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A Phylogenetic Study in Some Long-Horned Beetles (Coleoptera: Cerambycidae) Using Mitochondrial COI Gene and 16S rRNA Sequences

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Two regions of mtDNA genome, cytochrome oxidase subunit I (COI) and 16S ribosomal RNA (16S rRNA) genes, were sequenced for 15 species of the long-horned beetle belonging to four subfamilies and geographic samples of mulberry longicorn beetle, *Apriona germari*, from two localities in Korea. Ten samples of *A. germari* collected from Suwon and Busan revealed three COI haplotypes ranging in nucleotide divergence of 0.3% to 0.5%, and the two populations shared one common COI haplotype (80%). The sequence divergence among 15 species of the long-horned beetle was much higher in COI gene (12.3%~39.4%) than 16S rRNA gene (7.2% to 23.1), and the maximum value in the COI gene is exceptional compared with other relevant studies, including that of Coleoptera. The greatly increased divergence in the COI gene, in fact, was stemmed from a peculiar sequence of *Prionus insularis* belonging to Prioninae, divergence of which ranges from 31.2% to 39.3% from other species. We discussed possible reason of the divergence in this species. Due to the abnormality of COI gene divergence, decrease in phylogenetic signal was severe in COI nucleotide and, subsequently, the converted amino acid sequences, rendering us to put more confidence on the 16S rRNA gene data. Although the molecular phylogeny confidently supports the monophyletic origin of Lepturinae, the presence of discrepancy between molecular data and traditional taxonomic view also is a testable hyothesis. One such discrepancy includes taxonomic position of *Sophronica obrioides* and *Theophilea cylindricollis* belonging to Lamiinae.

Key words : Long-horned beetle, Mulberry longicorn beetle, MtDNA, COI gene, 16S rRNA gene, Phylogeny

Introduction

The Cerambycidae, commonly known as long-horned beetles and sometimes as longicornia is one of the largest groups in Coleoptera. The family have about 20,000 species throughout the world and about 300 species belonging to 43 tribes occur in Korea (The Entomological Society of Korea and Korean Society of Applied Entomology, 1994; Lee, 1987). The family is mainly tropical but has representatives throughout the world. Most species of the family are wood-borers, usually attacking dead or dying trees, although some of them are considered as serious pests in timber and wood products in the tropics (Lee, 1987; Daly *et al.*, 1998).

The species of Cerambicidae characteristically have very long antennae, often longer than the body, and variable in their length and size (*e.g.*, 85-120 mm in length in male *Callipogon relictus* and 6-8 mm in adult *Dinoptera minuta*, excluding the antennae). Another peculiar feature of the family is cylindrical, fleshy white, and legless larvae. Larvae generally bore into wood, though some are restricted to the softer roots and stems of herbaceous plants. In most species the larval stages last for several years (Crowson, 1981; The Entomological Society of Korea and Korean Society of Applied Entomology, 1994; Gillott, 1980).

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There is a substantial body of literature on the phylogenetic studies of insects using molecular markers (Ferraris and Palumb, 1996), and mitochondrial DNA has been subjected to a popular candidate to infer phylogeny among insect taxa (Avise, 1994; Funk *et al.*, 1995; Farrell, 1998; Kelly and Farrell, 1998). However, phylogenetic study on the Cerambicidae alone is not currently available as far as we know, on the basis of our extensive effort made in the GenBank and throughout reference search. One of the relevant study, which can be cited here is a phylogenetic study of the Coleoptera, which includes a few long-horned species in their analysis, using mitochondrial cytochrome oxidase I gene (COI) sequence data (Howland and Hewitt, 1995). In the analysis, they suggested that COI might be an informative gene at lower taxonomic levels, or in other insects, but improved resolution for the phylogeny will require a more highly conserved sequence. This is highly plausible in that the Coleoptera, including long-horned beetles, is an ancient group of insects, undergone considerable evolutionary time.

In this study, we analyzed 15 long-horned beetle species to construct phylogeny using two regions of mtDNA genome, mitochondrial cytochrome oxidase subunit I (COI) and 16S ribosomal RNA (16S rRNA) genes and discussed the utility of the two genes for the inference of Cerambycidae phylogeny. Additionally, we sequenced COI gene from a few individual mulberry longicorn beetles, *Apriona germari*, collected from two localities in Korea to test if any geographic variation exists among them. Although this species is known as a mulberry pest in eastern Asia including Korea (Hua, 1982; Lee, 1987; Yoon *et al.*, 1997), information on the molecular aspect, including molecular phylogenetic relationships among the species of the subfamily, Lamiinae, is not available at present.

Materials and Methods

Samples

Fifteen species of long-horned beetles and one false blister beetle, *Xanthochroa luteipennis*, which was utilized as an outgroup in the phylogenetic analysis, were collected from eight localities in Korea and one in Tsushima, Japan from 1997 to 2000. Collection information of the samples is listed in Fig. 1 and Table 1. *Psacothea hilaris* was kindly provided by a person at the Department of Sericulture and Entomology, NIAST, RDA in Korea. *Apriona germari* was collected from two regions, six samples in Suwon and four in Busan (Table 2). The collected samples were frozen at 70°C for molecular analysis after the measurement of a brief external morphology and taking of the picture.

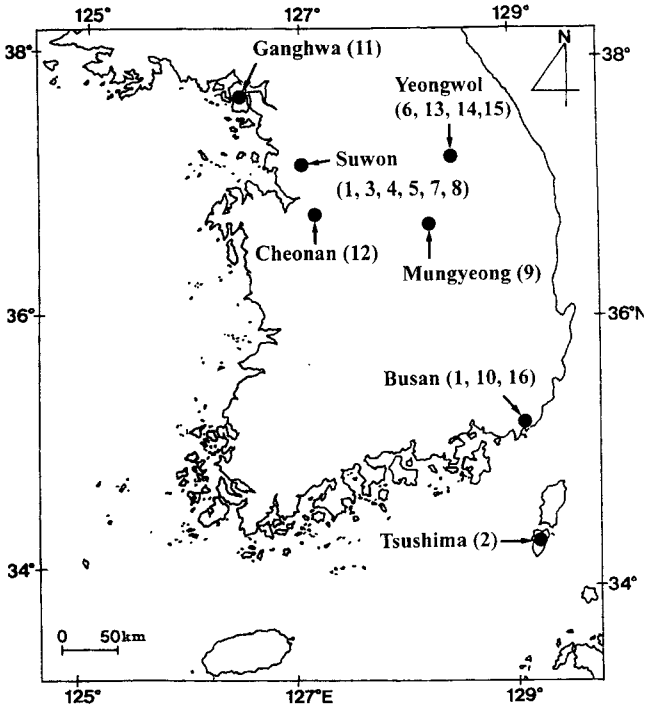

Fig. 1. Sampling locations of long-horned beetles and false blister beetle, *Xanthochroa luteipennis*. Numbers in parenthesis were introduced to indicate species collected at each locality as follows: 1, *Apriona germari*; 2, *Theophilea cylindricollis*; 3, *Anoplophora malasiaca*; 4, *Psacothea hilaris*; 5, *Moechotypa diphysis*; 6, *Thyestilla gerbleri*; 7, *Sophronica obrioides*; 8, *Massicus raddei*; 9, *Chlorophorus diadema*; 10, *Demonax transilis*; 11, *Plagionotus christophi*; 12, *Prionus insularis*; 13, *Anastrangalia sequensi*; 14, *Corymbia rubra*; 15, *Leptura aethiops*; 16, *X. luteipennis*.

MtDNA amplification and sequencing

Total DNA was extracted from the tissue samples by using Wizard Genomic DNA Purification Kit (Promega) following the manufacturers recommendation. The primers used for PCR amplification and sequencing of a portion of mitochondrial COI and 16S rRNA genes are described for their nucleotide sequences in Table 3. PCR amplifications were performed for 40 cycles under the following conditions: denaturation at 94°C for 30 s, annealing at 50°C for 40 s, and primer extension at 72°C for 45 s. To ascertain successful DNA replication, electrophoresis was carried out on 1% agarose gel. The PCR product was then purified with PCR purification Kit (QIAGEN) following manufacturers instruction. DNA sequencing was performed using ABI 377 Genetic Analyzer (PE Applied Biosystems). Sequ-

Table 1. Collecting information of each species and GenBank accession numbers of the mtDNA

Species	Collecting locality	Animal number	Collecting date	GenBank accession number	
				COI	16S rRNA
Family Cerambycidae					
Subfamily Lamiinae					
Apriona germari	Suwon-si, Gyeonggi-do	HA12	1999	AF332943	AF332927
Theophilea cylindricollis	Tsushima Island, Kyushu ; Japan	HA62	2000	AF332946	AF332930
Anoplophora malasiaca	Suwon-si, Gyeonggi-do	HA7	1999	AF332945	AF332929
Psacothea hilaris	NIAST, RDA, Suwon-si, Gyeonggi-do	HA15	1999	AF332944	AF332928
Moechotypa diphysis	Suwon-si, Gyeonggi-do	HA4	2000	AF332942	AF332926
Thyestilla gebleri	Yeongwol-gun, Gangwon-do	HA72	2000	AF332941	AF332925
Sophronica obrioides	Suwon-si, Gyeonggi-do	HA87	2000	AF332940	AF332924
Subfamily Cerambycinae					
Massicus raddei	Suwon-si, Gyeonggi-do	HA17	1999	AF332951	AF332935
Chlorophorus diadema	Mungyeong-si, Gyeongsangbuk-do	HA74	2000	AF332950	AF332934
Demonax transilis	Busan-si	HA58	2000	AF332949	AF332933
Plagionotus christophi	Ganghwa-gun, Gyeonggi-do	HA61	2000	AF332948	AF332932
Subfamily Prioninae					
Prionus insularis	Cheonan-si, Chungcheongnam-do	HA3	1998	AF332947	AF332931
Subfamily Lepturinae					
Anastrangalia seqensi	Yeongwol-gun, Gangwon-do	HA81	2000	AF332939	AF332923
Corymbia rubra	Yeongwol-gun, Gangwon-do	HA71	2000	AF332938	AF332922
Leptura aethiops	Yeongwol-gun, Gangwon-do	HA85	2000	AF332937	AF332921
Family Oedemeridae					
Xanthochroa luteipennis	Busan-si	HA88	2000	AF332936	AF332920

