0.501 (vs. CS23)
		0.536 (vs. SS1)
SC7	0.115 (vs. SC19)	0.337 (vs. CS23)
		0.407 (vs. SSl)
CS23	0.147 (vs. CS45)	0.330 (vs. SS1)
SSl	0.189 (vs. SS22)	
	0.530 (vs. SS4)	

genus *Rhizoglyphus* was summarized in Table 3 as a typical example. The distances within strains were almost the same as those between strains, e.g. RH12. For RH6, the distance within strain (0.002–0.007) was not almost the same as those between strains (0–0.016), but they were an order of magnitude smaller than those between species, e.g. 0.140 (RH6 vs. RH12). The genetic distances between genera were relative higher than those between congeneric species as summarized in Table 4. For example, the distance between *Rhizoglyphus* species was 0.135 (RH3 vs. RH12). On the other hand, between genera, mean distance was more than 0.294 (RH3 vs. SC7).

Phylogenetic analysis

The molecular phylogenetic tree of the suborder Astigmata was constructed by using all of the species examined. The bootstrap value of the clades consisting of closely related species was relatively high. However, the relationships between distantly related taxa were not determined due to very low bootstrap support (data not shown). This result suggested that the utility of the ITS2 region for phylogenetic study of astigmatid mites was limited to the relationships among lower taxa. Thus, the phylogeny of the subfamily Rhizoglyphinae was reconstructed using the ITS2 sequences. A total of 635 characters were used in the phylogenetic analysis. The molecular phylogenetic trees obtained from three different methods [NJ, MP, and ML methods] were congruent for the most part except for a few minor differences. The molecular phylogenetic tree of the subfamily Rhizoglyphinae inferred from the ITS2 sequences using the NJ method is shown in Figure 1. *T. putrescentiae* and *T. neiswanderi* (TY41 and TY90) were used as an outgroup. The bootstrap values obtained from the other two methods (MP and ML methods) were put in Figure 1. In the NJ tree, the subfamily Rhizoglyphinae was separated into three major groups. The first group (group 1) consisted of three genera [*Histiogaster* (HS9, HS10, HS11 and HS72), *Rhizoglyphus* (RH3, RH6, RH12, RH34, RH51, RH73, RH79, RH86 and RH87) and *Schwiebea* (SC7, SC13, SC15, SC16, SC17, SC19, SC25, SC29, SC52 and SC84)]. The second group (group 2) consisted of two genera: *Cosmoglyphus* (CS23, CS39 and CS45) and *Sancassania* (SS1, SS2, SS18, SS20, SS21, SS22, SS24, SS27, SS28, SS31, SS33, SS35, SS36, SS37, SS43, SS56, SS67, SS68, SS76, SS83, SS88 and SS89), except for *Sancassania* sp. 6 (SS4). The third group comprised *Sancassania* sp. 6 (SS4). The monophyly of group 2 (*Cosmoglyphus* and *Sancassania* species) was well supported with a bootstrap value of 81%. Within this group, the monophyly of congeneric species was highly supported by bootstrap value. On the other hand, the monophyly of group 1 (*Histiogaster, Rhizoglyphus* and *Schwiebea* species) was only weakly supported with a bootstrap value of 57%. However, the monophyly of two genera (*Histiogaster* and *Schwiebea*) was highly supported with bootstrap values of 100 and 99%, respectively, and that of the genus *Rhizoglyphus* was also well supported (86%). MP analysis indicated that 144 characters were constant and 457 characters were parsimony informative. The tree obtained from MP analysis by treating gaps as missing data was very similar to this (data not shown). The minor differences of tree topology obtained from MP and NJ analyses were the relationships among three genera (*Histiogaster, Rhizoglyphus* and *Schwiebea*) and the position of *Sancassania* sp. 6 (SS4). In the MP tree, two genera *Rhizoglyphus* and *Schwiebea* composed one clade within group 1, and the genus *Histiogaster* was the sister group of this clade. SS4 was a sister group of group 1. The monophyly of the clade consisting of *Cosmoglyphus* and *Sancassania* species (group 2) was well supported with bootstrap values of 87%, and that of the clade consisting of *Histiogaster*, *Rhizoglyphus* and *Schwiebea* species (group 1) was moderately supported with

