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Differentiation of Three Phenotypically Similar *Blattella* spp.: Analysis with Polymerase Chain Reaction-Restriction Fragment Length Polymorphism of Mitochondrial DNA

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ABSTRACT Differentiation of *Blattella asahinai* Mizukubo, *Blattella vaga* Hebard, and *Blattella germanica* (L.) was investigated using polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP). DNA fragments corresponding to mitochondrial CO1, CO1/TL2 (small and large fragments), and ITS2 regions were amplified using PCR. The universal primers used for amplifying mitochondrial CO1 and CO1/TL2 (small and large fragments) were CO1-J-1718/CO1-N-2191, CO1-J-2441/TL2-N-3014, and CO1-J-1751/TL2-N-3014. Amplification was observed with all primer combinations, but diagnostic patterns were found only with *AluI* digested amplicons of CO1 and CO1/TL2 (large fragments) regions. Both inter/intraspecific polymorphism was observed for the 517 bp CO1 region between German (A, B) and Asian (A, B, C) cockroach populations. Polymorphic form B of the Asian cockroach (374 and 179 bp fragments) was nearly identical to the field cockroach (370 and 178 bp fragments). The CO1 amplicon (517 bp) differentiated German and field cockroaches but not Asian and field cockroaches. The CO1/TL2 amplicons (1275 bp) digested with *AluI* clearly differentiated all three species. The Asian cockroach had a distinct 540 bp fragment, whereas the field cockroach had a 403 bp fragment and the German cockroach lacked both fragments. Intraspecific polymorphs for CO1/TL2 amplicons (1275 bp) were observed with the German cockroach only. Portions of the CO1 and TL2 genes were sequenced, revealing 76% identity among German, Asian, and field cockroaches. Sequence data also revealed that German and Asian cockroaches were more closely related to each other than to the field cockroach.

KEY WORDS *Blattella asahinai*, *Blattella germanica*, *Blattella vaga*, polymerase chain reaction–restriction fragment length polymorphism, mitochondrial DNA

IN NORTH AMERICA, three morphologically similar *Blattella* spp., namely the Asian cockroach, *B. asahinai* Mizukubo, the field cockroach, *B. vaga* Hebard, and the German cockroach, *B. germanica* (L.), are known to occur. They can be easily misidentified (Benson and Zungoli 1997). Flock (1941) reported morphological similarities between German and field cockroaches, and Roth (1986) and Hall (1987) noted that Asian and German cockroaches are difficult to differentiate from each other. Currently, morphological characters, such as size and shape of tergal glands (Roth 1986, Appel 1995) and the genital hook of males, are used to differentiate Asian, German, and field cockroaches (Atkinson et al. 1991, Appel 1995). Ross and Mullins (1988) differentiated first instars, large nymphs, and oothecae of German and Asian cockroaches using size and color patterns. Lawless (1999) described five additional characters (setae on mandibles, coloration of right tegmen, number of veins in the hind wing and size and shape of marginal bristles on cerci) to differentiate Asian and German cockroach. A major limitation for the use of morphological characters is the required expertise of a trained entomologist (Appel

1995, Lawless 1999). Moreover, the use of morphological characters for species identification causes difficulty when damaged specimens are used, a situation commonly encountered by pest control professionals.

Behavioral keys based on flight and response to light is another approach to differentiate German, Asian, and field cockroaches (Appel 1995). Generally, Asian cockroaches are strong fliers and are attracted to light (Benson and Zungoli 1997). These characters have led to the establishment of the Asian cockroach population in an area 900 km inland from Tampa, FL, the probable point of its entry into the United States (Roth 1986, Brenner et al. 1988). Thus, the spread of Asian cockroaches into areas already infested with the German cockroach creates identification and control problems for pest control professionals (Hall 1987). This is further complicated by the observation of Asian and German cockroaches inside homes (Brenner et al. 1988). No insecticide resistance in Asian cockroaches has been reported, whereas German cockroaches have developed resistance to various classes of insecticides (Hall 1987). German and Asian cockroaches can interbreed and produce viable offspring, which can make identification of hybrids extremely difficult and also increases the concern for resistance devel-

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Table 1. Primer combinations used in the amplification of 4 DNA fragments in Asian, field, and German cockroaches

Forward Primer	Sequence 5'-3'	Reverse Primer	Sequence 5'-3'
CI-J-1718 ^a	GGAGGATTTGAAAATTGATT AGTTCC	CI-N-2191 ^a	CCCGGTA AAAATTA AAAATATAA ACTTC
CI-J-2441 ^a	CCAACAGGAATTA AAAATTTT TAGATGATTAGC	TL2-N-3014 ^a	TCCAATGCACTAATCTGCCATA TTA
CI-J-1751 ^a	GGATCACCTGATATAGCATT CCC	TL2-N-3014 ^a	TCCAATGCACTAATCTGCCATA TTA
ITS2 ^b	ATATGCTTAAATTCAGCGGG	ITS2 ^b	GGGTCGATGAAGAACGCAGC

^a Simon et al. (1994).