Table 2. A list of trapping localities, animal numbers, mitochondrial COI haplotypes, and GenBank accession numbers of *Apriona germari*

Collecting locality (no. of individuals)	Collection date	Animal number	COI haplotype	GenBank accession number
1. Suwon-si, Gyeonggi-do (6)	1999	HA12	AG1	AF332943
		HA11	AG2	AF335528
	2000	HA46	AG1	AF335529
		HA47	AG1	AF335530
		HA48	AG1	AF335531
		HA49	AG1	AF335532
2. Busan-si (4)	2000	HA41	AG1	AF335533
		HA42	AG1	AF335534
		HA43	AG3	AF335535
		HA45	AG1	AF335536

Table 3. Amplification and sequencing primers used in this study

Gene	Primer	Length (mer)	Sequence
COI			
Forward	CI-J-1718	23	5'-GGAGCTCCTGACATAGCATTCCC-3'
Reverse	CI-N-2191	26	3'-CCCGGTAAAATTAAAATATAAACTTC-3'
16S rRNA			
Forward	LR-J-12887	22	5'-CCGGTCTGAACTCAGATCACGT-3'
Reverse	LR-N-13398	20	5'-CGCCTGTTTATCAAAAACAT-3'

Note. Nomenclature of the primers follows the standard given by Simon *et al* (1994).

	30					60
***Apriona germari* (HA12)**	CGAATAAATA	ATATAAGATT	TTGATTGTTA	CCCCCATCAT	TAACTTTATT	AATTATAAGA
***Apriona germari* (HA11)**	.....	.....	.....	.....	.....	.....
***Apriona germari* (HA43)**	.....	.....	.....	.....	.....	.....
Theophilea cylindricollis	.....	.C.....	....C.T..G	.....T..CC	.....GC.	.....
Anoplophora malasiaca	.....	.C.....	.....AC.T	..A..T....	...TA...C.	.C.A.....
Psacothea hilaris	.....	....G.....	C...C.T...	C	.....C.TC.	G.....
Moechotypa diphysis	.....G..C.	.....	....C.T..G	.....T..TC	.TCT.C.C..	.....
Thyestilla gebleri	.....	.C.....	...G..A...	.....T..CC	...GAC.T..	.....
Sophronica obrioides	.....	.....	....C.T...	..A..T....	.....	.....
Massicus raddei	.....	.....	C...C.TC..	.....T...C	.....C.C..	.....G
Chlorophorus diadema	.....	.....G..	...GC.TC..	.....	...GGC..C.	.....
Demonax transilis	.....C.	.....	.....A...	..A..T..T.	..GGAC.C..	.....
Plagionotus christophi	.....C.	.....	...GC.T...	CC	.T...C.TC.	TC.A.....
Prionus insularis	A..T.....	....T..T..	C.....A...	..A..TAGT.	..TTA.....	.G.AT.CTCT
Anastrangalia seqensi	.....	.C.....	....C.T...	..A..T...C	....A.....	.....
Corymbia rubra	.....C.	.C.....	.....A..G	..A..T...C	.TT.C.....	.....
Leptura aethiops	.....	.C.....	.....A...	..A..T....	.G..AC.C..	.....
Xanthochroa luteipennis	.....	.....	...G..AC.T	..T.....TC	.....	.....

	90					120
***Apriona germari* (HA12)**	AGAATTGTAG	AAAATGGAGC	AGGAACTGGA	TGAACTGTTT	ACCCCCCTTT	AGCTGCTAAT
***Apriona germari* (HA11)**	.....	.....	.....	.....	.....	.....
***Apriona germari* (HA43)**	.....	.....	.....	.....	.....	.....
Theophilea cylindricollis	.....C....	....C..T..	.....A...	.....A....	.....CC.	TT.A..C...
Anoplophora malasiaca	.....	.T.GA..G..	.....A...	.....A....	.T..A..A..	.....
Psacothea hilaris	.....T.	.....	...G..A..T	.....A....	.....A..	.....
Moechotypa diphysis	.....	.....	T.....A..T	.....C..A.	....G..C..	.....A...
Thyestilla gebleri	.....C.	.T.....G..	.....A...	.....C....	.....A..	...A.....
Sophronica obrioides	.....	.....G..	.....G	.....A....	.T..T...C.	TT.A.....C
Massicus raddei	.....	...G...G..	T.....A...	.....A....	....T.....	C
Chlorophorus diadema	...G.A....	....C.....	T.....A...	.....	.....CC.	.T.CT.A...
Demonax transilis	.....T.	.....	C.....	.....A.	.T..A..AC.	.T.A..A...
Plagionotus christophi	.....	.....G..	C.....	....A....	....T...C.	TT.A..A...
Prionus insularis	GC.G..A...	..GG.....T	T..T..A...	.....AC...	....T.....	...C.GATTA
Anastrangalia seqensi	.....C....	...G.....	T.....A..T	..G..A..C.	....T.....	.T..T.....
Corymbia rubra	.....	...G...T..	T.....A...	.....A.	....A.....	.T.....
Leptura aethiops	...G.A..T.	...GA..C..	G.....A...	.....A....	....A.....	.T..T.....
Xanthochroa luteipennis	.....A....	.....	.....A...	.....A....	.T..A...C.	.T..T.....

	150					180
***Apriona germari* (HA12)**	GTAGCCCATA	GAGGTTCATC	CGTAGATTTA	GCAATTTTTA	GTCTTCATTT	AGCTGGAATT
***Apriona germari* (HA11)**	.....	.....	.....	.....	.....	.....
***Apriona germari* (HA43)**	.....	.....	.....	.....	.....	.....
Theophilea cylindricollis	.....C.	....A..T..	A.....C..	.....	..T.A...C.	...A..T...
Anoplophora malasiaca	..T..A....	.....T..	A..T.....	..T.....C.	.AT.A...C.	T.....
Psacothea hilaris	.....	.....G.T..	A.....C..	.....C.	.....	...A.....
Moechotypa diphysis	A.T.....C.	....CC.T..	T..G..C...	..T.....	..T.A...C.	...A..T...
Thyestilla gebleri	C.T.....	.....	T..T.....	..C.....	.A..A.....	C
Sophronica obrioides	A.T..T....	AT..C.....	A..T.....	.....	....A.....	...A..T...
Massicus raddei	A.C.....	....GG....	T.....C..	.....C.	.AT.A.....	...A..T...
Chlorophorus diadema	A.T.....	....A.....	A.....CC..	..C..C..C.	.AT.A..CC.	...A.....C
Demonax transilis	A.T.....	.T..AG....	T.....C...	.....	.A.....C..	...A.....
Plagionotus christophi	A.T..T....	....A..C..	T..T.....	..T.....C.	.A..A.....	...C..T...
Prionus insularis	CA.AGT..C.	.T..AC.TAG	T.....	.....G	C.T.A...C.	.T.A..GG.A
Anastrangalia seqensi	A.T..T...G	.T..A..T..	T.....	..T.....	.A.....	...A..T...
Corymbia rubra	A.T.....G	.T..A.....	T.....	..T.....	.A.....	...A.....C
Leptura aethiops	A.T..T..CG	.T..A..T..	A..T.....	..T.....	.A.....C.	...A.....
Xanthochroa luteipennis	A.T..T..C.	....A..C..	T.....C..	..T.....	.A.....C..	...A..T...

Fig. 2. COI nucleotide sequences (394 bases) of three *A. germari* haplotypes, 14 species of long-hornd beetles and one *X. luteipennis*. Only nucleotides that differ from *A. germari* (HA12) are indicated

ence alignment was performed using IBI MacVector (ver. 6. 5).

