Entomological Science, 2001, 4(3): 335–344

Genetic Variation of 16S rRNA Gene Observed in *Ceruchus lignarius* and *Dorcus rectus rectus* (Coleoptera: Lucanidae)

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Abstract. Genetic variation of 16S rRNA gene sequences were investigated in two Japanese lucanid beetles, *Ceruchus lignarius* and *Dorcus rectus rectus*, which have different habitats and distributional patterns. Present results indicate that the inter-populational sequence variation of 16S rRNA within three subspecies of *C. lignarius* are much smaller than those between all combinations of different subspecies, suggesting the monophylies of these three subspecies. Our results also suggest that sequence divergence within a single population is very low in this species and that differentiations increase in the order of intra-population (0–0.11%), intra-subspecies (0–2.26%), intra-species (2.80–7.99%) and interspecies (7.34–10.05%). Likewise, intra-subspecific nucleotide replacements of *D. r. rectus* (0–1.06%) were much smaller than the interspecific divergence (14.82–19.95%).

Key words: *Ceruchus lignarius, Dorcus rectus rectus*, mitochondrial DNA, 16S rRNA, genetic variation, Lucanidae.

Introduction

The family Lucanidae, which consists of about 1,000 species, is widely distributed in the world and particularly abundant in Asian humid tropics (Benesh, 1960; Mizunuma & Nagai, 1994). Most of the species in this family show remarkable sexual dimorphism, especially in the shape of the mandibles. However, the phylogeny of the family, as well as its ecology, remains uncertain, mainly due to the difficulty in the coding of morphological characters for cladistic analyses. Thus, phylogenetic analyses on the basis of molecular data are expected to much contribute to the solution on phylogeny of this family.

Recently, mitochondrial DNA (mtDNA) sequences have became a standard tool for the phylogenetic inference of various insect groups (Simon *et al.*, 1994). Among the mtDNA genes, cytochrome oxidase subunit I (COI), NADH dehydrogenase subunit 5 (ND5) and 16S ribosomal RNA (16S rRNA) have been used for the phylogenetic inference of some beetles (e.g., Juan *et al.*, 1996; Su *et al.*, 1998; Kambhampati & Charlton, 1999). Whereas there is some understanding of COI, little is known about the sequence divergences of 16S rRNA, although some studies have revealed that the latter gene evolved more slowly than the former (Funk *et al.*, 1995).

Although there have been a number of debates on insect phylogeny based on the molecular data, only a few studies addressed genetic variation within a taxon (Xiong & Kocher, 1993; Vogler *et al.*, 1993). Most authors (e.g., Fang *et al.*, 1993; Juan *et al.*, 1996; Kambhampati & Charlton, 1999) examined only a few individuals, mostly one, as the representative of the operational taxonomic unit (OTU), mostly a species or population. However, some authors have revealed morphspecies did not exclusively form a single cluster (e.g., Coleoptera *Prodontria capito*: Emerson *et al.*, 1995; Orthoptera *Laupala* spp.: Shaw, 1996; Lepidoptera *Heliconius melpomene*: Brower, 1996). Therefore, analyses of intra-specific or intra-populational variation based on many individuals are expect to contribute to solution of phylogenetic inference.

In the present study, we investigated the genetic variation of 16S rRNA sequences in two Japanese lucanid beetles, *Ceruchus lignarius* and *Dorcus rectus rectus*. These two species have different habitats and distributional patterns. *Ceruchus lignarius* is distributed in Hokkaido, Honshu, Shikoku and Kyushu in Japan. This species is confined only to montane environments. *Ceruchus lignarius* consists of three subspecies (Fujita, 1987; Mizunuma & Nagai, 1994), i.e., *C. l. lignarius* [Hokkaido and north eastern

Honshu], *C. l. monticola* [central Honshu] and *C. l. nodai* [Shikoku and Kyushu] (Fig. 1a). On the other hand, *D. rectus*, which is found in East Asia (China, Korea, Taiwan and the Japanese islands), is widely distributed from open lowlands including secondary forests to deep primary forests in the highlands. *Dorcus rectus* consists of three subspecies (Mizunuma & Nagai, 1994), *D. r. kobayashii* [Tokara islands], *D. r. miekoae* [Hachijo island] and *D. r. rectus* [other regions] (Fig. 1b). A comparison of such species, which have different habitats and distributional patterns, gives fruitful information to study genetic divergences within species or subspecies. Our purposes are to estimate: 1) the polymorphism within a single population of *C. lignarius* and *D. r. rectus*; 2) the genetic variation among populations of *C. lignarius* and *D. r. rectus*; 3) the sequence divergences among species of *Ceruchus* and *Dorcus*; and 4) the phylogeny and biogeography of these two taxa.

Materials and Methods

Insects

We followed the taxonomic system of Mizunuma & Nagai (1994). A total of 19 beetles from nine populations belonging to three subspecies of *C. lignarius*, including three forms of *C. l. monticola* (see Fig. 1a), and a total of 27 beetles from 15 populations of *D. r. rectus* were examined for DNA analysis (Fig. 1b: see Appendix for further details). We selected *D. striatipennis striatipennis, D. titanus pilifer* and *C. piceus* as outgroups for our analyses.

DNA extraction, amplification and sequencing

Small amounts of muscle, removed from anesthetized or dead specimens (stocked in 99.5% ethanol or in dried condition), were homogenized in an extraction buffer (10 mM NaCl, 10 mM Tris-HCl [pH 8.0], 25 mM EDTA, 0.5% sodium dodecyl sulfate). After digesting samples with Proteinase K (0.1 mg/ml) at 37℃ for eight hours, DNA was extracted with phenol (one time), 25 : 24 : 1 of phenol/chloroform/isoamylalcohol (two times) and 24 : 1 of chloroform/isoamylalcohol (one time) and precipitated in ethanol with a 1/25 volume of 3.0 M sodium acetate. Samples were resuspended in a TE buffer followed by ethanol precipitation.