^b Navajas et al. (1998).

opment in the Asian cockroach (Roth 1986, Ross 1992).

A third approach for differentiating cockroach species is cuticular hydrocarbon analysis (Carlson and Brenner 1988). These authors used the technique to differentiate Asian, field, and German cockroaches from one another and observed significant differences in hydrocarbon component patterns among males, females, and nymphs. Although analysis of cuticular hydrocarbons is useful for species discrimination, they vary in composition based on sex, age, population origin, environmental condition, and physiological state of a species (Hadley 1977, Blomquist et al. 1987, Carlson and Brenner 1988, Toolson et al. 1990, Golden et al. 1992).

Use of the PCR has revolutionized the field of molecular biology, and it is now possible to differentiate species using small amounts of DNA (Simon et al. 1994). Both nuclear and mitochondrial genes have proven to be useful for molecular diagnostics (Moritz et al. 1987, Kirby 1990). PCR-RFLP has been used for species discrimination of *Aedes* spp. (West et al. 1997) and *Anopheles* spp. (Beebe and Saul 1995) mosquitoes, whereas direct RFLP of mitochondrial DNA has been used for species discrimination of termites (Broughton 1995). Further, PCR amplified products were used for direct sequencing of previously unknown regions. Kambhampati (1995, 1996) used PCR products of mitochondrial ribosomal genes for sequence determination and to examine the phylogenetic relationship of cockroaches, termites and mantids.

The primary objectives of our research were to differentiate *B. asahinai*, *B. germanica*, and *B. vaga* based on mitochondrial DNA using PCR-RFLP analysis; and to sequence the amplified fragments of cytochrome oxidase I (COI)/tRNA-Leucine (TL2) gene. DNA sequencing was performed to determine the base sequence of the amplified fragment and to construct a dendrogram based on the sequence identity.

Materials and Methods

Test Insects and DNA Isolation. Adult males from three laboratory-cultured colonies of *B. germanica*, *B. asahinai*, and *B. vaga* were used. *B. germanica* ($n = 15$) was a laboratory colony from Lincoln, NE, and *B. asahinai* ($n = 15$) and *B. vaga* ($n = 7$) were laboratory

colonies from Auburn, AL. Voucher specimens are maintained at the Department of Entomology, University of Nebraska, Lincoln, NE.

The insects were frozen at -20°C for 2 d before DNA extraction. DNA was isolated from the thorax using a phenol-chloroform extraction method (Maniatis et al. 1982), as modified by Taylor et al. (1996). The extraction process included homogenizing a thorax in 200 μl of lysis buffer (100 mM NaCl, 100 mM EDTA, 100 mM of Tris, 0.5% SDS, pH 7.0) followed by incubation steps after addition of proteinase K (4 μl of 20 mg/ml) at 55°C for 3 h followed by RNAase (2 μl of 10 mg/ml) at 37°C for 20 min. DNA pellets were precipitated in 100% ethanol and stored overnight at -20°C . Finally, the DNA was washed in 70% ethanol, centrifuged, and vacuum dried. DNA was then resuspended in 100 μl of TE buffer (10 mM Tris, pH 8.0 and one mM EDTA) and stored at -20°C .

PCR Amplification. PCR amplification was done with a programmable thermal controller (PTC-100) (MJ Research, Waltham, MA); and a total of four mitochondrial and nuclear genes were amplified. We did not separate the mitochondrial and nuclear DNA. Instead the primers used in the amplification process are designed either to amplify the insect mtDNA (Simon et al. 1994) or nuclear rDNA (Navajas et al. 1998). The three amplified regions were as follows: (1) a central portion of the mitochondrial COI gene amplified using the primers CI-J-1718 and CI-N-2191; (2) two (large and small) portions of mitochondrial COI and an adjoining portion of the tRNA-Leucine (TL2) gene amplified using the primers CI-J-1751 and TL2-N-3014, and CI-J-2441 and TL2-N-3014, respectively; and (3) the nuclear ribosomal ITS2 region amplified using the primers listed by Navajas et al. (1998). The primer sequences are presented in Table 1. Composition of the reaction mixture (50 μl) used for amplification with the primers, CI-J-1718, CI-N-2191, CI-J-2441, and TL2-N-3014 was 28.5 μl water, 5 μl 10 \times PCR buffer II (PE Applied Biosystems, Branchburg, NJ), 800 μM dNTP mix (PE Applied Biosystems), 0.8 μM of each primer, 2.5 U AmpliTaq DNA polymerase (PE Applied Biosystems), 2 μl DNA, and 3 mM MgCl_2 . Amplification was performed using the following temperature profile: 94°C for 2 min, 35 cycles of 94°C for 45 s, 48°C for 1 min, and 72°C for 1.5 min. After a final extension step at 72°C for 5 min, PCR products were stored at 4°C .