Phylogenetic analysis using PAUP and PHYLIP

PAUP (Phylogenetic Analysis using Parsimony) ver. 3.1

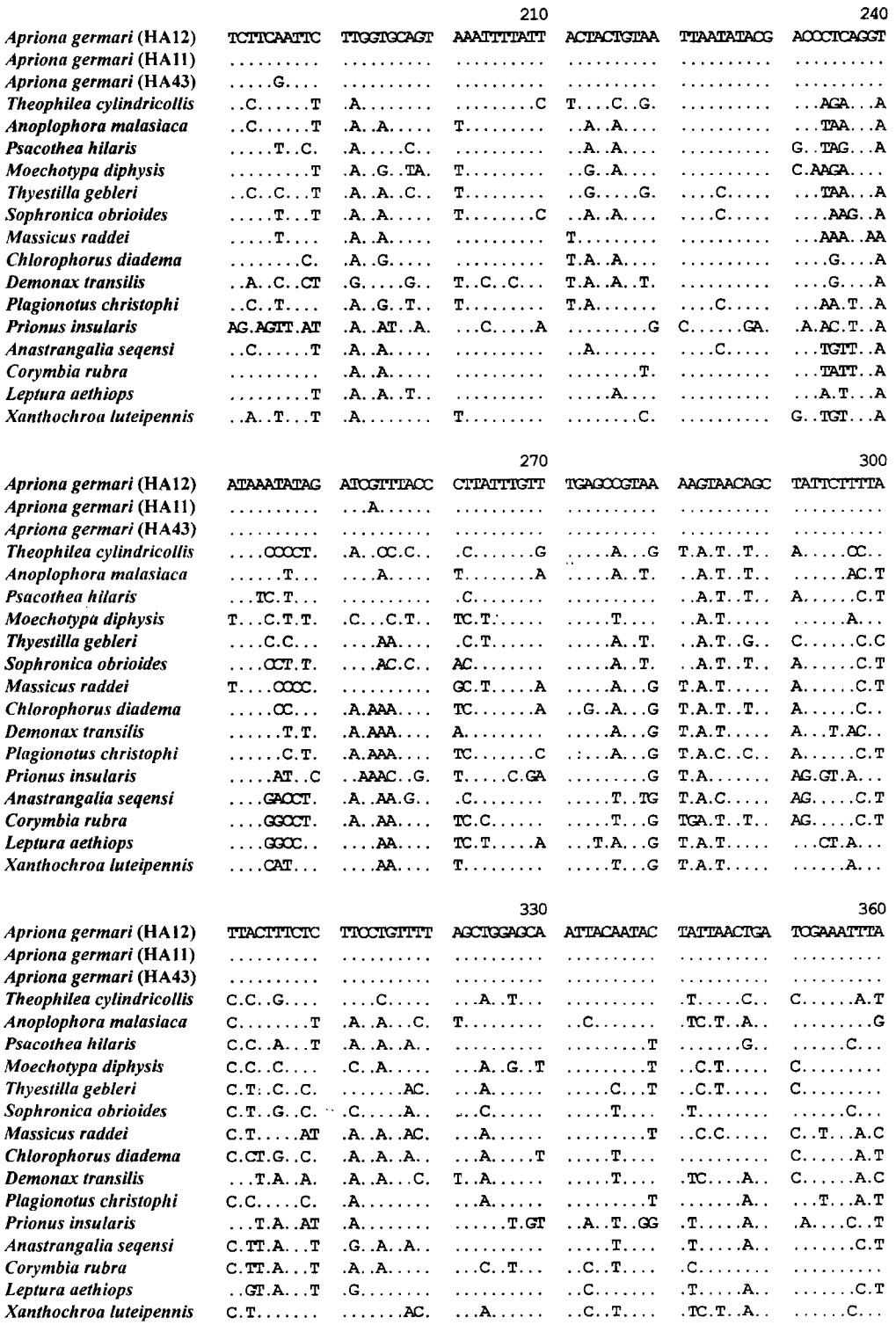

Fig. 2. Continued

(Swofford, 1990) was used to infer possible phylogenetic relationships among the matralines of the long-horned beetle sequences. *X. luteipennis* was used as an outgroup. PAUP analysis was performed using an equal weighting of transitions and transversions as well as several ratios up to and including 1:20. Also, the nucleotide sequences of COI gene were converted into amino acid sequence for phylogenetic analysis. For both cases, heuristic searches

			390	394
***Apriona germari* (HA12)**	AATACTTCTT	TTTTTGACCC	CGCAGGAGGA	GGAG
***Apriona germari* (HA11)**	.....	.....	.....	....
***Apriona germari* (HA43)**	.....	.....	.....	....
Theophilea cylindricollis	.....A..C.	.....	.....	....
Anoplophora malasiaca	.....A.	.C.....T..	A.....	..T.
Psacothea hilaris	.....A.	.....	G.....	....
Moechotypa diphysis	.....	.....T..	G..T.....	....
Thyestilla gebleri	.....A..A.	.....T..	A..C.....	....
Sophronica obrioides	.....	....C.....	A..T.....	....
Massicus raddei	.....A..C.	.C.....	T..G..G...	..T.
Chlorophorus diadema	..C.....A.	.....T..	A.....	....
Demonax transilis	.....A..A.	.C.....T..	T..T.....T	....
Plagionotus christophi	.....A..A.	.....T..	A..T.....	..T.
Prionus insularis	.....A.	.C....AGT	A..T..T...	..T.
Anastrangalia seqensi	.....	.C.....	A.....T	..G.
Corymbia rubra	.....	.....	TT.T..T..T	....
Leptura aethiops	.....	.C.....T..	A..T.....T	....
Xanthochroa luteipennis	.....C..A.	.....	A.....T	..G.

Fig. 2. Continued

were performed and reliability of the topology was tested by bootstrapping (1,000 iterations). As an alternative to the form of parsimony analysis, we used Neighbor-Joining method in PHYLIP (Phylogeny Inference Package) ver. 3.5c (Felsenstein, 1993). To obtain phylogenetic tree, the data set was first iterated 100 times using the subprogram SEQBOOT. Each iterated data set was run with the subprogram DNADIST or PROTDIST to obtain distance matrix between pairs of nucleotide or amino acid sequences. For nucleotide sequences Kimuras 2-parameter method was employed to obtain distance matrix (Kimura, 1980). Individual trees from each distance matrix were obtained using the subprogram NEIGHBOR. The homologous sequences of *X. luteipennis* were included in the analysis to root the trees. Finally, a consensus tree representing reliability at each branch in the tree was obtained using the subprogram CONSENSE.

Results

COI gene sequences

Fig. 2 shows the nucleotide sequences of 18 samples for a 394 bp region of the COI gene. No insertion or deletion was found is this region. These sequences were deposited in the GenBank, and accession numbers are shown in Table 1. The GC-contents for the COI gene of 16 samples are presented in Table 4.

Table 4. Sequence length of the mitochondrial COI genes and 16S rRNA genes analyzed and their GC-contents

Species	COI DNA sequence		16S rRNA sequence	
	Sequence length (bp)	GC-content (%)	Sequence length(bp)	GC-content (%)
Apriona germari (HA12)	394	33.0	507	26.2
Theophilea cylindricollis	394	40.6	504	28.8
Anoplophora malasiaca	394	32.7	508	25.6
Psacothea hilaris	394	36.8	507	26.4
Moechotypa diphysis	394	37.1	508	25.2
Thyestilla gebleri	394	38.3	507	27.6
Sophronica obrioides	394	35.3	505	26.3
Massicus raddei	394	37.6	510	32.2
Chlorophorus diadema	394	38.6	504	30.2
Demonax transilis	394	34.8	510	27.1
Plagionotus christophi	394	36.3	507	27.4
Prionus insularis	394	33.2	505	28.1
Anastrangalia seqensi	394	35.3	510	24.5
Corymbia rubra	394	34.8	510	23.9
Leptura aethiops	394	33.8	510	23.5
Xanthochroa luteipennis	394	34.0	502	23.9

	30					60
Apriona germari (HA12)	RMNNMSFWLL	PPSLITLLIMS	SIVENGAGTG	WTVYPPLAAN	VAHSGSSVDL	AIFSLHLAGI
Theophilea cylindricollis	....	....	....	.......S..	....	....
Anoplophora malasiaca	....	....M..L..	...DS.....	....	....	....
Psacothea hilaris	....	....	....	....	.....A....	....
Moechotypa diphysis	....	....L.....	....	....	I....P....	....
Thyestilla gebleri	....	....S.....	...D......	....	L.........	....
Sophronica obrioides	....	....	....	.......S..	I..N......	....
Massicus raddei	....	....	....S.....	....	I....A....	....
Chlorophorus diadema	....	....S.....	.V........	.......SS.	I.........	....
Demonax transilis	....	....G.....	....	.......S..	I....A....	....
Plagionotus christophi	....	.......L..	....	.......S..	I.........	....
Prionus insularis	SL..I.....	....L..VF.	AVM.G.V...	..L.....GL	QS...P....	...A...S.V
Anastrangalia seqensi	....	....	....S.....	.......SS.	I..G......	....
Corymbia rubra	....	....S.....	....S.....	.......S..	I..G......	....
Leptura aethiops	....	....	.V..S.....	.......SS.	I..G......	....
Xanthochroa luteipennis	....	....	.M........	.......SS.	I.........	....

	90					120
Apriona germari (HA12)	SSILGAVNFI	TTVINMRPSG	MNMDRLPLFV	WAVKVTAILL	LLSLPVLAGA	ITMLLTDRNL
Theophilea cylindricollis	....	S.......E.	.TPE......	...VI.....	....	I
Anoplophora malasiaca	....	........K.	..L.......	....I.....	....	....
Psacothea hilaris	....	....	.SL.......	....I.....	....	....
Moechotypa diphysis	......I...	.......QE.	LTF.......	....I.....	....	....
Thyestilla gebleri	....	........K.	.TL..M....	....I.....	....	....
Sophronica obrioides	....	........K.	.TF.......	....I.....	....	....
Massicus raddei	....	S.......KE	L.P.......	...VI.....	....	I
Chlorophorus diadema	....	S.......A.	..LEQM....	...VI.....	....	I
Demonax transilis	....	S.......A.	..FEQM....	...VI.....	....	I
Plagionotus christophi	....	S.......T.	..LEQM....	...VI.....	....	I
Prionus insularis	..L..SM..M	...A..STP.	.KLHK.A..G	...VM..V..	.........G	M..V...S.F
Anastrangalia seqensi	....	........V.	.SPE.M....	...VI..V..	....	....
Corymbia rubra	....	........I.	.SPE.M....	...VI..V..	....	....
Leptura aethiops	....	........L.	.SP..M....	.S.VI.....	....	....
Xanthochroa luteipennis	....	........V.	.TL..M....	...VI.....	....	....