A part of the mitochondrial 16S rRNA gene was

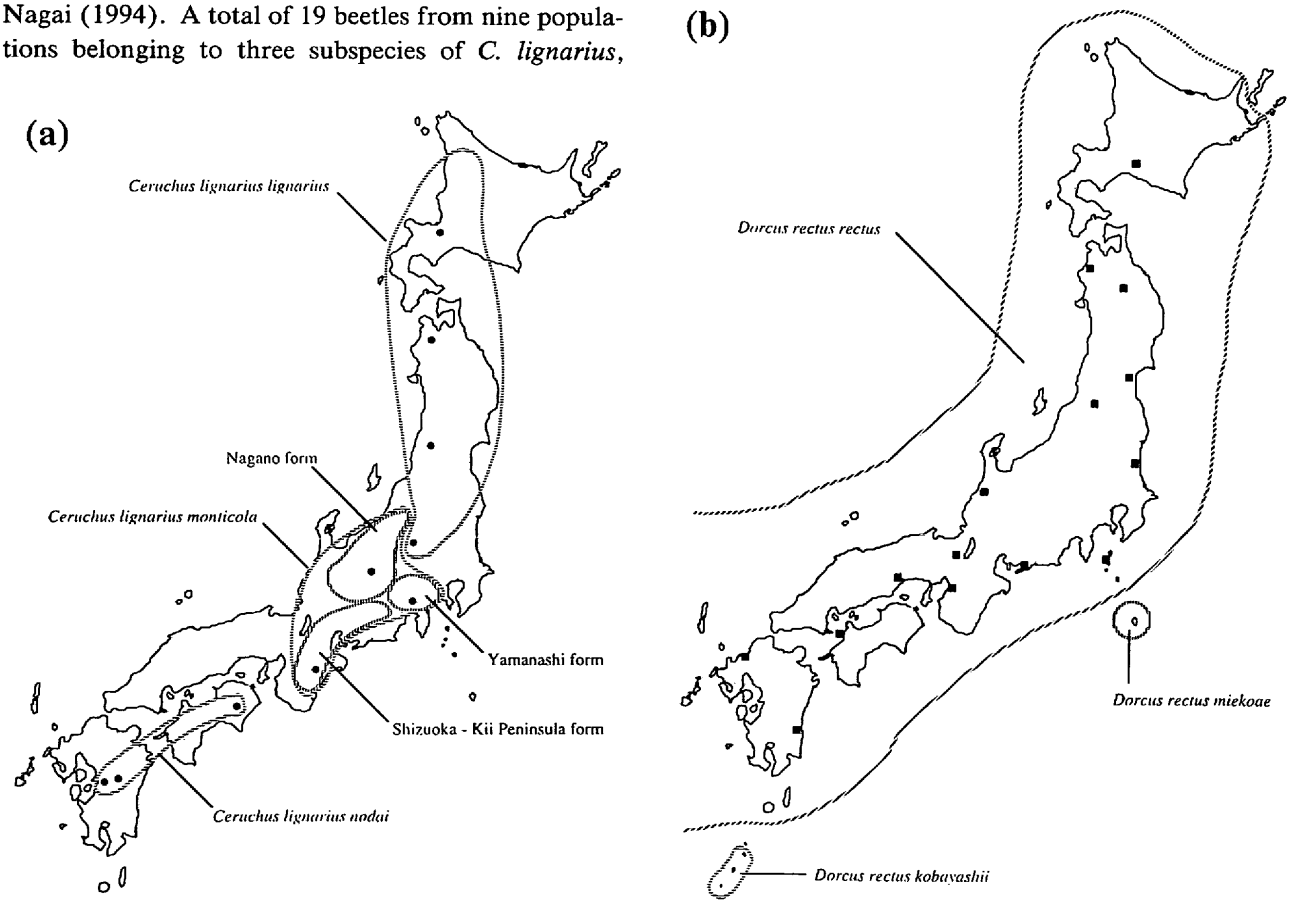
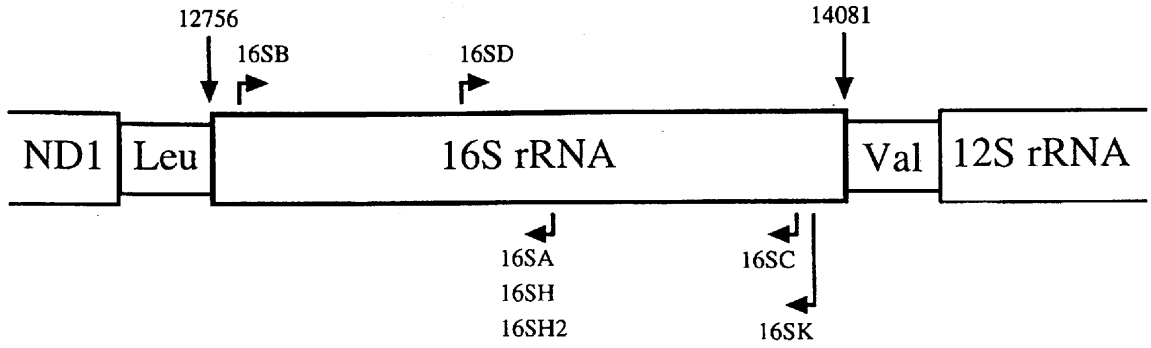


Fig. 1. (a) Approximate geographic ranges (e.g., Fujita, 1987) and collection sites of *C. lignarius*. (b) Approximate geographic ranges (e.g., Mizunuma & Nagai, 1994) and collection sites of *D. r. rectus* in Japan.