Composition of the reaction mixture (50 μ l) used for amplification of the primers C1-J-1751 and TL2-N-3014 was 21.5 μ l water, 5 μ l 10 \times PCR buffer II, 1,200 μ M dNTP mix, 0.8 μ M of each primer, 2.5 U AmpliTaq DNA polymerase, 6 μ l DNA template, and 3.5 mM MgCl₂. Amplification was done using the following profile: 94°C for 2 min, 40 cycles of 94°C for 1 min, 52°C for 30 s, and 72°C for 1.5 min. After a final extension step at 72°C for 8 min, PCR products were stored at 4°C.

The reaction mixture (25 μ l) used for amplification of ITS2 primers was as follows: 12.75 μ l water, 2.5 μ l 10 \times PCR buffer II, 800 μ M dNTP mix, 0.8 μ M of each primer, 2.5 U AmpliTaq DNA polymerase, 3 μ l DNA template, and 2.5 mM MgCl₂. Amplification was done using the following profile: 94°C for 5 min, 35 cycles of 94°C for 1 min, 57°C for 30 s, and 72°C for 1.5 min. After a final extension step at 72°C for 7 min, PCR products were stored at 4°C. A negative control containing no DNA template was used for each amplification cycle.

Restriction Digestion. After amplification, samples were stored at 4°C until they were ready for digestion with restriction enzymes. For restriction digestion, the amplified samples were used directly with restriction enzymes and buffers. Four restriction enzymes, *Mfe*I, *Mse*I, *Alu*I, and *Msp*I, were used for the screening procedure. Restriction digestion with enzymes *Alu*I, *Mse*I, and *Msp*I was conducted using 1 \times Buffer 2 (50 mM NaCl, 10 mM Tris-HCl [pH 7.9], and 1 mM DDT [New England Biolabs, Beverly, MA,]), whereas 1 \times Buffer 4 (50 mM potassium acetate, 20 mM Tris acetate, 10 mM magnesium acetate, 1 mM DDT, pH 7.9 [New England Biolabs]) was used for *Mfe*I. The reaction mixtures (20 μ l) used for various restriction enzyme digestions were as follows: *Mfe*I digestion-4 units enzyme, 2.0 μ l 1 \times Buffer 4, and 8.0 μ l DNA template; *Mse*I digestion-4 units enzyme, 2.0 μ l 1 \times Buffer 2, 0.2 μ l BSA, and 8.0 μ l DNA template; *Msp*I digestion-4 units enzyme, 2.0 μ l 1 \times Buffer 2, and 8.0 μ l DNA template; and *Alu*I digestion-10 units enzyme, 2 μ l 1 \times Buffer 2, and 9.3 μ l DNA template. Digestion was conducted at 37°C for 16 h using a Perkin Elmer Cetus model 9600 thermocycler (Perkin Elmer, Branchburg, NJ) and the digested products were stored at 4°C until fractionation.

Electrophoresis. PCR amplification was confirmed by loading the PCR product onto a 1.0% agarose TBE (0.089 M Tris, 0.089 M boric acid, 0.5 M EDTA [pH 8.0]) gel containing ethidium bromide (0.6 mg/ml). Samples were electrophoresed at 80 V for 20 min and visualized using a UV illuminator. Restriction digests were separated on 9% polyacrylamide gel (PAGE) in a gel unit (16 by 20 cm by 1.5 mm) at 200 V for 4.5 h. After electrophoresis, restriction digestions were visualized with silver staining (Amersham Pharmacology Biotech, Piscataway, NJ) and scanned into a gel documentation program (Genomic Solutions, Ann Arbor, MI).

DNA Sequencing. PCR amplicons for all three species were amplified using the procedure described earlier and purified by Gene Clean II Spin Kit (Bio 101, La Jolla, CA) using the manufacture protocol. The

cleaned amplicons were sequenced directly using a LI-COR model 4000 DNA Sequencer (LI-COR, Lincoln, NE) at the DNA Sequencing Core Facility, University of Nebraska, Lincoln, NE. The sequences were aligned using PILEUP (Genetics Computer Group [GCG], Madison, WI). The final sequence reported here was determined by comparing the strands produced by both primers with each other, and the consensus sequences were compared with the sequence from the same regions of other known insect species. Further, this research was not focused on phylogenetic analysis of cockroach species. To illustrate similarity, Jukes-Cantor distances were calculated using the unweighted pair group method of arithmetic averages algorithm, with the resulting matrix data used to draw a similarity tree in the Growtree program (GCG).