	131
Apriona germari (HA12)	NTSFFDPAGG G
Theophilea cylindricollis	....
Anoplophora malasiaca	....
Psacothea hilaris	....
Moechotypa diphysis	....
Thyestilla gebleri	....
Sophronica obrioides	....
Massicus raddei	....
Chlorophorus diadema	....
Demonax transilis	....
Plagionotus christophi	....
Prionus insularis	.....EV... .
Anastrangalia seqensi	....
Corymbia rubra	.......S.. .
Leptura aethiops	....
Xanthochroa luteipennis	....

Fig. 3. COI amino acid sequences (131 amino acids) of 15 species of long-hornd beetles and one *X. luteipennis*. Only amino acids that differ from *A. germari* (HA12) are indicated.

In the ten samples of *A. germari* collected from Suwon and Busan, three haplotypes (AG1 AG3) were obtained from the nucleotide sequence of COI gene (Table 2). Sequence alignment revealed two variable nucleotides

Table 5. Pairwise comparisons of COI gene sequences among three haplotypes of *Apriona germari*, 14 species of long-horned beetles and *Xanthochroa luteipennis*

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1. *Apriona germari* (HA12)	-	0.003	0.003	0.188	0.183	0.137	0.198	0.178	0.173	0.195	0.203	0.211	0.208	0.335	0.198	0.188	0.193	0.173
2. *Apriona germari* (HA11)	1	-	0.005	0.190	0.185	0.140	0.201	0.180	0.175	0.198	0.201	0.208	0.206	0.332	0.201	0.190	0.195	0.175
3. *Apriona germari* (HA43)	1	2	-	0.190	0.185	0.137	0.201	0.178	0.173	0.195	0.206	0.211	0.208	0.335	0.201	0.190	0.195	0.173
4. *Theophilea cylindricollis*	74	75	75	-	0.218	0.190	0.201	0.206	0.178	0.190	0.175	0.228	0.188	0.371	0.190	0.208	0.211	0.198
5. *Anoplophora malasiaca*	72	73	73	86	-	0.188	0.223	0.168	0.190	0.203	0.218	0.216	0.208	0.338	0.198	0.211	0.175	0.180
6. *Psacothea hilaris*	54	55	54	75	74	-	0.201	0.183	0.178	0.175	0.193	0.234	0.201	0.363	0.198	0.211	0.226	0.198
7. *Moechotypa diphysis*	78	79	79	79	88	79	-	0.198	0.211	0.211	0.228	0.226	0.218	0.368	0.234	0.221	0.223	0.213
8. *Thyestilla gebleri*	70	71	70	81	66	72	78	-	0.175	0.190	0.208	0.208	0.180	0.393	0.208	0.206	0.218	0.185
9. *Sophronica obrioides*	68	69	68	70	75	70	83	69	-	0.198	0.208	0.213	0.175	0.353	0.165	0.190	0.211	0.183
10. *Massicus raddei*	77	78	77	75	80	69	83	75	78	-	0.203	0.226	0.180	0.355	0.195	0.198	0.221	0.211
11. *Chlorophorus diadema*	80	79	81	69	86	76	90	82	82	80	-	0.190	0.178	0.360	0.213	0.218	0.213	0.206
12. *Demonax transilis*	83	82	83	90	85	92	89	82	84	89	75	-	0.201	0.343	0.213	0.201	0.201	0.190
13. *Plagionotus christophi*	82	81	82	74	82	79	86	71	69	71	70	79	-	0.363	0.190	0.211	0.221	0.201
14. *Prionus insularis*	132	131	132	146	133	143	145	155	139	140	142	135	143	-	0.332	0.340	0.312	0.345
15. *Anastrangalis sequensi*	78	79	79	75	78	78	92	82	65	77	84	84	75	131	-	0.122	0.142	0.165
16. *Corymbia rubra*	74	75	75	82	83	83	87	81	75	78	86	79	83	134	48	-	0.157	0.188
17. *Leptura aethiops*	76	77	77	83	69	89	88	86	83	87	84	79	87	123	56	62	-	0.180
18. *Xanthochroa luteipennis*	68	69	68	78	71	78	84	73	72	83	81	75	79	136	65	74	71	-

Numbers above the diagonal are mean distance values; numbers below the diagonal are absolute distance values.

Table 6. Pairwise comparisons of amino acid sequences for the region of mitochondrial COI gene among 15 species of long-horned beetles and *Xanthochroa luteipennis*

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1. *Apriona germari* (HA12)	-	0.069	0.053	0.031	0.076	0.061	0.053	0.084	0.107	0.099	0.092	0.336	0.099	0.107	0.099	0.076
2. *Theophilea cylindricollis*	9	-	0.092	0.069	0.092	0.084	0.061	0.069	0.069	0.061	0.053	0.334	0.076	0.084	0.092	0.069
3. *Anoplophora malasiaca*	7	12	-	0.053	0.092	0.046	0.069	0.084	0.115	0.115	0.092	0.336	0.107	0.107	0.107	0.092
4. *Psacothea hilaris*	4	9	7	-	0.069	0.053	0.053	0.076	0.107	0.092	0.092	0.328	0.092	0.099	0.092	0.069
5. *Moechotypa diphysis*	10	12	12	9	-	0.076	0.061	0.092	0.130	0.107	0.122	0.328	0.122	0.122	0.122	0.092
6. *Thyestilla gebleri*	8	11	6	7	10	-	0.053	0.099	0.092	0.099	0.092	0.344	0.099	0.092	0.099	0.061
7. *Sophronica obrioides*	7	8	9	7	8	7	-	0.084	0.099	0.084	0.084	0.351	0.076	0.084	0.076	0.053
8. *Massicus raddei*	11	9	11	10	12	13	11	-	0.099	0.076	0.084	0.351	0.099	0.107	0.099	0.099
9. *Chlorophorus diadema*	14	9	15	14	17	12	13	13	-	0.038	0.038	0.336	0.084	0.092	0.084	0.061
10. *Demonax transilis*	13	8	15	12	14	13	11	10	5	-	0.038	0.351	0.092	0.092	0.107	0.084
11. *Plagionotus christophi*	12	7	12	12	16	12	11	11	5	5	-	0.344	0.084	0.092	0.099	0.069
12. *Prionus insularis*	44	45	44	43	43	45	46	46	44	46	45	-	0.344	0.351	0.351	0.336
13. *Anastrangalis sequensi*	13	10	14	12	16	13	10	13	11	12	11	45	-	0.031	0.038	0.053
14. *Corymbia rubra*	14	11	14	13	16	12	11	14	12	12	12	46	4	-	0.061	0.084
15. *Leptura aethiops*	13	12	14	12	16	13	10	13	11	14	13	46	5	8	-	0.053
16. *Xanthochroa luteipennis*	10	9	12	9	12	8	7	13	8	11	9	44	7	11	7	-

Numbers above the diagonal are mean distance values; numbers below the diagonal are absolute distance values.

(nucleotide positions 186 and 254, respectively), and both of them were transitionally substituted (A⇔G) (Fig. 2). In terms of variations at the codon position, nucleotide position 186 was the third place of a codon, and 254 was the second place of a codon, but these all designated identical amino acids, respectively (Fig. 3). The sequence divergence among three haplotypes in pairwise comparisons ranged from 0.3% (1 bp) to 0.5% (2 bp) (Table 5). Dis-

	30					60
Apriona germari (HA12)	GGCTTTTTGT	ATATAATTTA	AAGTCTGGCC	TGCCCACTGA	AGTTTTAAAG	-GGCCGCGGT
Theophilea cylindricollis	...C.C...A	T.....G	.G....A...	.....G....	TT.A.....T	.....
Anoplophora malasiaca	.....A	TA.....	.G.....A..	.....	G.AA..T..A	....T.....
Psacothea hilaris	...C.A...A	GA..G....T	.G.....	.....	TT....TG.A	T.....
Moechotypa diphysis	...C.....	TA.A.....	.G....A...	...T.G....	G.AG.....	.....
Thyestilla gebleri	.....A	TA.....	.G.....	.....	..A.....T	.....
Sophronica obrioides	...CG.....	TAT.T.....	.G....AA..	.....C....	GAA...T...	.....A.
Massicus raddei	...C.A...A	T...T....T	.G....T...	.....	GTAA.....	.....
Chlorophorus diadema	.T.C.....C	T...G.....	.G....A...	.....TA...	.T.-.....T	.....
Demonax transilis	.....C.A	G.....	.....AA..	.....TA...	.TA.....T	.....
Plagionotus christophi	...C.....A	.....	.G....AA..	...T.TA...	TT.A.....T	.....
Prionus insularis	...C.....A	T.....	.G....A...	.....	GTA.....T	.....
Anastrangalia seqensi	...C..AC..	TG.....	.G....A...	.....	.AA.....T	.....A..
Corymbia rubra	.....	T.....	.G....AA..	.....C....	.AA.....T	.....A..
Leptura aethiops	...C.....	T..A.....	.G....TA..	.....	.TAA.....T	.....A..
Xanthochroa luteipennis	.T.....A	T.....	.....A..	...T..A...	.AA..A-..T	.A.....A..