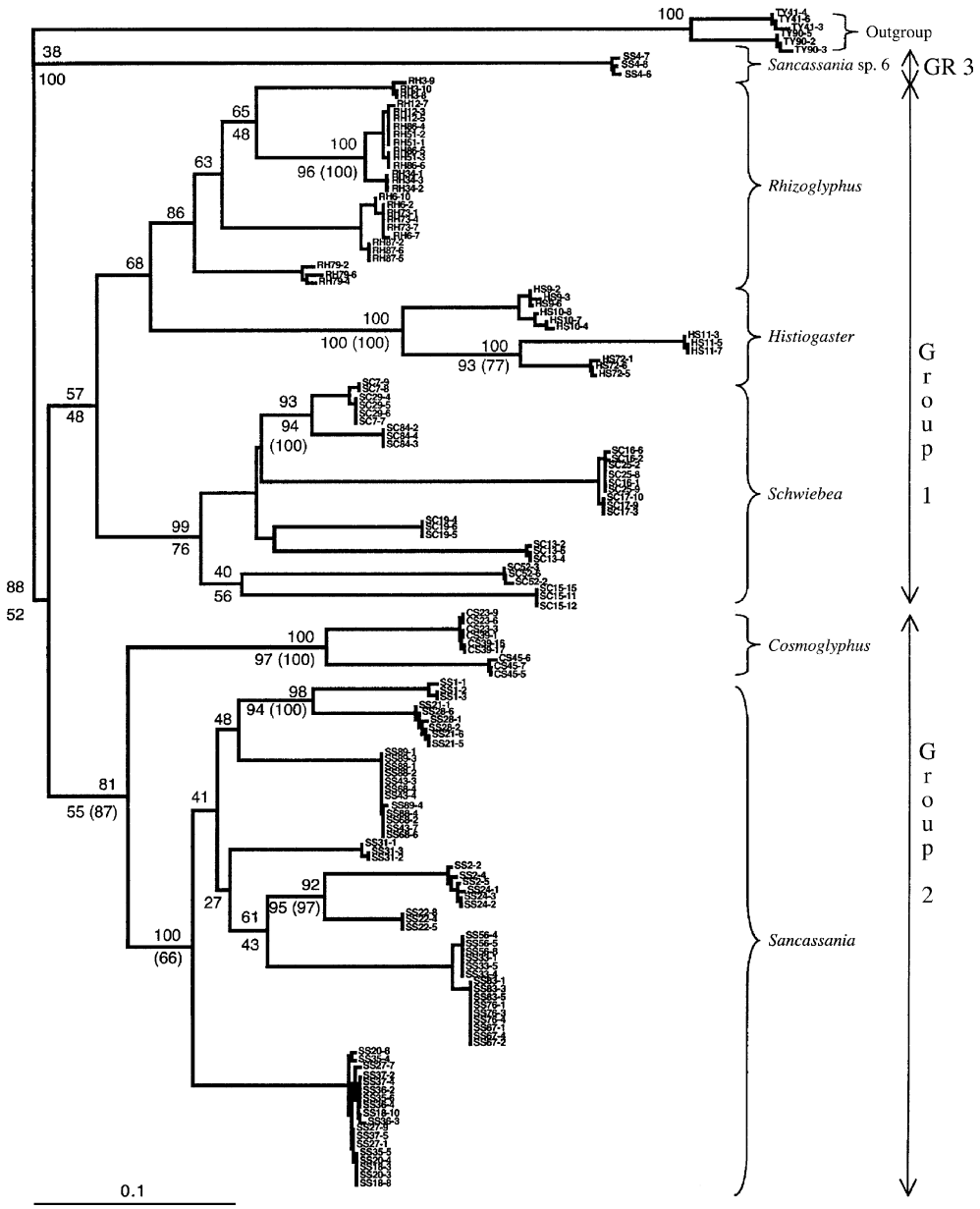

Figure 1. The molecular phylogenetic tree inferred from the ITS2 sequences of the subfamily Rhizoglyphinae by the NJ method. *T. putrescentiae* (TY41) and *T. neiswanderi* (TY90) were used as an outgroup. The scale of distances is shown under the tree. The tree separated into three major groups (group 1, 2 and SS4) corresponding to the morphological classification by Zachvatkin. Each species was clearly distinguishable. The numbers at nodes represent percentage of bootstrap confidence level obtained from three different methods (above: NJ method, below: ML method and in parentheses: MP method).

bootstrap values of 79%. The monophyly of two genera *Cosmoglyphus* and *Histiogaster* was supported by high bootstrap values (100%, each), and that of the genus *Rhizoglyphus* was also well supported (86%). The monophyly of the genus *Schwiebea* was moderately supported by a bootstrap value of 72%, and that of the genus *Sancassania* was only weakly supported (66%). The tree topology obtained from ML and NJ analyses was highly congruent except for the monophyly of the genus *Rhizoglyphus* under ML analysis. Within the ML tree, the genus *Rhizoglyphus* did not form a monophyletic group. Though the monophyly of two genera (*Cosmoglyphus* and *Histiogaster*) was supported by high bootstrap value (97 and 100%, respectively), the monophyly of group 1 (*Histiogaster*, *Rhizoglyphus* and *Schwiebea*), group 2 (*Cosmoglyphus* and *Sancassania*) and the genus *Sancassania* was only weakly supported by low bootstrap value.