Results

Amplification of mtDNA was observed with all primers in the initial screening of German and Asian cockroaches. For German and Asian cockroaches, the sizes of the amplified products were 517 bp for C1-J-1718/C1-N-2191 (Fig. 1), 550 bp for C1-J-2441/TL2-N-3014, and 579 bp for the ITS2 region. *Alu*I digestion of 517 bp CO1 amplicons showed both inter/intraspecific polymorphisms between German and Asian cockroaches. Two intraspecific polymorphisms were observed for the German, and three polymorphisms were observed for the Asian cockroaches. The same 517 bp CO1 amplicon digested with *Msp*I and *Mse*I showed no intra- or interspecific polymorphisms. Similarly, the 550 bp CO1/TL2 amplicon of German and Asian cockroaches showed no intra- or interspecific polymorphisms when digested with *Alu*I, *Msp*I, and *Mse*I. Two polymorphisms were observed for Asian cockroaches with *Alu*I, *Msp*I, and *Mse*I digests of ITS2 amplicon; however, one of the polymorphisms of Asian cockroaches was identical to the polymorphisms observed in German cockroaches. *Mfe*I digest of ITS2 amplicons also showed no intra- or interspecific polymorphisms between German and Asian cockroach.

Subsequently, the 517 bp CO1 amplicon of German, Asian and field cockroaches was digested with *Alu*I. Diagnostic interspecific polymorphism was observed with the German cockroach but there was no diagnostic interspecific polymorphism for Asian and field cockroaches. The band patterns observed for the German cockroach were clearly distinct from those of the Asian and field cockroach. Fragment sizes for all three species, including the polymorphic forms, based on the migration in polyacrylamide gel are presented in Table 2. Four of 15 German cockroaches showed polymorphic form A, whereas 11 specimens had polymorphic form B (Fig. 2). In the Asian cockroach, polymorphism A was observed only in one individual, and polymorphism B was seen in four individuals, and polymorphism C in 10 individuals. The polymorphic form B of the Asian cockroach closely resembles that of field cockroach with 4 bp separating the top band and 1 bp separating the bottom band. The fragment

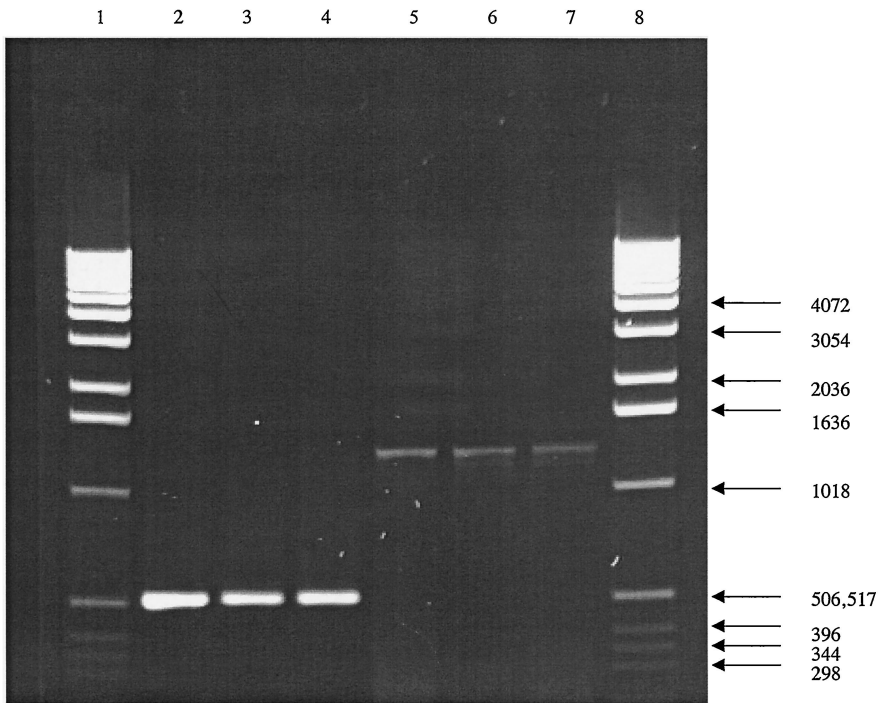


Fig. 1. Undigested amplicons visualized on 1% Agarose gel stained with ethidium bromide. Lanes 1 and 8: 1 kb markers. Lane 2: *B. germanica* (517 bp). Lane 3: *B. asahinai* (517 bp). Lane 4: *B. vaga* (517 bp). Lane 5: *B. germanica* (1275 bp). Lane 6: *B. asahinai* (1275 bp). Lane 7: *B. vaga* (1275 bp).

sizes were determined using the gel documentation program and overall eight gels were analyzed.