16SA (22bp : 13396-13427) 5'-CGC CTG TTT AAC AAA AAC ATG T-3'

16SB (22bp : 12866-12887) 5'-CCG GTT TGA ACT CAG ATC ATG T-3'

16SC (22bp : 13882-13903) 5'-TAC CTT GTG TAT CAG GGT TTA T-3'

16SD (22bp : 13311-13332) 5'-ATT ATG CTA CCT TTG CAC GGT C-3'

16SH (21bp : 13397-13427) 5'-TGC CTG TTT A(A/T)T AAA AAC ATG -3'

16SH2 (21bp : 13397-13427) 5'-TGC CTG TTT ATT AAA AAC ATG -3'

16SK (25bp : 13889-13913) 5'-TAA TTT ATT GTA CCT T(G/T)T GTA TCA G-3'

Fig. 2. Diagram of mitochondrial 16S rRNA gene and flanking tRNA, showing position of PCR and sequencing primers. Numbering is according to the *Drosophila yakuba* mitochondrial genome (Clary & Wolstenholme, 1985).

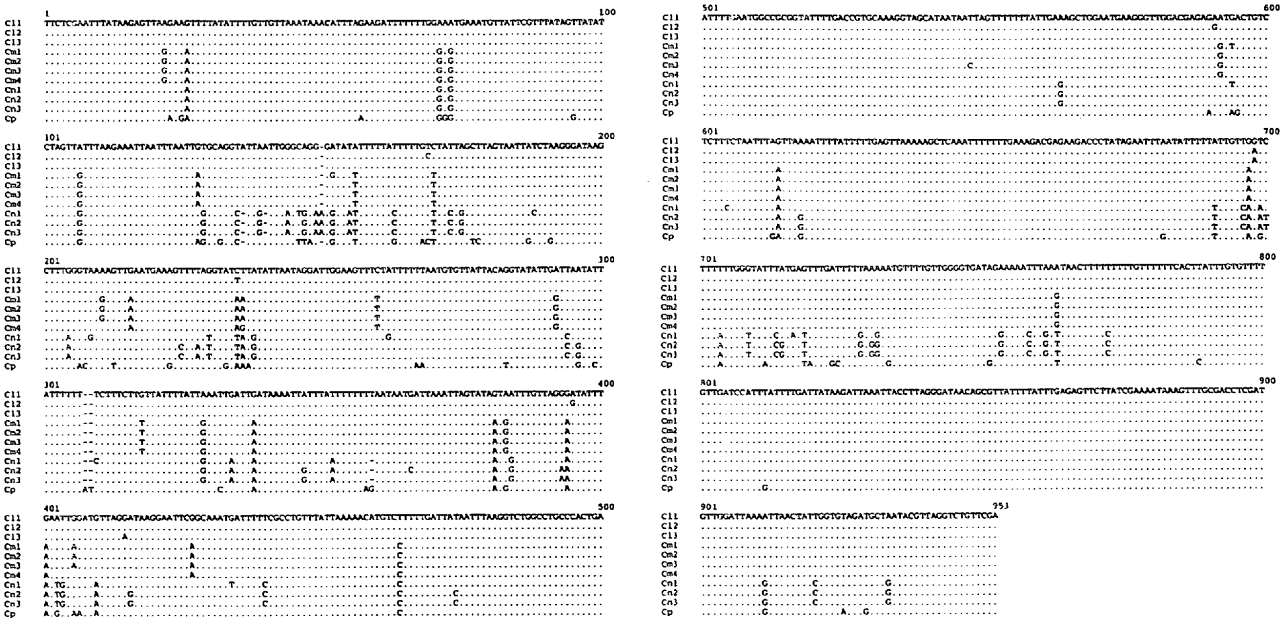


Fig. 3. Aligned sequences of mitochondrial 16S rRNA gene of *C. lignarius* and *C. piceus* (Cp). The haplotypes of *C. lignarius* are described in Table 1. Dots indicate identity with the first sequence; dashes denote a gap. Asterisk indicates bases identical to all individuals.

amplified by polymerase chain reaction (PCR) (Saiki *et al.*, 1988) using six primers: 16SA and 16SH (modified LR-N-13398 in Simon *et al.*, 1994), 16SB (modified LR-J-12887 in Simon *et al.*, 1994), and 16SC, 16SD and 16SK (presented here) (Fig. 2). Amplification proceeded in 50 μl of reaction solution containing 10 mM Tris-HCl, 50 mM KCl, 2 mM $MgCl_2$, 0.2 mM each dNTP, 25 pmol each primer, template DNA (1–5 μg) and 1.25 U ExTaq polymerase (Takara Shuzo, Otsu, Japan). The temperature regimen of 35 cycles was five minutes at 94℃ (initial step), one minute at 94℃, one minute at 45–50℃, three minutes at 72℃ and five minutes at 72℃ (final step). Amplified DNA was purified by electrophoresis in 2% agarose gel.

Nucleotide sequences were determined for both

strands with a Thermo sequenase cycle sequencing kit (USB, Cleveland, USA) and a LI-COR Model 4200 Automated DNA Sequencer (LI-COR, Lincoln, USA) or with a dye terminator cycle-sequencing FS Ready Reactions Kit and an ABI PRISM 377 DNA Sequencer (Perkin-Elmer Corp., Norwalk, USA) using 16SA, 16SH2 (modified LR-N-13398 in Simon *et al.*, 1994), 16SB, 16SC and 16SD primers (Fig. 2).

Analysis

Sequence data was aligned by CLUSTAL W 1.8 (Thompson *et al.*, 1994) at DNA Data Bank of Japan (DDBJ), using default gap penalties, and some modification by eye. Genetic distances among taxa were calculated based on Kimura's two-parameter model (Kimura, 1980). The neighbor-joining (NJ) method was performed using PAUP 4.0b4a (Swofford, 1998). The confidences levels of each branch were estimated using 1,000 bootstrap replications (Felsenstein, 1985).

We also incorporated the published data for COI, ND5 and 16S rRNA into the analyses (Vogler *et al.*, 1993; Vogler & DeSalle, 1993; Funk *et al.*, 1995; Juan *et al.*, 1996; Su *et al.*, 1996; Juan *et al.*, 1998; Su *et al.*, 1998) in order to compare the sequence divergences between genes.