Discussion

In the present study of Astigmata, the length of the ITS2 sequences varied considerably (282–592 bp). These results were consistent with the results of *Tetranychus* species (Navajas et al. 1998) and *Ixodes* species (Wesson et al. 1993). Almost no variation in the ITS2 region was detected within conspecific individuals and among geographical isolates, while the region demonstrated a relatively high level of variation among certain species. More than 95% of specimens examined exhibited less than 3% intraspecific variation based on consensus sequences. Although the variation ranged from 4.5 to 5.7% in the case of *Aleuroglyphus ovatus* (AL47), the values slightly higher than for other mites, there were no sequences similar to AL47 among all specimens examined. The minimum interspecific variation of ITS2 sequences among astigmatid mites was 4.1% [between *T. similis* (TY42) and *T. longior* (TY55)]. This was approximately the same as the highest intraspecific values of AL47, as mentioned. However, the sequences of TY42 and TY55 were obviously different and could be used to distinguish the species (see GenBank Accession No. AB104978 and AB104997). The interspecific variation between *Rhizoglyphus* sp. 2 (RH34) and *R. robini* (RH12, RH51 and RH86) was 6.4%, again nearly the same as the intraspecific variation in AL47. In this case, these two species were distinguished based on the length of their ITS2 region: 447 bp for RH34 and 467 bp for RH12, RH51 and RH86. However, more than 9.6% interspecific variation [between *Schwiebea* sp. 5 (SC84) and *S. elongata* (SC7 and SC29)] was observed among astigmatid mites. Our results are consistent with the report of other arthropod species, including mites.

In *Psoroptes*, two rDNA classes were found and the distribution of these classes correlated with neither the geographic origin of the mites nor the body sampling site of skin scrapings. The corresponding identities were 94.8% (class 1), 99.7% (class 2) and 96.1 % (between consensus sequences of the two rDNA class) (Zahler et al. 1998). In the mosquito *Aedes aegypti* and related species,

the intraspecific variation in the ITS2 region was not remarkable, and thus this region was reliable enough to be employed for predicting divergence among closely related mosquito populations (Wesson et al. 1992). Very little variation, 2–3%, was found among individuals and geographically distant conspecific individuals in some *Orius* species in ITS1 sequences (Honda et al. 1998). On the other hand, the intra-isolate genotypic variation with pairwise identities of 97.8% or higher were detected in the ITS2 sequences of *Sarcoptes* (Zahler et al. 1999) and *Otodectes* (Lohse et al. 2002). The intra-isolate variations of *Sarcoptes* and *Otodectes* were slightly lower than the intra-individual and/or intraspecific variations of Astigmata.

The homogeneity of the ITS2 sequences among Astigmata might be smaller than in other mite species (*Sarcoptes* and *Otodectes*). Intraspecific variation among the ITS2 sequences of *Aleuroglyphus ovatus* (AL47) was slightly higher among than those of other astigmatid mites and other mite species (*Sarcoptes* and *Otodectes*). This suggests the possibility that the ITS2 region of AL47 has not been homogenized completely within individuals and/or between individuals. Rich et al. (1997) revealed heterogeneity of the ITS2 region within individual deer ticks and recommended caution in utilizing highly variable portions of rDNA, such as the ITS regions. The different order of homogenization of the ITS2 region among Astigmata is interesting. The variation between geographically separated strains was almost the same as that within strains. Thus variation of the ITS2 region derived from differences in geographical origin does not to appear to exist among Astigmata included here. This suggests that the ITS2 region among Astigmata is well conserved within species. Based on these findings, we conclude that the ITS2 region of Astigmata is sufficiently conserved among conspecific individuals to be effective for species identification of Astigmata, though AL47 may require a little more attention.

The ITS2 region is known to be applicable to the phylogenetic analysis of closely related species (Hillis and Dixon 1991). The present results support this. The phylogeny, containing many taxa at different hierarchical levels, inferred by the ITS2 region was unreliable as determined by low bootstrap values. However, phylogenetic relationships between members of the Rhizoglyphinae, based on the ITS2 region were highly supported by bootstrapping. The family Acaridae is the largest among free-living mites and the most diverse group in the suborder Astigmata, consisting of over 79 genera. However the definition of this family is now ambiguous (OConnor 1982). The family is separated into two subfamilies: Acarinae and Rhizoglyphinae. According to Zachvatkin's classification (1941), the subfamily Rhizoglyphinae consists of two tribes: Acotyledonini and Rhizoglyphini, based on the presence or absence of the seta *Ve*. The former tribe comprises of the two genera (*Cosmoglyphus* and *Sancassania*) and the latter three genera (*Histiogaster*, *Rhizoglyphus* and *Schwiebea*).