The 1275 bp COI/TL2 amplicon (Fig. 1) digested with *AluI* had distinct migration patterns that clearly differentiated all three *Blattella* species. Two intraspecific polymorphs (A and B) were observed with German cockroach (Fig. 2). Polymorph A was observed in four individuals, whereas polymorph B was observed in 11 individuals. No intraspecific polymorphism was observed with *AluI* digests of the COI/TL2 amplicon in both the Asian and field cockroach. Inter- and intra-specific fragment sizes for Asian, field and German cockroach are presented in Table 2.

Table 2. Primers, species and polymorphs identified by fragment size for the German, Asian and field cockroach using 517 bp and 1275 bp amplicons digested by restriction enzyme *AluI*

Primers combinations	Species-polymorphs	Fragment sizes, bp
CI-J-1718 and CI-N-2191	<i>Blattella germanica</i> -A	344, 126
CI-J-1718 and CI-N-2191	<i>Blattella germanica</i> -B	318, 125
CI-J-1718 and CI-N-2191	<i>Blattella asahinai</i> -A	374, 321, ^b 180
CI-J-1718 and CI-N-2191	<i>Blattella asahinai</i> -B	374, 179
CI-J-1718 and CI-N-2191	<i>Blattella asahinai</i> -C	315, 152
CI-J-1718 and CI-N-2191	<i>Blattella vaga</i>	370, 178
CI-J-1751 and TL-2-N-3014	<i>Blattella germanica</i> -A	462, 381 ^a
CI-J-1751 and TL-2-N-3014	<i>Blattella germanica</i> -B	471, 397, 374, 118 ^b
CI-J-1751 and TL-2-N-3014	<i>Blattella asahinai</i>	540, 374 ^a
CI-J-1751 and TL-2-N-3014	<i>Blattella vaga</i>	481, 403, 377, 236 ^b

^a Interpreted as double band.

^b Faint band detected due to silver staining or partial digestion.

The alignment of the COI/TL2 sequence for all three species is depicted in Fig. 3. There are 23 bases in the field cockroach and 64 bases in the Asian cockroach that lie before the sequences listed in Fig. 3. Our sequence information is available through the

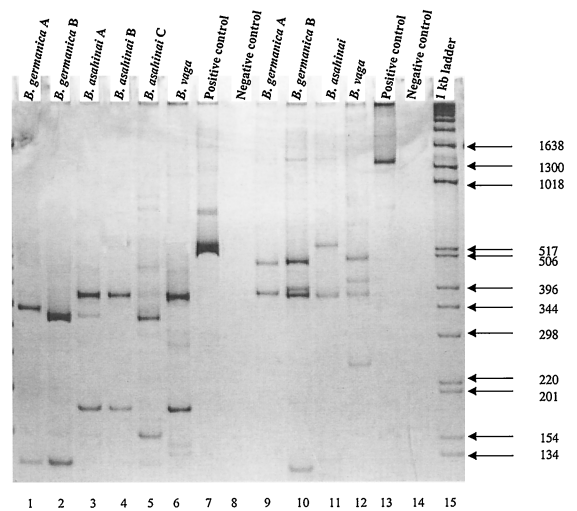


Fig. 2. *AluI* digests of *Blattella* spp. on 9% PAGE. Lanes 1-7: 517 bp COI amplicons. Lanes 9-13: 1275 bp COI/TL2 amplicons. Lanes 8 and 14: negative control. Lane 15: 1 kb marker.

