Pattern of Morphological Diversification in the *Leptocarabus* Ground Beetles (Coleoptera: Carabidae) as Deduced from Mitochondrial ND5 Gene and Nuclear 28S rDNA Sequences

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Most of the mitochondrial NADH dehydrogenase subunit 5 (ND5) gene and a part of nuclear 28S ribosomal RNA gene were sequenced for 14 species of ground beetles belonging to the genus *Leptocarabus.* In both the ND5 and the 28S rDNA phylogenetic trees of *Leptocarabus,* three major lineages were recognized: (1) *L. marcilhaci*/*L. yokoae*/*Leptocarabus* sp. from China, (2) *L. koreanus*/*L. truncaticollis*/*L. seishinensis*/*L. semiopacus*/*L. canaliculatus*/*L. kurilensis* from the northern Eurasian continent including Korea and Hokkaido, Japan, and (3) all of the Japanese species except *L. kurilensis.* Clustering of the species in the trees is largely linked to their geographic distribution and does not correlate with morphological characters. The species belonging to different species groups are clustered in the same lineages, and those in the same species group are scattered among the different lineages. One of the possible interpretations of the present results would be that morphological transformations independently took place in the different lineages, sometimes with accompanying parallel morphological evolution, resulting in the occurrence of the morphological species belonging to the same species group (=type) in the different lineages.

Introduction

Leptocarabus is a well-known higher taxon in the subtribe Carabina of the family Carabidae. Although often ranked as one of the subgenera of the grand genus *Carabus,* we treat it as a distinct genus for convenience. The genus contains 20 species (Imura and Mizusawa 1996; Kwon and Lee 1984). All of the *Leptocarabus* species are hindwingless and can move only by walking. They are distributed throughout the northern and eastern Eurasian continent including the Korean Peninsula, the Japanese Islands, Sakhalin, and the Kurils. Most of the species are large and elongate in shape and black to brown in color, while some others are characterized by a smaller size and metallic tint to the body (fig. 1). The *Leptocarabus* species have been well studied taxonomically and are classified into several subgenera (see below) (Nakane 1961, 1962; Ishikawa 1972, 1992; Kwon and Lee 1984; Imura and Mizusawa 1996). According to Imura and Mizusawa (1996), they are classified into five species groups (hereinafter called ''types'' for convenience), *Procerulus* (P) (=subgenus *Leptocarabus* [s. str.]), *Semiopacus* (S) (=subgenus *Adelocarabus*), *Koreanus* (K) (=subgenus *Weolseocarabus*), *Canaliculatus* (C) (=subgenus *Aulonocarabus*), and *Truncaticollis* (T) (no proposed subgenus), based on the morphological characters (table 1). The P type contains *Leptocarabus marcilhaci, Leptocarabus yokoae,* and *Leptocarabus* sp. from central China and *Leptocarabus procerulus, Leptocarabus kumagaii, Leptocarabus hiurai,* and *Leptocarabus kyushuensis* from Japan. This type is said to be closely related to the K type based on morphological characters (Ishikawa 1972, 1989). The S type contains *Leptocarabus semiopacus, Leptocarabus seishinensis,* a few other species mainly from Korea, and *Leptocarabus arboreus* from Japan and Sakhalin (Kwon and Lee 1984; Ishikawa 1992; Imura and Mizusawa 1996). The K type is composed of a single species, *Leptocarabus koreanus,* which inhabits the Korean Peninsula (Kwon and Lee 1984; Imura and Kezuka 1992). The C type species are widely distributed in the eastern Eurasian continent, Sakhalin, Hokkaido, and the Kurils. The T type contains four species which are widely distributed from the northeastern part of the Eurasian continent to Alaska (Imura and Mizusawa 1996).

The taxonomic and phylogenetic studies for the genus *Leptocarabus* based on morphology need to be reexamined with more explicit procedures, such as molecular evolution studies, because discrepancies have often been reported between morphology and molecular studies for some other carabid group (Su et al. 1996*b,* 1998). In the present paper, phylogenetic relationships have been estimated by analyzing a large part of the ND5 gene sequences and a reasonable length of the nuclear 28S rDNA of 52 examples covering the representative *Leptocarabus* species and geographic races from their known localities. The molecular phylogeny uncovered an interesting evolutionary history of *Leptocarabus* that cannot be reached by cladistic analysis that depends on morphology alone.

Materials and Methods

Isolation of DNA

Names and localities of the specimens used are shown in table 2 and figure 2. To prevent degradation of DNA, the specimens were immediately killed in 95% ethanol and stored in ethanol until use. A single adult individual of each species was used for DNA extraction. Total DNA was extracted from thorax muscle (10–25 mg) using the QIAamp tissue kit (QIAGEN GmbH, Germany).

Key words: *Leptocarabus* ground beetles, mitochondrial ND5 gene, nuclear 28S rDNA, phylogeny, parallel evolution.