Results

Aligned sequences from the mitochondrial 16S rRNA gene of *C. lignarius* and *D. r. rectus* are presented in Fig. 3 and 4, respectively. All sequences in the present study showed a substantial bias for adenine (A) and thymine (T) (72.6–76.3%). Several observations demonstrated the existence of such a strong AT

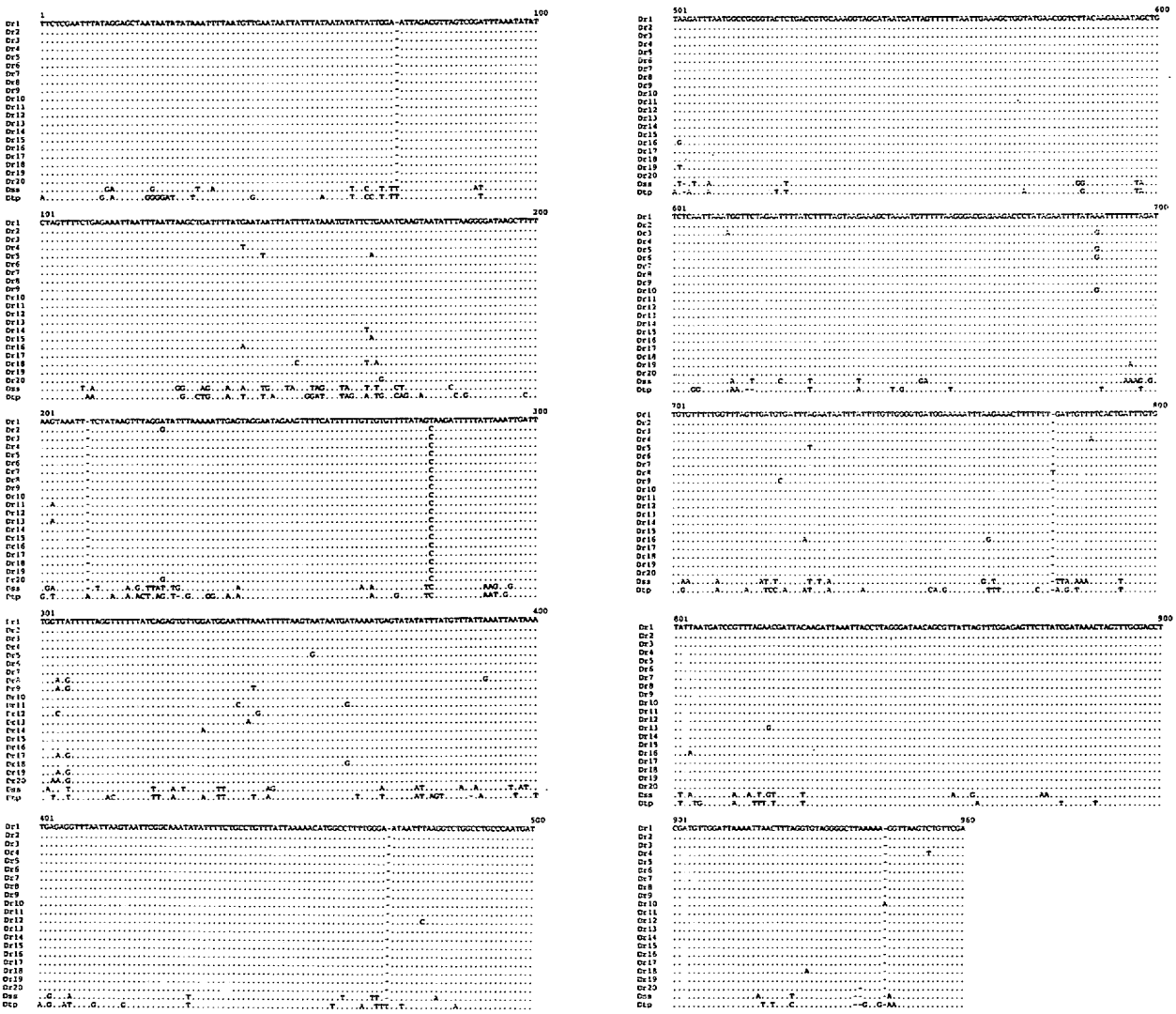


Fig. 4. Aligned sequences of mitochondrial 16S rRNA gene of *D. r. rectus, D. s. striatipennis* (Dss) and *D. titanus pilifer* (Dtp). The haplotypes of *D. r. rectus* are described in Table 1. Dots indicate identity with the first sequence; dashes denote a gap. Asterisk indicates bases identical to all individuals.

bias in the insect mitochondrial genome (Simon *et al.*, 1994).

The mitochondrial 16S rRNA gene fragment of *C. lignarius* consisted of 951 total aligned sites, 99 of which were variable. The sequence of *C. l. nodai* included 1-base pair (bp) insertion and three-bp deletions. The population of *C. lignarius* from Fuji (ten individuals) had two sequence haplotypes (Table 1). The two haplotypes differed in 1 bp (0.11%) (Table 2). Number of substitutions in the obtained sequences among four populations of *C. l. lignarius*, three populations of *C. l. monticola* and three populations of *C. l. nodai* varied from 0 bp (0%: Aomori vs. Yamàgata) to 5 bp (0.53%: Hokkaido vs. Aomori or Yamagata, and Aomori or Yamagata vs. Gunma), 2 bp (0.21%: Nagano vs. Fuji [Cm2]) to 5 bp (0.53%: Nagano vs. Mie) and 1 bp (0.11%: Kumamoto vs. Miyazaki) to 21 bp (2.26%: Tokushima vs. Kumamoto), respectively. Inter-subspecific nucleotide substitutions among three subspecies of *C. lignarius* ranged from 26 bp (2.80%: Hokkaido vs. Mie) to 71 bp (7.99%: Aomori or Yamagata vs. Kumamoto). Sequence divergences among forms of *C. l. monticola* (0.21–0.53%) were lower than those among subspecies of *C. lignarius* (2.80–7.99%) and as large as those among populations of *C. l. lignarius* (0–0.53%). Interspecific nucleotide substitutions of *C. lignarius* and *C. piceus* were observed from 66 bp (7.34%) to 88 bp (10.05%).