1	TAAATAATAT	AAGTTTTTGA	CTTTTACCTC	CATCTCTATC	TCTTCTATTA	*G
1C...	..A.....A.	.T..AT....	A...T....G	*F
1C...	..A.....A.	.T..AT....	A...T....G	*A
51	GCTAGTAGCC	TTGTTGAAAG	AGGAGCTGGG	ACTGGTTGAA	CCGTATACCC	*G
51T.	.G.....	...T.....	..A.....	.T.....T..	*F
51T.	.G.....	..A.....	..T.....	.C.....C..	*A
101	TCCCTTAGCT	AGAGGAATTG	CTCATGCTGG	AGCTTCAGTA	GATTTAGCTA	*G
101	A..TC.....T....	T..A.....TA.	*F
101	T..CT.....A....	A..T.....AT.	*A
151	TTTCTCATT	ACATCTTGCA	GGTGTCTCAT	CAATTTTAGG	TGCCGTA AAT	*G
151	...T..C..	...T.A...	..A..T....T..T...	*F
151	...T..A..	...C.T...	..T..A....C..A...	*A
201	TTTATTTCAA	CAATTATTAA	CATGAAACCA	ATTAATATAA	GACCTGAACG	*G
201	T..A.....TC....	AA.....	*F
201	C..G.....AT....	GC.....	*A
251	AATTCCTTTA	TTGTTTGGT	CAGTAGGTAT	TACTGCATTA	TTATTATTAT	*G
251	G.....CC.TA..A.	.T.....	..A..T...	...C.T....	*F
251	A.....TT.AT..G.	.A.....	...T..A...	...T.A....	*A
301	TATCCTTACC	AGTTCTTGCA	GGTGCAATTA	CAATATTATT	AACAGACCGA	*G
301	...A.....	T..AT.A...	..G..T....T..T..T	*F
301	...C.....	A..TC.T...	..T..A....A..C..A	*A
351	AATTTAAATA	CTTCATTTTT	TGACCCTGCA	GGGGGAGGTG	ATCCTATTTTT	*G
351A.....CT.	...T.....T	..T..T....	...A.....	*F
351T.....TC.	...C.....A	N.G..A....	...T.....	*A
401	ATATCAACAT	TTATTTTGAT	TCTTTGGACA	TCCAGAAGTT	TATAT-TTTAA	*G
401C....	C.....T..	.T.CN....A	T...TC.....	*F
401TN...	T.....A..	.T.AN...T	C...CNNNN..	*A
451	TTTTACC-AGG	G-TTTGGGTAT	GA--TTTCTCAT	ATCATTTGCC	ATGAAAGAGG	*G
452C.G.	.A.TT.....	..AT.....	..TA..N.T.	..T..N....	*F
452NNT.	.-.CN.....	..TT...AT..	T.AC..C.T.	..G.NA....	*A
501	TAAAAA--GGAA	GC--TTTTGGAA	-ATTTAXGGAAXT	AAT-TTTTGCT	ATATTA-GCAA	*G
507	G.A.A.AG...N	G.CN.T.C....	A..NNCNGG..A.	...-CNT...CGC...	*F
506	T.N.N.--...A	A.CT.N.T....	N..TTACNN..T.	...ANCC...TCG...	*A
551	TTGG--T-TTATT	A-GGAT-TTGTT	G--TTTGAGCTC	A-TCATA-TAT-T	TAC-TGTA-GGA	*G
565	..G.GC.-.N..T	ANGAA.N...A.	GNTT.T...N.	AA..A.-...--	-.-NN..A-G..	*F
564	..T.GN.N.C..N	NTNGN.-...-	CGTN.A...C.	NT..T..T...CT	A.CTT..NTN..	*A
601	A-TA-GATGTGG	--ATACC-CGAGC	CTA--TTTFA-CT	TC-AGCTACTA	TAAT-TATTGC	*G
625	.-.-N.T..A.	--A...T-...G.	A..T-.T.-...-	..AG.....	...TA.....	*F
626	.T..GN.C..C.	GNT...NG...C.	C..TC..A..C..	..TA.....	...AN.....	*A

Fig. 3. DNA sequence of COI gene of *B. germanica*, *B. Vaga* and *B. asahinai*. Periods represent identical base; -, gap; and x indicates the end of the sequence. *G, German cockroach; *F, field cockroach; *A, Asian cockroach.

GENBANK assigned as accession numbers: AF228733 for the Asian cockroach, AF228734 for the German cockroach, and AF228735 for the field cockroach. There were no undetermined bases in German cock-

roaches but there were 17 and 59 undetermined bases in field and Asian cockroaches, respectively. Most undetermined bases were between the 400 and 800 bp regions. There was 76% identity among German, Asian