Molecular phylogenetic trees obtained from three different methods [MP, ML, and NJ methods] were highly congruent. Using all three methods, the subfamily Rhizoglyphinae was composed of three clusters of congeneric

species; the first (group 1) was made up of *Histiogaster*, *Rhizoglyphus* and *Schwiebea*, corresponding to tribe Rhizoglyphini. The second cluster (group 2) consisted of *Cosmoglyphus* and *Sancassania*, corresponding to tribe Acotyledonini. The third consisted of only one species [*Sancassania* sp. 6 (SS4)]. SS4 was preliminary identified as one of the *Sancassania* species based on its morphology, but is excluded not only from the cluster comprising *Sancassania* and *Cosmoglyphus* species, but also from group 1 (*Histiogaster*, *Rhizoglyphus* and *Schwiebea*). Further study will be required to establish the taxonomic status of SS4.

Monophyly of group 2 (*Cosmoglyphus* and *Sancassania* species) was well supported by MP and NJ analyses, with bootstrap values of 87 and 81%, respectively. On the other hand, the monophyly of group 1 (*Histiogaster*, *Rhizoglyphus* and *Schwiebea* species) was suggested using all three methods and was reasonably supported by MP (79%). It was not supported under NJ (57%) and ML (48%). However, this work supports the hypothesis that the groups of the mites belonging to the tribe Rhizoglyphini are independent of the tribe Acotyledonini. A robust phylogeny comes from multiple characters, such as morphological characters, the information from various independent genes and, especially in Astigmata, chemical compounds from the opisthonotal glands. The present results reveal that the ITS2 region is useful for species identification and examining the taxonomical relationships in lower taxa of Astigmata.

References

Felsenstein J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39: 783–791.

Felsenstein J. 1995. PHYLIP (Phylogeny Inference Package) Version 3.57c. Department of Genetics, University of Washington, Seattle, USA.

Gotoh T., Gutierrez J. and Navajas M. 1998. Molecular comparison of the sibling species *Tetranychus puerariicola* Ehara et Gotoh and *T. urticae* Koch (Acari: Tetranychidae). Entomol. Sci. 1(1): 55–57.

Hillis D.M. and Dixon M.T. 1991. Ribosomal DNA: molecular evolution and phylogenetic inference. Quart. Rev. Biol. 66: 411–453.

Honda J.F., Nakashima Y., Yanase T., Kawarabata T. and Hirose Y. 1998. Use of internal transcribed spacer (ITS-1) region to infer *Orius* (Hemiptera: Anthocoridae) species phylogeny. Appl. Entomol. Zool. 33(4): 567–571.

Kuperus W.R. and Chapco W. 1994. Usefulness of internal transcribed spacer regions of ribosomal DNA in Melanopline (Orthoptera: Acrididae) systematics. Ann. Entomol. Soc. Am. 87(6): 751–754.

Kuwahara Y. 1999. Chemical ecology of astigmatid mites. In: Hidaka T., Matsumoto Y., Honda K., Honda H. and Tatsuki S. (eds.), Environmental Entomology. University of Tokyo Press, Tokyo (in Japanese), pp. 380–393.

Kuwahara Y., Ishii S. and Fukami H. 1975. Neryl formate: alarm pheromone of the cheese mite, *Tyrophagus putrescentiae* (Schrank) (Acarina, Acaridae). Experientia 31(10): 1115–1116.

Kuwahara Y., Mori N., Shimizu K., Tanaka C. and Tsuda M. 1998. Pheromone studies on astigmatid mites: Recent progress – A comparison of molecular phylogeny, distribution and function of female sex pheromone in *Caloglyphus* spp. (Acarina: Acaridae). J. Asia-Pacific Entomol. 1(1): 9–15.

Kuwahara Y., My-Yen L.T., Tominaga Y., Matsumoto K. and Wada Y. 1982. 1,3,5,7-Tetramethyldecyl formate, Lardolure: aggregation pheromone of the acarid mite, *Lardoglyphus konoi* (Sasa et Asanuma) (Acarina: Acaridae). Agric. Biol. Chem. 46(9): 2283–2291.