The NJ dendrogram of *C. lignarius* derived from 16S rRNA sequences is shown in Fig. 5. The ingroup portion of this dendrogram could be divided into two clusters, of which one, consisting of *C. l. lignarius* and *C. l. monticola*, was supported in high bootstrap values (99%). This cluster was further split into two subclusters consisting of populations of *C. l. lignarius* (100%) and populations of *C. l. monticola* (100%). The other cluster, supported in 100% of bootstrap values, contained populations of *C. l. nodai*.

The mitochondrial 16S rRNA gene fragment of *D. r. rectus* consisted of 957 total aligned sites, 38 of which were variable. Sequences of Miyagi (Dr8) and Yamagata included a 1-bp insertion. Sequences of Miyazaki included a 1-bp deletion. The population of

Table 1. List of *Ceruchus lignarius* and *Dorcus rectus rectus* populations and mtDNA haplotypes. Number of individuals found in each population are given in parentheses.

Taxon name	Location	mtDNA haplotypes
Ceruchus lignarius lignarius	Hokkaido	Cl1
	Aomori	Cl2
	Yamagata	Cl2
	Gunma	Cl3
Ceruchus lignarius monticola	Nagano	Cm1
	Fuji	Cm2 (9), Cm3 (1)
	Mie	Cm4
Ceruchus lignarius nodai	Tokushima	Cn1
	Kumamoto	Cn2
	Miyazaki	Cn3
Dorcus rectus rectus	Hokkaido	Dr1
	Aomori (10)	Dr1, Dr2, Dr3, Dr4, Dr5, Dr6 (2), Dr7 (3)
	Iwate	Dr6
	Miyagi (2)	Dr8, Dr9
	Yamagata	Dr10
	Ibaraki	Dr11
	Toshima	Dr12
	Aichi	Dr13
	Ishikawa	Dr7
	Kyoto (2)	Dr14, Dr15
	Osaka	Dr16
	Okayama (2)	Dr7, Dr17
	Ehime	Dr18
	Fukuoka	Dr19
	Miyazaki	Dr20

Table 2. Numbers of nucleotide replasements (below disgonal) and sequence divergence (%; above disgonal) based on Kimura's two-parameter model for 16S rRNA gene sequences in *Ceruchus lignarius* haplotypes. See Table 1 for the haplotype abbreviations.

	Cl1	Cl2	Cl3	Cm1	Cm2	Cm3	Cm4	Cn1	Cn2	Cn3
Cl1	—	0.53	0.21	3.25	3.03	3.14	2.80	7.27	7.63	7.52
Cl2	5	—	0.53	3.70	3.47	3.59	3.25	7.63	7.99	7.88
Cl3	2	5	—	3.47	3.25	3.36	3.03	7.51	7.87	7.76
Cm1	30	34	32	—	0.21	0.32	0.53	6.56	7.14	7.03
Cm2	28	32	30	2	—	0.11	0.32	6.79	7.15	7.04
Cm3	29	33	31	3	1	—	0.42	6.91	7.27	7.16
Cm4	26	30	28	5	3	4	—	6.67	7.03	6.92
Cn1	65	68	67	59	61	62	60	—	2.26	2.15
Cn2	68	71	70	64	64	65	63	21	—	0.11
Cn3	67	70	69	63	63	64	62	20	1	—

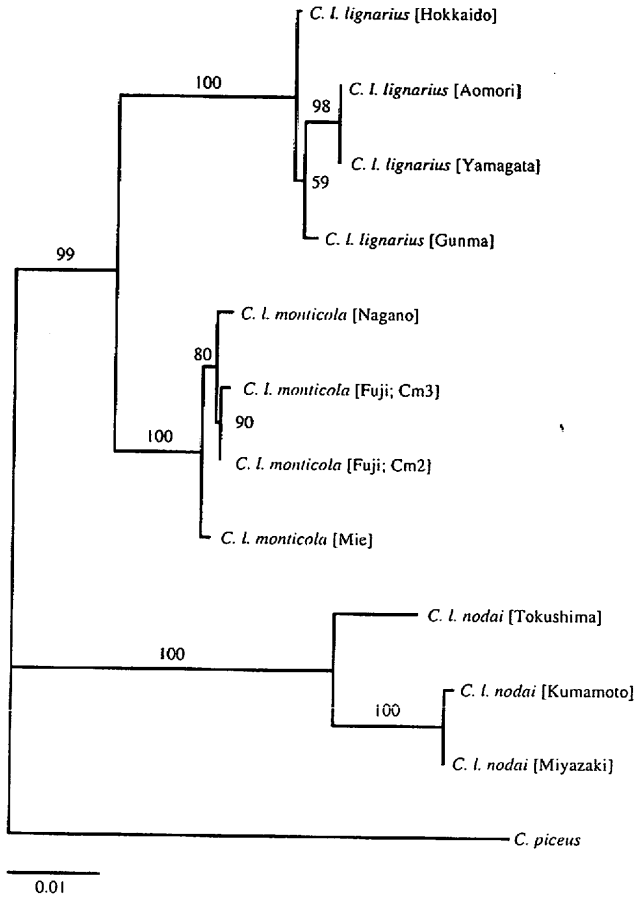


Fig. 5. NJ dendrogram for populations belonging to *C. lignarius* derived from 16S rRNA gene sequences. Bootstrap values are given for each branch. Bar equals 0.01 Kimura's two-parameter distance.

D. r. rectus from Aomori (ten individuals) had seven different sequence haplotypes (Table 1). The maximum nucleotide substitution among the haplotypes of Aomori was 8 bp (0.84%) (Table 3). Nucleotide replacements between two samples of Miyagi, Kyoto and Okayama involved 3 bp (0.32%), 3 bp (0.32%) and 2 bp (0.21%), respectively. Nucleotide substitutions between 15 populations of *D. r. rectus* varied from 0 bp (0%: Hokkaido vs. Aomori [Dr1], Aomori vs. Iwate [Dr6], and Aomori vs. Ishikawa vs. Okayama [Dr7]) to 10 bp (1.06%: Osaka or Ehime vs. Miyazaki). Interspecific nucleotide substitutions of *D. r. rectus, D. s. striatipennis* and *D. titanus pilifer* were observed from 128 bp (14.82%) to 166 bp (19.95%).