	90					120
Apriona germari (HA12)	AACTTGACCG	TGCTAAGGTA	GCATAATCAT	TAGTTTTTTA	ATTGAAAGCT	GGTATGAATG
Theophilea cylindricollis	.TT.....	...A.....	.....A..	.....	.....	..A.....
Anoplophora malasiaca	..A.....	.A.....	.....	.....	.A..G.....	..A.....
Psacothea hilaris	..T.....	.....	.....	.....	.....	.....
Moechotypa diphysis	.TT.....	.....	.....	.....C...	...AG.....	..A.....
Thyestilla gebleri	.TT.....	.....	.....	.....	.....	..A.....G.
Sophronica obrioides	.TT....T..	.....	.....A	.....C...	....G.....	..A.....
Massicus raddei	.T.....	...A.....	.....A..	.....C...	....G.....	..C.....C.
Chlorophorus diadema	.TT.....	...A.....	.....A..	.....C...	...AG.....	..A.....
Demonax transilis	.TT.....	...A.....	.....A..	.....CC...	...AGG....	..A.....
Plagionotus christophi	.T.C.....	...A.....	.....A..	.....	.....	..A.....
Prionus insularis	.CAC.....	...A.....	.....	.....	.....	..A.....A.
Anastrangalia seqensi	.TT.....T.	...A.....	.....	.....	.....	..A.....A.
Corymbia rubra	.TT.....T.	...A.....	.....	.....	...A.....	..A.....A.
Leptura aethiops	.TT.....T.	...A.....	.....	.....	.....	..A.....G.
Xanthochroa luteipennis	.TA.....T.	...A.....	.....	.....	.....	..A.....

	150					180
Apriona germari (HA12)	GTTTGATGAA	AAAATAACTG	TCTCTGATTT	ATTTTAATT-	GAATTTTATA	TTTAAGTAAA
Theophilea cylindricollis	.C.G.....G	GG...T....	.....TT..A	.A...TTA-.	.....T	....G.....
Anoplophora malasiaca	...G.....	.....	.....AT..A	.....A..	.....T	.....
Psacothea hilaris	...AA..A.G	.....	.....A...A	.....T....	.....T	.....
Moechotypa diphysis	...G.....	.....T....	.T..AA...A	...GAT....	T.....C	.....
Thyestilla gebleri	...G.....	....GT....	.....A.A..	....G..AA.	.....T	.....
Sophronica obrioides	...GA..A.G	......G...	......G...	...A....G.	-.....T	.....
Massicus raddei	..AG.....G	.G...G....	.....AG..A	.....	.....	.....G
Chlorophorus diadema	...GA.....	.....T....	.....TTC..	.G..ATTA-.	.....T	.....G
Demonax transilis	...GA....G	.....T....	...T.TT..A	.....TT...	.....T	.....G
Plagionotus christophi	...GA....G	.....G....	.....TT..A	......TA-.	.....T	...T.....G
Prionus insularis	.C.GA.CA.G	.....	.....AT..A	..A.CT.G-.	-.....T	.....
Anastrangalia seqensi	...GA....G	....A.....	.....AT..A	.A..ATTAAA	.....T	.....T..
Corymbia rubra	...GA....G	....ATT...	......T..A	....AT..AA	A.....T	.....T..
Leptura aethiops	...GA.....	....A.....	.....AT..A	....AT.AAA	T.....T	.....T..
Xanthochroa luteipennis	....A..A.G	....ATT...	....AAT...	.A.-.TT.G.	-.....T	.....T..

Fig. 4. 16S rRNA sequences (504-510 bases) of 15 species of long-hornd beetles and one *X. luteipennis*. Only nucleotides that differ from *A. germari* (HA12) are indicated.

tribution and frequency of haplotypes are listed in Table 2. Haplotype AG1 was found in both localities (eight samples), and AG2 and AG3, respectively, were found either in Suwon or Busan as a single individual.

```
                                                  210                                                 240
Apriona germari (HA12)      AAAGCTTAAA  TTTTTTTAAA  AGACGAGAAG  ACCCTATAGA  GTTTTATAAA  TTT-A-AATG
Theophilea cylindricollis   ..........  ....A.....  ..........  .....T....  .........TT  ...T.T...A
Anoplophora malasiaca       ........T.  ...A......  ..........  ..........  ..........  A...T...AT
Psacothea hilaris           .........T  .AA....G..  ..........  ..........  ....A.....  AA.GT.G.GT
Moechotypa diphysis         ..........  ..........  ..........  ..........  ..........  A.AG.T.TAA
Thyestilla gebleri          ..........  ..........  ..........  ..........  .........TG  AA.TGG.TAT
Sophronica obrioides        ..........  .A.AA.....  ..........  ..........  .........GT  ...A..TTAT
Massicus raddei             ..........  .AA.A.....  ..........  .....G....  A........T.  GAGTTGT..T
Chlorophorus diadema        ..........  .G..A.....  ..........  ..........  .........TT  .AAATT.G.T
Demonax transilis           ..........  ...AA.....  ..........  ..........  ........TTT  C..A.A.G.T
Plagionotus christophi      ......G...  .AA.A.....  ..........  ..........  ....G....T  ..AT.GTT.T
Prionus insularis           ..........  .G..A.....  ..........  ..........  ....A...TT  ...T.TTG.A
Anastrangalia seqensi       ..........  ..........  ..........  ..........  ..........  A..T.....A
Corymbia rubra              ..........  ..........  ..........  ..........  ....A...T.  A.AT...TAA
Leptura aethiops            ..........  ..G.......  ..........  ..........  ....A.....  A..T...T.A
Xanthochroa luteipennis     ..........  ......A...  ..........  ..........  ..........  .A.A-.TTAT

                                                  270                                                 300
Apriona germari (HA12)      TTAGTACTTT  TAGGATTTTT  ACTTTTAAAA  -TTTAAA-TT  TATTTGATTG  GGGTGATTGA
Theophilea cylindricollis   ..TT.TT..-  ........--A  TT..G...T.  T.G....A..  ......G...  ...C......
Anoplophora malasiaca       ..T...T...  .......A..  .T...AT..T  ...AG.T...  ..........  ...C.....G
Psacothea hilaris           ..TAA.T...  ..........  .A...A...T  .AAA.TT...  ..........  ..........
Moechotypa diphysis         ...AA.T...  ...A.A.A..  .AA.....TT  ....A..T...  ..........  ........A.
Thyestilla gebleri          ...TAGT.--  -...TA....  TAAG.A..TG  T..ATTT...  ......G...  ......C...
Sophronica obrioides        ..TT.TT...  ...T.....A  .AA....T.T  T.AA.--.AG  ......G...  ........A.
Massicus raddei             AATC.T.C.A  ...T......  .G..GGTT..  TGGA.TTTAG  ......G...  .......C..
Chlorophorus diadema        .GT.GTT..-  .G.T....-.  .AA.G.T-..  T.GGTTTA..  ......G...  ...C....A.
Demonax transilis           AATTATT..-  ...T....-A  TT..AA.T..  G...T..GAA  .G........  ...C......
Plagionotus christophi      .ATA.TT..G  .T.T....-A  .AAAG..-..  TAC.T.TAA.  .....TG...  ...C...G..
Prionus insularis           .A.A.TT..-  -..A...A..  .....GT--.  T......A.C  .....AG...  ...A......
Anastrangalia seqensi       A.GAAGT...  ...A.....A  .T.....TT.  G..A.TT...  ..........  ..........
Corymbia rubra              A.GAATT...  ...A.....A  .T.....TGG  G..A.TT..A  .....AG...  ........A.
Leptura aethiops            ..TAA.T...  ..TA.A....  .AA..AG.TT  A..A..T...  ..........  .....G....
Xanthochroa luteipennis     AATT.TTA..  A..A--...A  .A...A...T  TA.G.TTTA.  .....TG...  .......AT.

                                                  330                                                 360
Apriona germari (HA12)      AAAATTTAGT  AAACTTTTTT  TTTAT-TAGA  ATATTAATTA  GTAAGTTTTT  GATCCA-TTA
Theophilea cylindricollis   ......G.A.  ..........  ..-....TA.  .C.......G  A.GGT..A..  ........-.T
Anoplophora malasiaca       .......GA.  T.........  .A..AA.TTT  .C..A.....  A.G..AAA..  ........A.
Psacothea hilaris           ......A.A.  T........C  .......TTT  .C..A....T  A.G.A.....  ........AG
Moechotypa diphysis         ........A.  ..........  ..-.....T.  .C......AT  A.G.T.....  ..........
Thyestilla gebleri          ....C.A.T.  ..........  .A.T..A.A.  CC...C...T  A.G.AAA...  ........AG
Sophronica obrioides        ......A...  ..........  .G.....TA.  .C.......T  A.G.T.A...  .......G.T
Massicus raddei             ......AGAA  ..........  ..A.....TT  .C...T...T  A.G.AAAA..  ..........T
Chlorophorus diadema        ......A.T.  ..........  A.-T...GTT  .C...T...T  A.G.A..G..  .....G...T
Demonax transilis           ......A.T.  ..........  ..A.A..TA.  .C.......T  A.GGTA....  ......C..T
Plagionotus christophi      ......G.T.  ..........  ..GT...TTT  .C...T...T  A.G.AC.A..  ..........T
Prionus insularis           ........A.  T.........  .......GCT  .C...G....  A.G....A..  .......A.T
Anastrangalia seqensi       ........A.  ..........  ....A.A.A.  .C.......T  A.G.A.A...  ..........
Corymbia rubra              ........AG  ..........  ......A.A.  .C.......T  A.G.A.....  ..........
Leptura aethiops            ........A.  ..........  ..A...A.T.  .C.......T  A.G.AAA...  ..........
Xanthochroa luteipennis     ..........  G........A  ......G.A.  TC..A....T  A.GTA.AA..  ....TT.AAT
```