651	TGTACCCACT	G-GTATTAAAA	TTTTCAGTTG	ACTAGCTACC	TAT-ATATG-GA	*G
679A.CA	..A.....T...TG	..T..CAG-	..T-.....-	*F
686C.NT	.A.T.....C...GA	..N..NTA-	..NA.....NN.	*A
701	TCTCA-ATTAA	C-TTACAGAGC	ACC--TTG--TTTA	TGA-GCTCTAG	GATTT---GTCT-T	*G
730	...AA...A	.C.T-T.....	.C.--N..-G..T.	...TC.AC... ..T.T---.A.-.		*F
740	...NG...G	.T.NAC.....	.C.NTT..TN..A.	...ANNTN... ..A.NANG..C.N.		*A
751	C--TTATTTAC-A	GTAGG-AGGAT	TAA-CTGGA--GT	AGTT----CTTGCT	AATTCATC-AA	*G
785	.G--N..TTN.CT	...-.C...T	...-...GT--..T---A.AGCA	A...CATCA.T	*F
802	.GTTA..NNA.AA	...G.A...N	...N...NNAG..TCCNG.GNTA	G...GNNNC.A	*A
801	TTGATA-TTGT	T-CTTCATGAT	ACATATTATG	TAGTCGCCCA	TTTCCACTAT	*G
839	.T....A.T..	..T.A.....T..T..	C..T.....	*F
865	.A....N.G..	.GA.T.....T..C..	T..C.....	*A
851	GTACTATCTA	TAGGAGCAGT	ATTTGCAATC	ATAGCAGGGT	TTATTC AATG	*G
891	..AT....A.T..	...T.....TC..T.	*F
918	..GT....T.A..	...A.....CA..A.	*A
901	ATATCCATTA	TTTACTGGTT	TATCTTTAAA	TCCAAAGTGA	TTAAAAATTC	*G
942	A....T...A..A.	...A...C.	...T..A...	*F
969	G....A...T..T.	...T...A.	...A..G...	*A
951	-AATTTTCAAT	TATATTTTTA	GGAGTAAATT	TAACATTTTT	TCCTCAACAT	*G
993	-A...A...C	A.....	..T...A...	C.....	*F
1020	GN...T...T	T.....	..A...G...	T.....	*A
1001	TTTCTTGGTT	TAGCCGGAAT	GCCACGACGA	TATTCTGATT	ATCCTGACGC	*G
1044	...T.A..A.A.....	A..T..A..GA....	...C.GAT..	*F
1072	...C.T..T.C.....	G..A..G..AT....	...N.NNC..	*A
1051	TTATGCGAGCT	TGAAATGTTA	TTTCATCAAT	TGGATCAATA	ATTCATTTG	*G
1095	..T.A.T...T	.A..T.....T..C.	*F
1123	..A.G.A...A	.T..A.....A..T.	*A
1101	TAGCTGTATT	AATATTTATT	TTTATCATAT	GAGAAAGAAT	AACTACTAAC	*G
1146A...C.	T.....	..C..T....C..	.T.A..NN.T	*F
1174	...T...T.	A.....	..T..A....A..	.A.T..TA.T	*A
1151	CGACAAGTAT	TATTTCTTAC	TCAAACAAGA	AATTCTATTG	AATGATTTCA	*G
1197	..C....CTT..A..	A..AA.T...-X		*F
1225	..A....ACC..T..	T..CN.A...T....	*A
1201	AAATATTCCA	CCAGCTGAAC	ATAGTTATGC	TGAATTACCA	ACTATTTTCAT	*G
1276C...-	-.-..C..X		*A
1251	ATTAGATCCG	TT				*G

Fig. 3. Continued

and field cockroaches. Similarly, there were 79, 74, and 69% identities among *Drosophila yakuba* Burla and German, field, and Asian cockroaches, respectively (Clary and Wolstenholme 1985). In relation to the *D. yakuba* genome, the position of mtDNA sequences in the German cockroach corresponds to nucleotides 1757 through 3017, for the Asian cockroach it corresponds to nucleotides 1693 through 2988, and for the field cockroach it starts at 1735 and ends at 2941 (Clary

and Wolstenholme 1985). Further, the dentogram (Fig. 4), using sequence data from German, Asian, and field cockroaches, and the cricket *Gryllus pennsylvanicus* Burmeister, showed that the German cockroach is more closely related to the Asian cockroach than the field cockroach. The sequence data of the cricket (accession no. GPU88332) were used as an outgroup, and the sequence data of *B. germanica* are available as accession no. S72627.

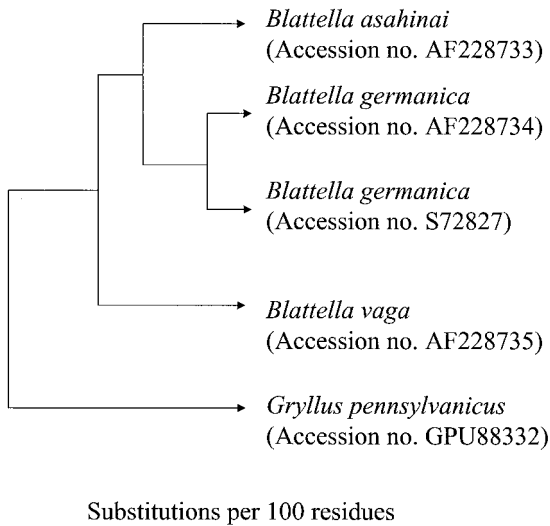


Fig. 4. Growtree dendrogram from the COI gene sequence of German, Asian, and field cockroach, and a cricket (outgroup) compiled by pileup program using the unweighted pair-group method with arithmetic average method.