The NJ dendrogram of *D. r. rectus* is shown in Fig. 6. In this dendrogram, populations of *D. r. rectus* exclusively constituted a clusters (100%), and two outgroups, *D. s. striatipennis* and *D. titanus pilifer*, were distantly located from this cluster. However, the relationships within *D. r. rectus* were not resolved in detail.

The percentages of sequence divergence are shown in Fig. 7. Differentiations of COI and ND5 sequence exceeded that of 16S rRNA.

Discussion

Sequence variation

Present results indicate that inter-populational sequence variations of 16S rRNA within three subspecies of *C. lignarius* are much smaller than those between all combinations of different subspecies, suggesting the monophylies of these three subspecies. Our results also suggest that sequence divergence within a single population is very low in this species (0.11%), and that differentiations increase in the order of intra-population (0–0.11%), inter-population (0–2.26%), inter-subspecies (2.80–7.99%) and inter-species (7.34–10.05%). In addition, intra-subspecific nucleotide replacements of *D. r. rectus* (0–1.06%) are much smaller than their interspecific divergence (14.82–19.95%). This result is consistent with the results for *C. lignarius*, suggesting that only a few specimens may represent the features of the sequence specific to subspecies when we choose an adequate portion of mtDNA.

Recent studies demonstrated that mutation occurs at a higher rate in the COI gene than in 16S rRNA (e.g., Coleoptera: Funk *et al.*, 1995; Hymenoptera: Dowton & Austin, 1997; Lepidoptera: Pollock *et al.*, 1998; Coleoptera: Garin *et al.*, 1999). This view was confirmed by the present results (Fig. 7). Analyses using genes with a relatively higher rate, such as COI, sometimes suffer from problems concerning the saturation of base substitution when we construct deep nodes in a phylogenetic tree (Howland & Hewitt, 1995). On the other hand, the 16S rRNA gene seems to be a good indicator to infer higher relationships.

Sequence divergences among species of *Ceruchus* (7.34–10.05%) and *Dorcus* (14.82–19.95%) are much larger than those of chrysomelid *Ophraella* (0–5.9%; Funk *et al.*, 1995), *Chrysolina* (0.78–10.2%; Garin *et al.*, 1999) and *Timarcha* (0–7.3%; Gómez-Zurita *et al.*, 2000), and cicindelid *Cicindela* (5.0–6.9%; Vogler & Person, 1996). These data may suggest that the diversification of each lucanid genus occurred earlier than those of other chrysomelid and cicindelid genera.

Phylogenetic relationships and biogeography

Ceruchus lignarius, which consists of three subspecies (Mizunuma & Nagai, 1994), is confined only to mountain environments. *Ceruchus l. lignarius* is dis-

Table 3. Numbers of nucleotide replasements (below disgonal) and sequence divergence (%; above disgonal) based on Kimura's two-parameter model for 16S rRNA gene sequences in *Dorcus rectus rectus* haplotypes. See Table 1 for the haplotype abbreviations.

	Dr1	Dr2	Dr3	Dr4	Dr5	Dr6	Dr7	Dr8	Dr9	Dr10	Dr11	Dr12	Dr13	Dr14	Dr15	Dr16	Dr17	Dr18	Dr19	Dr20
Dr1	—	0.21	0.32	0.42	0.63	0.21	0.11	0.42	0.53	0.21	0.42	0.42	0.42	0.32	0.21	0.63	0.32	0.63	0.53	0.63
Dr2	2	—	0.32	0.42	0.63	0.21	0.11	0.42	0.53	0.21	0.42	0.42	0.42	0.32	0.21	0.63	0.32	0.63	0.53	0.42
Dr3	3	3	—	0.53	0.53	0.11	0.21	0.53	0.63	0.11	0.53	0.53	0.53	0.42	0.32	0.74	0.42	0.74	0.63	0.74
Dr4	4	4	5	—	0.84	0.42	0.32	0.63	0.74	0.42	0.63	0.63	0.63	0.53	0.42	0.74	0.53	0.84	0.74	0.84
Dr5	6	6	5	8	—	0.42	0.53	0.84	0.95	0.42	0.84	0.84	0.84	0.74	0.42	1.06	0.74	1.06	0.95	1.06
Dr6	2	2	1	4	4	—	0.11	0.42	0.53	0.00	0.42	0.42	0.42	0.32	0.21	0.63	0.32	0.63	0.53	0.63
Dr7	1	1	2	3	5	1	—	0.32	0.42	0.11	0.32	0.32	0.32	0.21	0.11	0.53	0.21	0.53	0.42	0.53
Dr8	4	4	5	6	8	4	3	—	0.32	0.42	0.63	0.53	0.63	0.53	0.42	0.84	0.11	0.32	0.42	0.53
Dr9	5	5	6	7	9	5	4	3	—	0.53	0.74	0.63	0.74	0.63	0.53	0.95	0.21	0.95	0.42	0.53
Dr10	2	2	1	4	4	0	1	4	5	—	0.42	0.42	0.42	0.32	0.21	0.63	0.32	0.63	0.53	0.63
Dr11	4	4	5	6	8	4	3	6	7	4	—	0.63	0.42	0.53	0.42	0.84	0.53	0.63	0.74	0.85
Dr12	4	4	5	6	8	4	3	5	6	4	6	—	0.63	0.53	0.42	0.84	0.42	0.84	0.63	0.74
Dr13	4	4	5	6	8	4	3	6	7	4	4	6	—	0.53	0.42	0.84	0.53	0.84	0.74	0.85
Dr14	3	3	4	5	7	3	2	5	6	3	5	5	5	—	0.32	0.74	0.42	0.53	0.63	0.74
Dr15	2	2	3	4	4	2	1	4	5	2	4	4	4	3	—	0.63	0.32	0.63	0.53	0.63
Dr16	6	6	7	7	10	6	5	8	9	6	8	8	8	7	6	—	0.74	1.06	0.84	1.06
Dr17	3	3	4	5	7	3	2	1	2	3	5	4	5	4	3	7	—	0.74	0.21	0.32
Dr18	6	6	7	8	10	6	5	8	9	6	6	8	8	5	6	10	7	—	0.95	1.06
Dr19	5	5	6	7	9	5	4	3	4	5	7	6	7	6	5	8	2	9	—	0.53
Dr20	6	4	7	8	10	6	5	4	5	6	8	7	8	7	6	10	3	10	5	—