Fig. 4. Continued

The sequence divergence among 15 long-horned beetles in pairwise comparisons ranged from 12.2% (48 bp) to 39.3% (155 bp) in the COI gene (Table 5). All of the third codon positions were highly variable (96.18% of variation among species), whilst first position was somewhat conserved (50.38%) and second position was highly conserved (9.85%). The largest sequence divergence occurred in a comparison between *T. gebleri* of Lamiinae and *P.*

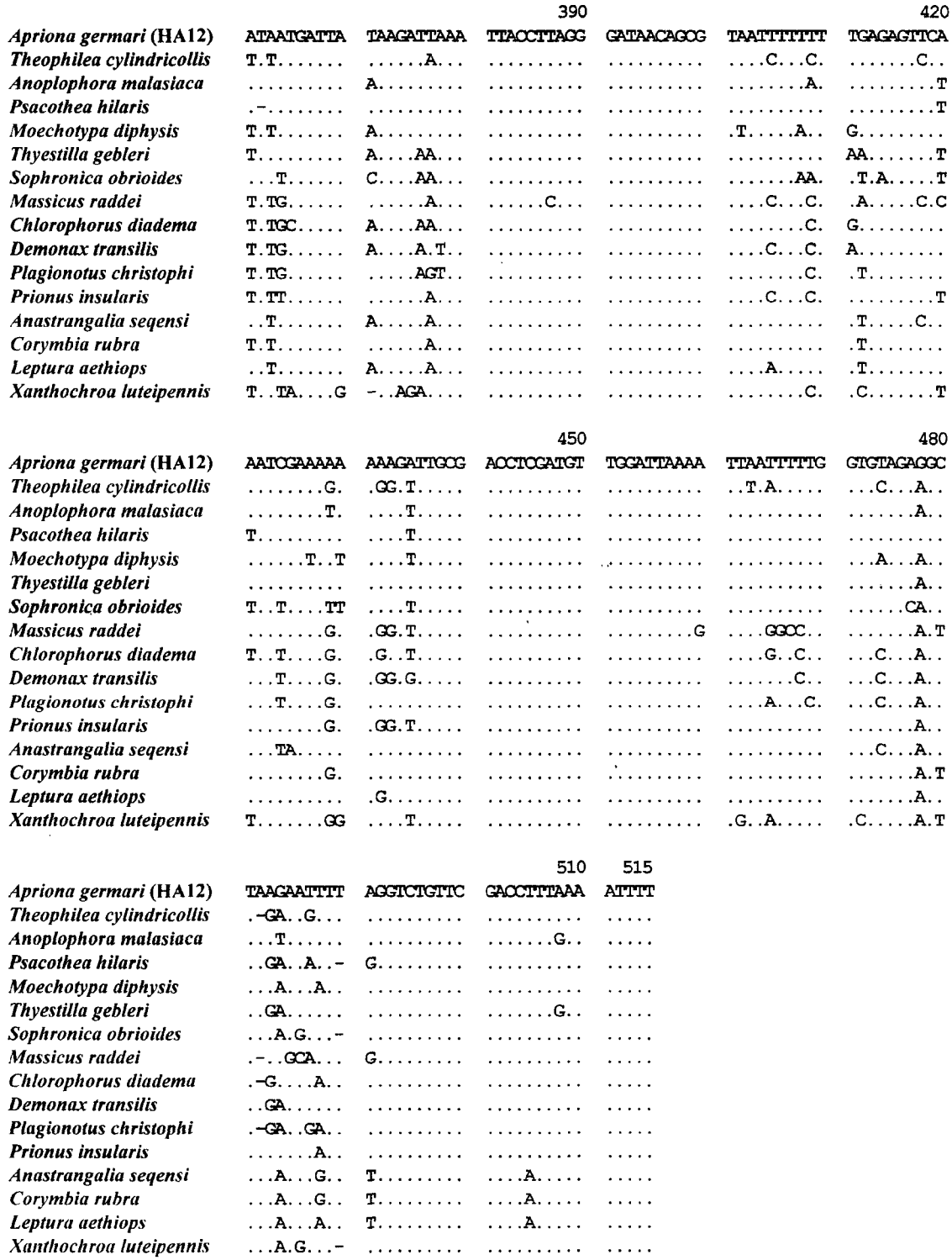

Fig. 4. Continued

insularis of Prioninae. In fact, the single species in the Prioninae was most divergent from all other ingroups (31.2% ~ 39.3%). Excluding this single species, the sequence divergence within Lamiinae, Cerambycinae and Lepturinae ranged from 13.7% (69 bp) to 22.3% (88 bp), 17.8% (70 bp) to 22.6% (89 bp) and 12.2% (48 bp) to 15.7% (62 bp), respectively. The deduced amino acid sequence divergence in COI gene ranged from 3.1% (4 amino acids) to

35.1% (46 amino acids) and the largest amino acid divergence also occurred when *P. insularis* was compared with several species of the other subfamilies (Table 6). The amino acid sequence divergence within the family was 12.2% (48 bp) ~ 23.4% (92 bp). The sequence divergence within Lamiinae, Cerambycinae and Lepturinae ranged from 3.1% (4 amino acids) to 9.2% (12 amino acids), 3.8% (5 amino acids) to 9.9% (13 amino acids) and 3.1% (4 amino acids) to 6.1% (8 amino acids), respectively. In both cases, sequence divergence was highest in Cerambycinae, Lamiinae next, and Lepturinae third.

16S rRNA gene sequences

Fig. 4 shows the nucleotide sequences of 16 samples for the 16S rRNA gene, including the outgroup taxon, *X. luteipennis*. These sequences were also deposited in the GenBank, and accession numbers are shown in Table 1. The length of the 16S rRNA gene from the 16 samples ranged from 502 bp to 510 bp (Table 4). Ranges of length variation within Lamiinae, Cerambycinae and Lepturinae were 504 bp ~ 508 bp, 504 bp ~ 508 bp, and all 510 bp, respectively, also showing the highest variation in Cerambycinae, next in Lamiinae, and homogeneity in Lepturinae (Fig. 4). The sequence divergence among fifteen long-horned beetles in pairwise comparisions ranged from 7.2% (37 bp) to 23.1% (119 bp) in the 16S rRNA gene (Table 7). The largest sequence divergence occurred in a comparison between *M. raddei* of Cerambycinae and *T. gebleri* of Lamiinae. The sequence divergence within Lamiinae, Cerambycinae and Lepturinae ranged from 12.6% (65 bp) to 17.9% (106 bp), 13.4% (69 bp) to 20.8% (107 bp) and 7.2% (37 bp) to 9.7% (50 bp), respectively. Thus, sequence divergence was highest in Cerambycinae, next in Lamiinae, and least in Lepturinae. In the comparison of the magnitude of divergence among genes, COI nucleotide sequences were most divergent, 16S rRNA gene next, and COI amino acid sequences least.

Phylogenetic relationships

PAUP and PHYLIP analyses were performed to investigate phylogenetic relationships among 15 species of long-horned beetles. Several weighting schemes, both by PAUP and PHYLIP, resulted in a variable topology in a few species, depending on different transversion weightings. Also, bootstrapping analysis weakly supported most nodes, although overall shape of the topology remained unchanged. However, bootstrap values were somewhat increased in many nodes in the analyses using COI amino acid and especially 16S RNA gene sequences. For the simplicity, here, we present unordered tree obtained by PAUP and PHYLIP. Fig. 5 shows the result of PAUP analysis using the nucleotide sequences of COI gene. As mentioned above most nodes were weakly supported and the members of Lamiinae, *S. obrioides* and *T. cylindricollis*, in this analysis grouped together with the subfamily Cerambycinae. Furthermore, the most divergent, single member of Prioninae, *P. insularis*, grouped together with the species of Lepturinae. This trend was mostly remained in the other weighting schemes, although species of Lepturinae often kept a monophyletic group with somewhat higher bootstrap estimates (data not shown). PHYLIP analysis from COI nucleotide sequence also revealed a similar feature in that the position of Cerambycinae species was fluctuating in their taxonomic positions. Also, *P. insularis* formed a monophyletic group with Lepturinae with somewhat higher bootstrap (55% bootstrap value) (Fig. 6).

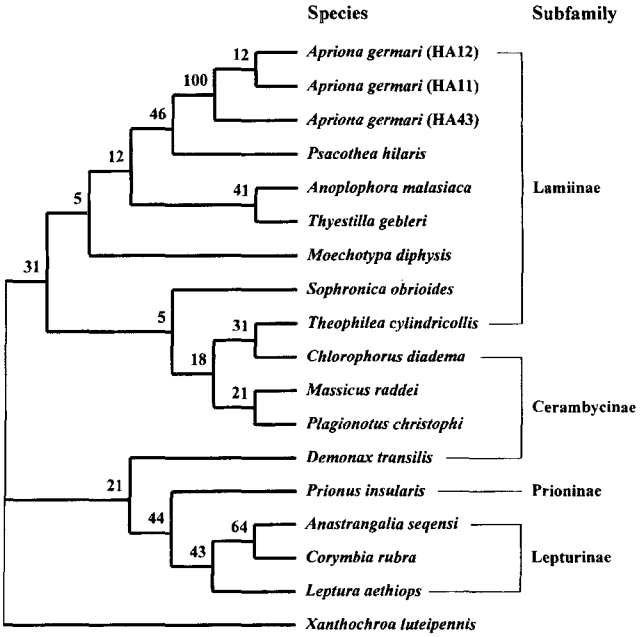

Fig. 5. PAUP analysis of mitochondrial COI sequences. The tree shown is majority-rule consensus of two equally parsimonious trees from the heuristic search using *X. luteipennis* as an outgroup. The numbers shown on the branches represent bootstrap values for 1,000 replicates. Tree length is 759 steps, Consistency Index is 0.445, and Retention Index is 0.392.