Discussion

We demonstrated that PCR-RFLP could be used to differentiate three closely related *Blattella* spp. *AluI* digested amplicons (517 bp COI and 1275 bp COI/TL2) were useful in discriminating German, Asian and field cockroaches. However, the usefulness of COI digested amplicons (517 bp) is limited because of the intraspecific polymorphism and band separation by few base pairs. Scott et al. (1993) stated that the PCR fragments diagnostic for species must differ by at least 50 nucleotides for unambiguous discrimination. In contrast to the 517 bp amplicons, the 1275 bp COI/TL2 amplicons that was digested by *AluI* showed consistent banding patterns in all gels. Although intraspecific polymorphism was observed in the 1275 bp amplicons of German cockroaches, the banding pattern of the German cockroach was clearly distinct from that of the Asian and field cockroach. There was no intraspecific polymorphism in the 1275 bp amplicons of the Asian and field cockroach. Furthermore, the 540 bp fragment is found only in the Asian cockroach, whereas the 403 bp is found in the field cockroach. Roehrdanz et al. (1994) and Szalanski and Powers (1996) also reported similar results. In the study by Szalanski and Powers (1996), intraspecific polymorphism was observed for the northern corn rootworm ($n = 15$) but the bands were discrete enough for differentiation of three corn rootworm species. Based on our study, species discrimination of the German, Asian, and field cockroaches can be performed by amplifying the mtDNA with CI-J-1751 and TL-2-N-3014 primer combinations and digesting the amplicons with the restriction enzyme *AluI*. Furthermore, current technology (PCR and DNA sequencer) has led to rapid and efficient sequencing procedures (Hoy 1994,

Martinez-Gonzales and Hegardt 1994) where sequences are determined directly from the PCR products (Kambhampati 1995, 1996). We found 99.2% identity between the COI gene sequences of German cockroaches that were sequenced directly from the PCR product and the sequence obtained in another study where cloning procedures were used (Martinez-Gonzales and Hegardt 1994). Variation between the two sequence procedures was caused by transition at 10 locations (295, 367, 397, 451, 1107, 1251, 1327, 1415, 1418, and 1488) and insertion at two locations (between 738 and 739, and 961 and 962).

We also observed differences in total length of the amplicons (in both 517 bp and 1275 bp amplicons) between the undigested and digested fragments. Disparity in the total length of the fragment size ranging from -426 to $+980$ bp between the undigested and digested fragments has also been reported by other researchers (Roehrdanz et al. 1994, Roehrdanz et al. 1997, Taylor and Szalanski 1999, Taylor et al. 1997). Presence of the double bands could result in the estimated length of the digested amplicon being lower than the undigested amplicons (Roehrdanz 1994, Taylor and Szalanski 1999, Taylor et al. 1997). In our study, this was observed with digested amplicons (1275 bp) of *B. germanica*-A and *B. asahinai*. However, total length of the digested fragments being higher than the undigested amplicons could have resulted from both partial digestion and the sensitivity of the silver staining method (Taylor et al. 1997). Because silver staining is three times more sensitive than the ethidium bromide staining, it is possible to detect bands of low intensity with the silver staining method (Merril 1990) in comparison to with ethidium bromide method. In our study, this was observed with 517 bp amplicon of *B. asahinai*-A, 1275 bp amplicon of *B. germanica*-B, and 1275 bp amplicon of *B. vaga* (Taylor et al. 1997). Even with increased enzyme concentration, we observed similar banding patterns of digested fragments by the silver staining method. Moreover, small differences in the total length of the amplicons between undigested and digested fragment might be a result of the secondary structure of the double stranded DNA, which affects migration of DNA in PAGE (Taylor et al. 1996).

In addition to morphologically based taxonomy, the application of allozyme electrophoresis, PCR-RFLP, RAPD-PCR, and SSCP are molecular techniques that can be used for species identification as well as evolutionary or phylogenetic studies (Hoy 1994, Black and DuTeau 1997). Success of the PCR-RFLP technique is based on the presence of restriction sites in the amplified region of either genomic DNA or mtDNA using specific restriction enzymes (West et al. 1997). Publication of the primer list for amplifying insect mtDNA (Simon et al. 1994) has led to the efficient and reliable use of PCR-RFLP for species identification using mtDNA. Most of the universal primer combinations used here were successful in amplifying a portion of the mtDNA. In addition to universal primers, PCR-RFLP can also be used for discriminating species with primers that amplify the internal transcribed spacers (ITS) and intergenic

spacers (IGS) of ribosomal DNA (Scott et al. 1993, Beebe and Saul 1995, and West et al. 1997). But, RFLP analysis of ITS amplicons of three *Blattella* spp. did not result in positive species discrimination patterns in our study. Single-strand conformation polymorphism (SSCP) is another technique that can be used for species discrimination but it requires silver staining for visualizations of the bands. Although silver staining is elaborate and more expensive than ethidium bromide staining, SSCP is very sensitive to single base pair changes. SSCP can be more efficient and less time consuming than PCR-RFLP or RAPD-PCR but the sensitivity of the SSCP analysis decreases as the base-pair number increases (Hiss et al. 1994, Black and DuTeau 1997). West et al. (1997) used SSCP analysis for species discrimination but observed identical patterns and attributed the failure to detect differences to the size of amplicons (1 kb). We suggest that for amplicons with 450 bp or more, PCR-RFLP may be the most efficient procedure for species identification of cockroaches using universal primers.

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