Kuwahara Y., Satou T. and Suzuki T. 1991. Chemical ecology on astigmatid mites. XXXI. Geranial as the alarm pheromone of *Histiostoma laboratorium* Hughes (Astigmata: Histiostomidae). Appl. Entomol. Zool. 26(4): 501–504.

Leal W.S., Kuwahara Y., Suzuki T. and Kurosa K. 1989a. β-Acaridial, the sex pheromone of the acarid mite *Caloglyphus polyphyllae*. Pheromone study of acarid mites XXI. Naturwissenschaften 76: 332–333.

Leal W.S., Kuwahara Y., Suzuki T. and Nakao H. 1989b. Chemical taxonomy of ecologically important *Tyrophagus* Mites (Acariformes, Acaridae). Agric. Biol Chem. 53(12): 3279–3284.

Lohse J., Rinder H., Gothe R. and Zahler M. 2002. Validity of species status of the parasitic mite *Otodectes cynotis*. Med. Vet. Ent. 16: 133–138.

Nakao H. and Kurosa K. 1988. Description of four species of acarid mites newly recorded from Japan, with reference to the damage caused to crops (Acari: Astigmata). Jpn. J. Appl. Ent. Zool. 32: 135–142. (In Japanese).

Navajas M., Lagnel J., Gutierrez J. and Boursot P. 1997. Species-wide homogeneity of nuclear ribosomal *ITS2* sequences in the spider mite *Tetranychus urticae* contrasts with extensive mitochondrial *COI* polymorphism. Heredity 80: 742–752.

OConnor B.M. 1982. Acari: Astigmata. In: Parker S.P. (ed.), Synopsis and Classification of Living Organisms, Vol. 2. McGrawHill, New York, pp. 146–169.

Porter C.H. and Collins F.H. 1991. Species-diagnostic differences in a ribosomal DNA internal transcribed spacer from the sibling species *Anopheles freeborni* and *Anopheles Hermsi* (Diptera: Culicidae). Am. J. Trop. Med. Hyg. 45(2): 271–279.

Rich S.M., Rosenthal B.M., Telford S.R.III, Spielman A., Hartl D.L. and Ayala F.J. 1997. Heterogeneity of the internal transcribed spacer (ITS-2) region within individual deer ticks. Insect Mol. Biol. 6(2): 123–129.

Sakata T., Shimano S. and Kuwahara Y. 2003. Chemical ecology of oribatid mites III. Chemical composition of oil gland exudates from two oribatid mites, *Trhypochthoniellus* sp. and *Trhypochthonius japonicus* (Acari: Trhypochthoniidae). Exp. Appl. Acarol. 29(3–4): 279–291.

Sasa M. 1965. Mites – An Introduction to Classification, Bionomics and Control of Acarina. University of Tokyo Press, Tokyo (In Japanese), pp. 368–382.

Swofford D.L. 2002. PAUP* 4.0 beta version: Phylogenetic Analysis and Using Parsimony (and Other Methods). Sinauer Associates Inc., Sunderland, Massachusetts, USA.

Thompson J.D., Higgins D.J. and Gibson T.J. 1994. Clustal W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positionspecific gap penalties and weightmatrix choice. Nucleic Acids Res. 22: 4673–4680.

Wada H. and Satoh N. 1994. Details of the evolutionary history from invertebrates to vertebrates, as deduced from the sequences of 18S rDNA. Proc. Natl. Acad. Sci. USA 91: 1801–1804.

Wesson D.M., McLain D.K., Oliver J.H., Piesman J. and Collins F.H. 1993. Investigation of the validity of species status of *Ixodes dammini* (Acari: Ixodidae) using rDNA. Proc. Natl. Acad. Sci. USA 90: 10221–10225.

Wesson D.M., Porter C.H. and Collins F.H. 1992. Sequence and secondary structure comparisons of ITS rDNA in mosquitoes (Diptera: Culicidae). Mol. Phylogenet. Evol. 1(4): 253–269.

Zachvatkin A.A. 1941. Tyroglyphoidea (Acari). Fauna of U. S. S. R. Arachnoidea. Vol. VI (1). English translation by A. Ratcliff and A.M. Hughes 1959, Amer. Inst. Biol. Sci., Washington, pp. 1–573.

Zahler M., Essig A., Gothe R. and Rinder H. 1998. Genetic evidence suggests that *Psoroptes* isolates of different phenotypes, hosts and geographic origins are conspecific. Int. J. Parasitol. 28: 1713–1719.

Zahler M., Essig A., Gothe R. and Rinder H. 1999. Molecular analyses suggest monospecificity of the genus *Sarcoptes* (Acari: Sarcoptidae). Int. J. Parasitol. 29: 759–766.