In terms of amino acid sequence, several nodes were somewhat highly supported by bootstrap value both by PAUP and PHYLIP (Fig. 7 and 8). For example, *C. diadema*, *D. transilis*, and *P. christophi* belonging to Cerambycinae grouped strongly together in both analyses (71% in PAUP and 82% in PHYLIP), although monophyletic grouping of the subfamily was not supported, because *T. cylindricollis* belonging to Lamiinae was included in the subfamiliy. Also, the species of Lepturinae in these anal-

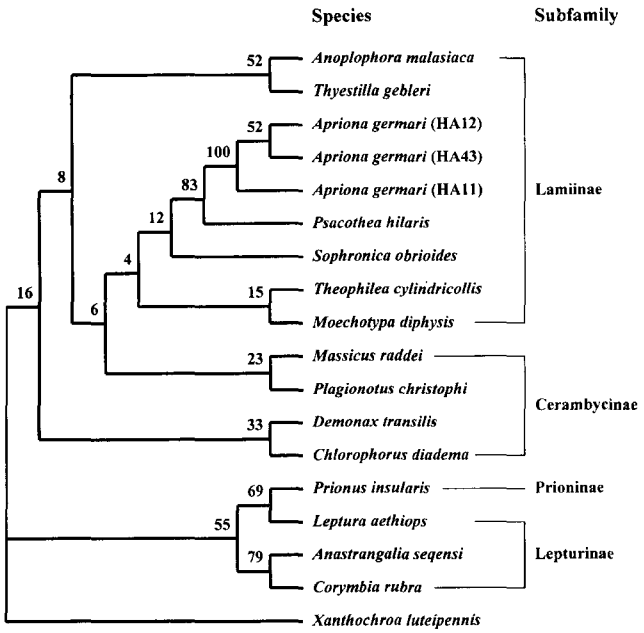

Fig. 6. PHYLIP analysis of mitochondrial COI sequences. The tree was obtained using the subprogram NEIGHBOR incorporated in PHYLIP with the option of Kimura's 2-parameter method (1980). The tree was rooted using *X. luteipennis*. The numbers shown on branches, which represent bootstrap values for 100 replications, was obtained using the subprogram CONSENSE.

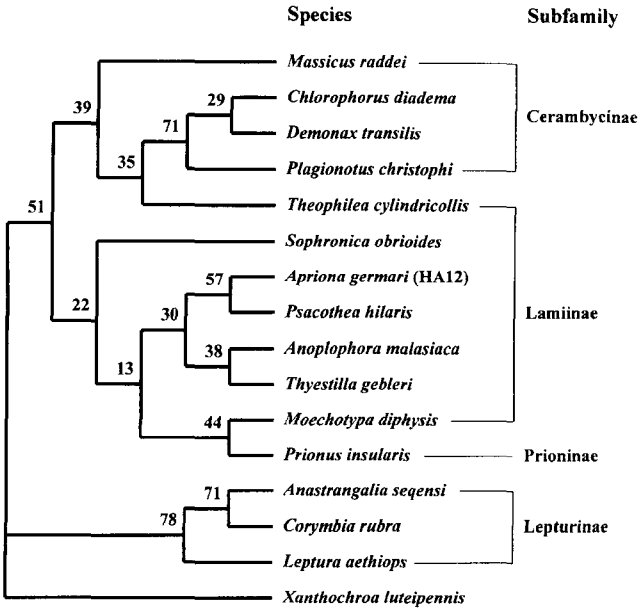

Fig. 7. PAUP analysis of mitochondrial COI amino acid sequences. The tree shown is majority-rule consensus of nine equally parsimonious trees from the heuristic search using *X. luteipennis* as an outgroup. The numbers shown on the branches represent bootstrap values for 1,000 replicates. Tree length is 109 steps, Consistency Index is 0.743, and Retention Index is 0.627.

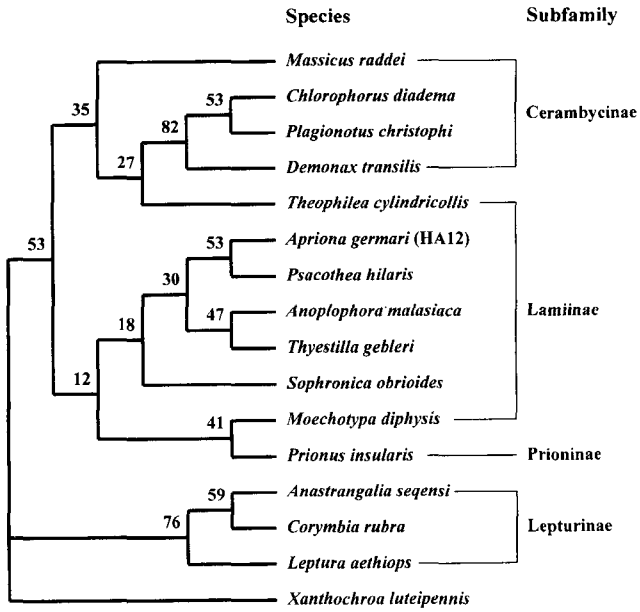

Fig. 8. PHYLIP analysis of mitochondrial COI amino acid sequences. The tree was obtained using the subprogram NEIGHBOR incorporated in PHYLIP. These trees were rooted using *X. luteipennis*. The numbers shown on branches, which represent bootstrap values for 100 replications, was obtained using the subprogram CONSENSE.

yses formed a monophyletic group with pretty high bootstrap values (78% in PAUP and 76% in PHYLIP), unlikely the analyses obtained by nucleotide sequences of COI gene (Fig. 5 and 6). Among the two species of Lamiinae, *S. obrioides* and *T. cylindricollis*, which were grouped together with Cerambycinae in the analyses using COI nucleotide (Fig. 5 and 6), *T. cylindricollis* collected in Japan still was grouped with Cerambycinae, although *S. obrioides* was not (Fig. 7 and 8).

Bootstrap analyses of 16S rRNA gene sequences both by PAUP and PHYLIP supported more nodes with higher values than those obtained with any other analyses (Fig. 9 and 10). For example, Lepturinae species, which formed a monophyletic group in the COI amino acid sequences (78% in PUAP and 76% in PHYLIP), were supported by 80% and 87% of bootstrap values in PAUP and PHYLIP, respectively (Fig. 9 and 10). Also, number of nodes supported by >50% of bootstrap value increased in these analyses than COI amino acid sequences. In these analyses again, *T. cylindricollis* was excluded from Lamiinae both in PAUP and PHYLIP. Collectively, our extensive phylogenetic analyses suggest better utility of 16S rRNA gene for the longed-horned beetles than the region of protein-coding gene, which reveals a high divergence. Lack of monophyletic relationships among Lamiinae and Cerambycidae, respectively, fluctuation of *P. insularis* of Pri-

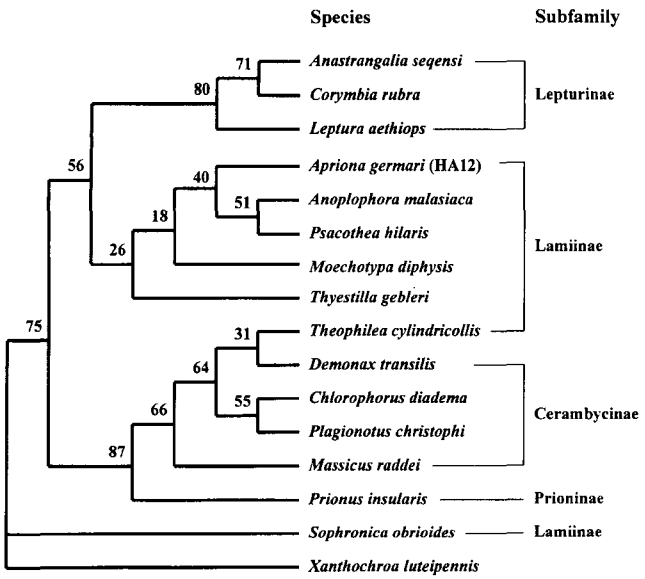

Fig. 9. PAUP analysis of mitochondrial 16S rRNA sequences. The tree shown is majority-rule consensus of two equally parsimonious trees from the heuristic search using *X. luteipennis* as an outgroup. The numbers shown on the branches represent bootstrap values for 1,000 replicates. Tree length is 761 steps, Consistency Index is 0.515, and Retention Index is 0.402.

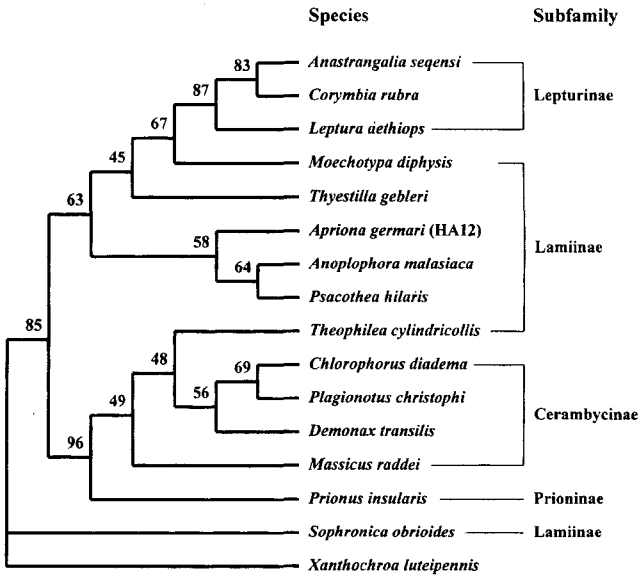

Fig. 10. PHYLIP analysis of mitochondrial 16S rRNA sequences. The tree was obtained using the subprogram NEIGHBOR incorporated in PHYLIP with the option of Kimura's 2-parameter method (1980). The tree was rooted using *X. luteipennis*. The numbers shown on branches, which represent bootstrap values for 100 replications, was obtained using the subprogram CONSENSE.

oninae in the taxonomic position, and formation of monophyletic group in the Lepturinae were characteristic.