tributed in Hokkaido and north-eastern Honshu, *C. l. monticola* in central Honshu and *C. l. nodai* in Shikoku and Kyushu (Fig. 1a). *Ceruchus l. monticola* was recognized in three forms (Fujita, 1987): the Nagano form, the Yamanashi form and the Shizuoka–Kii Peninsula form. The phylogenetic relationships of *C. lignarius* inferred in the present study correspond with its distributional pattern, seemingly due to its lower mobility, illustrating that *C. lignarius* is separated to constitute two lineages, namely *C. l. nodai* and the other two subspecies. These lineages occur in two isolated areas: Hokkaido and eastern-central Honshu (*C. l. lignarius* and *C. l. monticola*) and Shikoku and Kyushu (*C. l. nodai*). These two areas are divided by the Kii channel. Although *C. l. nodai* seems not to discern morphologically among populations, there is a large sequence divergence among populations. This divergence seems to have been increased geographically by the Bungo and Houyo channels between Shikoku and Kyushu.

Within the non-*nodai* lineage, we can recognize two phylogenetic lines, *C. l. lignarius* and *C. l. monticola*, which are distributed allopatrically, as well as *C. l. nodai*. With respect to other insect taxa, some authors have pointed out morphological similarities between populations from the Kii Peninsula and those from Shikoku and central Kyushu. Most of them are treated as a single taxon, such as *Platycerus sugitai* (Okuda & Fujita, 1987) and *Pidonia shikokuana* (Kuboki, 1984). In addition, these three areas are known as the "Sohayaki element" for Japanese flora (Koidzumi, 1931; Murata & Koyama, 1976). Murata & Koyama (1976) listed ten plant species distributed in these three areas, e.g., *Hydrangea sikokiana*. Contrary to such a pattern, present results indicate a closer affinity among *C. l. monticola* populations against Shikoku and Kyushu populations of *C. l. nodai*, although morphological diversifications of the former subspecies have been reported (Fujita, 1987). Such distinct relationships between Kyushu and Shikoku populations and Kii peninsula populations, Su *et al.* (1998) reported in populations of *Damaster* (Calabid beetle).

On the other hand, *C. l. lignarius* appears to be separated by the Tsugaru channel between Hokkaido and northeastern Honshu. However, the sequence divergence between these two populations of *C. l. lignarius* is very low (0.21–0.53%). This agrees with a study on *Damaster* that reported low divergence between the Hokkaido and northeastern Honshu populations (Su *et al.*, 1998).

The nucleotide divergence observed within the Aomori population of *D. r. rectus* (0–0.84%) is as large as those among populations (0–1.06%). Some haplotypes of Aomori were observed in other populations (Dr1: Hokkaido, Dr6: Iwate, Dr7: Ishikawa and Okayama). Moreover, the phylogenetic relationships of *D. r. rectus* could not be resolved well, suggesting an

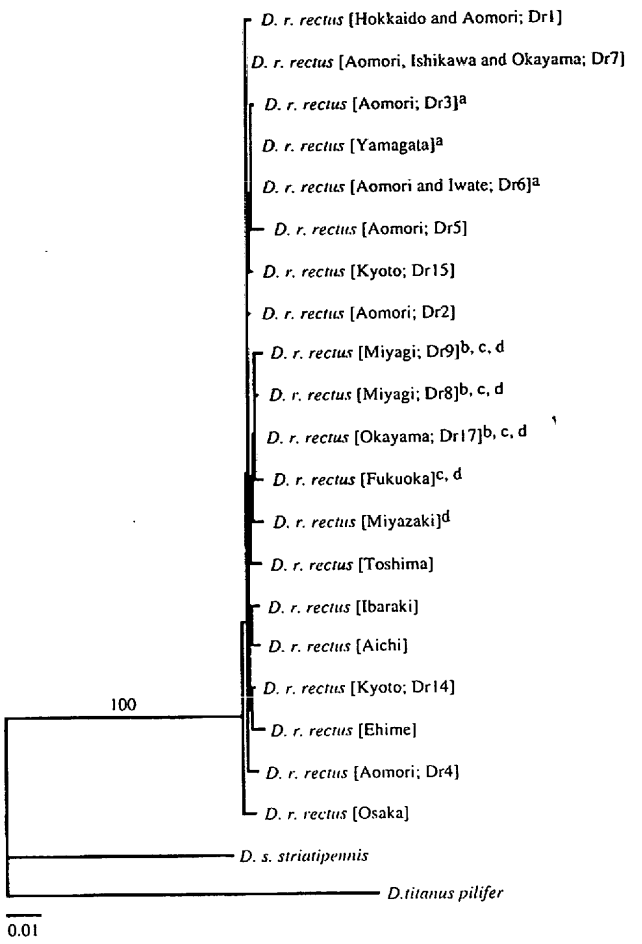


Fig. 6. NJ dendrogram for populations belonging to *D. r. rectus* derived from 16S rRNA gene sequences. Bootstrap values are given for each branch. Four cluster were supported in > 50% of bootstrap value; a 60%, b 63%, c 54% and d 54%. Bar equals 0.01 Kimura's two-parameter distance.