Discussion

To date, COI and 16S rRNA mitochondrial gene sequences have been used in many phylogenetic studies of insects (Howland and Hewitt, 1995; Juan *et al.*, 1995; Kim *et al.*, 2000a, b, c; Suzuki, 1997; Vogler and Pearson, 1996). However, these genes as well as other mitochondrial genome have never been subjected to phylogenetic analysis for long-horned beetles, Cerambycidae, even thought some species in this family were included for phylogenetic analysis of Coleoptera (Howland and Hewitt, 1995). Thus, as far as we know, this is the first report on the molecular phylogeny of Cerambycidae using direct sequences, although our sample size is far less to complete molecular phylogeny of Cerambycidae.

Sequence divergence in COI and 16S rRNA genes

The data in the present paper clearly indicate that maximum sequence divergence among 15 long-horned beetles in the COI gene was higher than anticipated (39.3%; Table 5). In the study of the Coleoptera based on COI sequence data, the maximum sequence divergence of 15 families, in which one ~ seven species were included, was 18% within family, and 27% between families (Howland and Hewitt, 1995). Thus, the divergence of COI gene sequence data is far greater than the upper limit of Coleoptera and even greater than the estimate of between-family. This inevitably lowered the bootstrap value and, resultantly, phylogenetic signal. Howland and Hewitt (1995) also encountered similar problem.

However, the greatly increased maximum sequence divergence of the family in this gene was mainly stemmed from *P. insularis* belonging to Prioninae. Sequence divergence of this species from others ranges from 31.2% to 39.3% (Table 5), but, excluding this species, the maximum sequence divergence is 23.4%, although the estimate is still higher than those of Coleoptera (Howland and Hewitt, 1995). In fact, we sequenced this portion of COI gene from *P. insularis* four times with two independent PCR products and carefully examined the external morphology of the species to find out any possible mistake, but no evidence was found. Nevertheless, the peculiarity of the COI sequence of *P. insularis* is obvious when the sequence was compared with those of the GenBank-registered insect sequences. In those comparisons, the sequence divergence between species of Cerambycidae and other insects such as leaf beetles, fruit flies, mosquitoes, locust, and honey bees ranged from 18.5% (73 bp) to 36.8% (145 bp), suggesting *P. insularis* is still highly

Table 7. Pairwise comparisons of 16S rRNA gene sequences among 15 species of long-horned beetles and *Xanthochroa luteipennis*

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1. *Apriona germari* (HA12)	-	0.173	0.126	0.138	0.136	0.157	0.179	0.212	0.208	0.192	0.200	0.163	0.144	0.153	0.148	0.216
2. *Theophilea cylindricollis*	89	-	0.194	0.200	0.183	0.194	0.206	0.186	0.153	0.146	0.144	0.138	0.163	0.177	0.183	0.237
3. *Anoplophora malasiaca*	65	100	-	0.138	0.153	0.157	0.181	0.214	0.219	0.202	0.217	0.175	0.167	0.186	0.153	0.219
4. *Psacothea hilaris*	71	103	71	-	0.167	0.171	0.181	0.206	0.216	0.221	0.208	0.190	0.171	0.183	0.163	0.212
5. *Moechotypa diphysis*	70	94	79	86	-	0.159	0.173	0.231	0.186	0.200	0.212	0.181	0.144	0.138	0.120	0.231
6. *Thyestilla gebleri*	81	100	81	88	82	-	0.190	0.227	0.192	0198	0.208	0.191	0.153	0.157	0.144	0.210
7. *Sophronica obrioides*	92	106	93	93	89	98	-	0.219	0.204	0.196	0.202	0.192	0.184	0.173	0.194	0.194
8. *Massicus raddei*	109	96	110	106	119	117	113	-	0.192	0.208	0.190	0.188	0.219	0.216	0.214	0.256
9. *Chlorophorus diadema*	107	79	113	111	96	99	105	99	-	0.151	0.134	0.173	0.198	0.198	0.200	0.239
10. *Demonax transilis*	99	75	104	114	103	102	101	107	78	-	0.159	0.184	0.177	0.179	0.198	0.233
11. *Plagionotus christophi*	103	74	112	107	109	107	104	98	69	82	-	0.171	0.196	0.194	0.192	0.229
12. *Prionus insularis*	84	71	90	98	93	101	99	97	89	95	88	-	0.171	0.159	0.165	0.217
13. *Anastrangalis sequensi*	74	84	86	88	74	79	95	113	102	91	101	88	-	0.072	0.082	0.202
14. *Corymbia rubra*	79	91	96	94	71	81	89	111	102	92	100	82	37	-	0.097	0.184
15. *Leptura aethiops*	76	94	79	84	62	74	100	110	103	102	99	85	42	50	-	0.212
16. *Xanthochroa luteipennis*	111	122	113	109	119	108	100	132	123	120	118	112	104	95	109	-

Numbers above the diagonal are mean distance values; numbers below the diagonal are absolute distance values.

divergent from major insect species (data not shown). Furthermore, human COI sequence (GenBank accession number NC001807) is diversified from the species of Cerambycidae by 32.7% (129 bp) ~ 37.8% (149 bp). Considering saturation of mitochondrial protein-coding genes, especially in the third position of codons, such divergence is very hard to reconcile with any known comparative data, and no obvious answer is currently available, except for the possibility of pseudo gene encoded in the nuclear genome. We, therefore, carefully checked amino acid sequences to see if any termination codon suddenly appears within COI gene, but this effort was not successful. Until more scrupulous experimental scheme provides the evidence of nuclear-encoded mitochondrial genome, we tentatively regarded the sequences as a mitochondria-encoded COI gene as usual.

Unlikely COI gene sequence data, 16S rRNA gene data showed the maximum sequence divergence of 23.1% (Table 7), and this resultantly supported more branches with higher bootstrap values than any other analyses both in the PAUP and PHYLIP. In the study of the fireflies based on 16S rRNA gene, the maximum sequence divergence of 27 species belonging to four subfamilies was 24.2% within family and 26.5% between families (Suzuki, 1997). Thus, our data are somewhat similar to those of firefly species, Lampyridae, although we included less number of species in the analysis.

COI gene sequence divergence in *A. germari*

Ten samples of *A. germari* collected from Suwon and Busan revealed three haplotypes, ranging in sequence divergence from 0.3% to 0.5% (Table 5). Even though the two localities are separated more than 350 kilometers in distance, the two population shared one common haplotype, AG1, with a high frequency (80%) and the sequence divergence among haplotypes were substantially low (0.5%). In fact, the magnitude of the sequence divergence is low compared with other Korea-dwelling insect species that are available for the homologous sequences of mtDNA (Kim *et al.*, 2000a, b; Lee *et al.*, 2000). However, an extensive sampling may explore further divergent array of haplotypes, so we want to avoid any conclusive remark on this issue.

Phylogeny

Even under some phylogenetic limitation of COI nucleotide, COI amino acid, and 16S rRNA sequences *per se* and resultant fluctuation in many branches, 16S rRNA data supported more branches with higher bootstrap values, and was close to the current taxonomy. For example, monophyly of Lepturinae was either not supported by nucleotide sequence of COI gene or less strongly by COI amino acid sequence, but robust by 16S rRNA data. However, all the data set collectively suggested us the presence of discrepancy between molecular data and traditional taxonomic views. Such discrepancy includes the taxo-

nomic positions of *S. obrioides* and *T. cylindricollis* in both phylogenetic analyses. This was especially obvious when COI amino acid and 16S rRNA sequences were utilized for data matrix. Although our samples included only small number of species within tribes, a consistent result by both genes questions the taxonomic positions of the two species within the subfamily, Lamiinae. Because species of the long-horned beetles are highly diversified in their morphology by a long evolutionary time, and by convergent evolution, adaptive to host plants, possible misidentification based solely on morphology might be one source of discrepancy between data set. Another possible source of discrepancy could be limitation of one small portion of mitochondrial gene. In fact, many studies including Coleoptera phylogeny suggested several analytic schemes to better resolve taxonomic position using mitochondrial and nuclear genes (Edwards *et al.*, 1991; Irwin *et al.*, 1991; Brower, 1994; Howland and Hewitt, 1995; Kim *et al.*, 2000c). However, this effort was not always successful even if more than one gene was included, and several analytical techniques also were involved. Particularly, Howland and Hewitt (1995) pointed out that data from one sequence inevitably might be insufficient to resolve phylogenetic relationships at all levels within such a large group, such as Coleoptera. In fact, *A. malasiaca* and *P. hilaris* both belong to the same tribe (Tribe Agniini) within Lamiinae, but they only showed a sister relationship when 16S rRNA gene was utilized as data in both PAUP and PHYLIP analyses (Fig. 9 and 10), supporting Howland and Hewitts view. As more species and data from more genes are accumulated better resolution on the phylogeny of Cerambycidae will be possible.

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