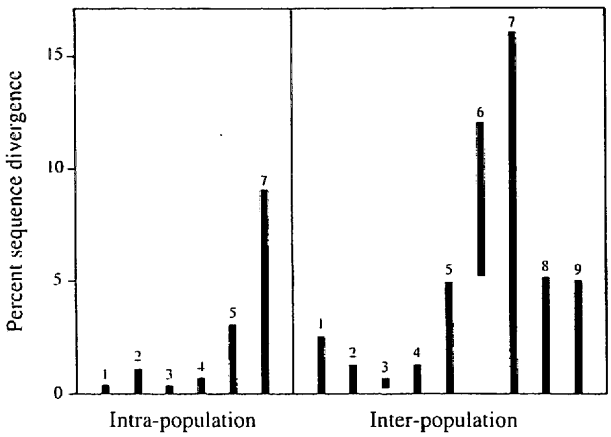


Fig. 7. Percentage mtDNA sequence divergences in Coleoptera species. Ranges of sequence divergence were represented by vertical lines. Pairwise sequence divergence was calculated between indiviuvals on intra-population and inter-population. Numbers indicate the following references (with the species and the sequenced genes given in parentheses): 1, present study (Lucanidae: *C. lignarius*; 16S rRNA); 2, present study (Lucanidae: *D. r. rectus*; 16 S rRNA); 3, Vogler *et al.* (1993) (Cincindelidae: *Cincindela dorsalis dorsalis*; COIII, ND1, $tRNA^{leu}$, 16S rRNA); 4, Vogler & DeSalle (1993) (Cincindelidae: *Cicindela dorsalis* subspecies; COIII, ND 1, $tRNA^{leu}$, 16S rRNA); 5, Funk *et al.* (1995) (Chrysomelidae: *Ophraella communa*, COI); 6, Juan *et al.* (1996) (Tenebrionidae: *Hegeter* species; COI); 7, Juan *et al.* (1998) (Tenebrionidae: *Hegeter politus*; COI); 8, Su *et al.* (1996) (Carabidae: *Ohomopterus* species; ND5, $tRNA^{Phe}$); and 9, Su *et al.* (1998) (Carabidae: *Damaster blaptoides* subspecies; ND5, $tRNA^{Phe}$).

almost concurrent radiation. These may reflect a high ability of migration and continuous distribution of *D. r. rectus* from lowlands to montane regions throughout Japan.

The larvae of these two lucanids have different food habit. The larva of *C. lignarius* is stenophagous for advanced decay stage of brown rot (Araya, 1993). On the other hand, the larva of *D. r. rectus* is euryphagous for both decay stages and decay types (Okajima & Yamaguchi, 1988). The dead wood in which *D. r. rectus* larvae occurred comprised many kinds of broad-leaved trees (e.g., *Quercus acutissima* and *Q. serrata*), widely distributed from lowlands to montane regions. This widely food habit of *D. r. rectus* may influences on continuous distribution and mix of haplotypes among populations.

Acknowledgments

We wish to thank A. Abe for providing materials and literatures and offering his valuable advice, S. Osawa, M. Matsui, T. Hikida, M. Kon, T. Yagi, Z.-H. Su and C.-G. Kim for various kinds of help, and K. Mizota and K. Nishikawa for providing us with samples. We also thank T. Hamaya, S. Kawada, T. Nomiya, S. Makita, A. Okumoto, M. Matsuda, Y. Kamidaira, N. Nakagawa and K. Iwata for their assistance in sample collection. This study was partially supported by a Grants-in-Aid from the Ministry of Education, Science, Sports and Culture of Japan (No. 11833007).

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Appendix

Samples

Localities and size of samples used in this study were given below.

Ceruchus lignarius lignarius Lewis, 1883: Maruyama Park, Sapporo, Hokaido (N=1); Mt. Ozaki, Hiraka, Aomori Pref. (N=1); Mt. Gassan, Yamagata Pref. (N=1); Mt. Akagi, Gunma Pref. (N=1). *Ceruchus lignarius monticola* Nakane, 1978: (Nagano form) Tobira, Matsumoto, Nagano Pref. (N=1); (Yamanashi form) Mt. Fuji (N=10); (Shizuoka–Kii Peninsula form) Mt. Kunimi, Mie Pref. (N=1). *Ceruchus lignarius nodai* Fujita, 1987: Minokoshi, Mt. Tsurugi, Tokushima Pref. (N=1); Shiratoriyama woodland pass, Izumi, Kumamoto Pref. (N=1); Mt. Shiraiwa, Miyazaki Pref. (N=1). *Ceruchus piceus* (Web., 1801): Toronto, Canada (N=1).

Dorcus rectus rectus (Motschulsky, 1857): Tomiuchi, Hobetsu, Hokkaido (N=1); Meya dam, Nishimeya Vil., Aomori Pref. (N=10); Tayama, Ashiro, Iwate Pref. (N=1); Nagamine, Taihaku, Sendai, Miyagi Pref. (N=2); Iide, Yamagata Pref. (N=1); Muramatsu, Tokai, Ibaraki Pref. (N=1); Mt. Miyatsuka, Toshima I. (N=1); Nishiakazawa, Toyohashi, Aichi Pref. (N=1); Ichinose, Ishikawa Pref. (N=1); Iwakura, Sakyo, Kyoto, Kyoto Pref. (N=2); Mt. Makio, Izumi, Osaka Pref. (N=1); Yoshinaga, Okayama Pref. (N=2); Mutsuki I., Ehime Pref. (N=1); Motooka, Nishi, Fukuoka, Fukuoka Pref. (N=1); Heiwa Park, Miyazaki, Miyazaki Pref. (N=1). *Dorcus striatipennis striatipennis* (Motschulsky, 1861): Tsuta, Aomori Pref. (N=1). *Dorcus titanus pilifer* (Vollenhoven, 1861): Kyoto Univ., Sakyo, Kyoto, Kyoto Pref. (N=1).

(Received February 7, 2001; Accepted June 25, 2001)