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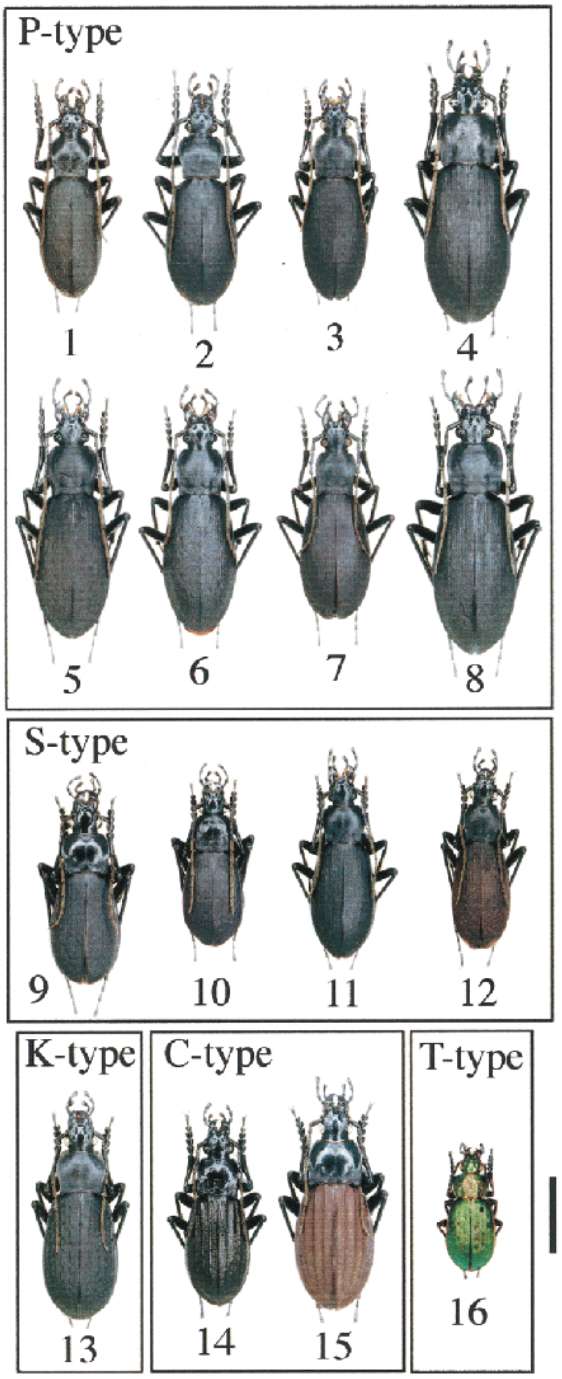

FIG. 1.—*Leptocarabus* ground beetles. 1—*L. marcilhaci*; 2—*L. yokoae*; 3—*L. kyushuensis kyushuensis*; 4—*L. kyushuensis cerberus*; 5—*L. hiurai*; 6—*L. procerulus procerulus*; 7—*L. procerulus miyakei*; 8—*L. kumagaii*; 9—*L. semiopacus*; 10—*L. seishinensis seishinensis*; 11—*L. arboreus arboreus*; 12—*L. arboreus gracillimus*; 13—*L. koreanus coreanicus*; 14—*L. kurilensis rausuanus*; 15—*L. canaliculatus jankowskiellus*; 16—*L. truncaticollis.* Scale bar: 10 mm.

PCR Amplification and DNA Sequencing

Altogether, 52 specimens from the Eurasian continent and the Japanese Islands were analyzed. Total DNA was used as a template for amplification of ND5 DNA and 28S rDNA fragments by the polymerase chain reaction (PCR) (Saiki et al. 1988). The 1,084-bp sequence

Table 1
Morphological Characters of the Five Species Groups (Types)

Species Group (Type)	Size	Proportion	Color	Setae of Submentum	Primary Costae of Elytra	Male Genitalia			
						Ostium Lobe	Basal Lobe	Median Lobe	Aggonoporius
Procerulus (P)	Large	Long and slender	Black and mat	Basically absent	Faint	Vestigial	Absent	Present	Often developed
Semiopacus (S)	Rather small	Slender, rather robust	Blackish brown and mat	Present	Faint	Vestigial	Present	Absent	Vestigial
Koreanus (K)	Large	Robust	Black and mat	Present	Strong	Well developed and bilobed	Absent	Present	Vestigial
Canaliculatus (C)	Medium	Rather slender, robust	Blackish or reddish brown	Present	Very strong	Small	Present	Absent	Vestigial
Truncaticollis (T)	Small	Rather robust	Black with metallic tint	Present	Weak	Vestigial	Present	Absent	Vestigial

Table 2
List of the *Leptocarabus* Specimens Used in this Study

Species Group (Type)	Species and Subspecies (by morphology)	Locality No.	Locality	DDBJ/EMBL/GenBank Accession No. (ND5/28S)
Procerulus (P).....	*marcilhaci*	1	Wudu, South Gansu, China	AB031425/AB031399
	sp.	2	Mt. Guangwu shan, northeast Sichuan, China	AB031426/AB031400
	sp.	3	Daba, northeast Sichuan, China	AB031427/AB031401
	yokoae yokoae	4	Shennongjia, west Hubei, China	AB031428
	kyushuensis cerberus	5	Kure, Hiroshima, Japan	D50356[a]/AB031402
	kyushuensis cerberus	6	Mt. Kanmuriyama, Hiroshima, Japan	AB031430
	kyushuensis nakatomii	7	Kurayoshi, Tottori, Japan	AB031431
	hyushuensis nakatomii	8	Matsue, Shimane, Japan	AB031432
	kyushuensis kyushuensis	9	Gokase, Miyazaki, Japan	AB031433
	kyushuensis kyushuensis	10	Otsu, Kumamoto, Japan	AB031434
	kyushuensis kyushuensis	11	Hitoyoshi, Kumamoto, Japan	AB031435/AB031403
	hiurai	12	Saijo, Ehime, Japan	AB031436/AB031404
	hiurai	13	Hongawa, Kochi, Japan	AB031437
	hiurai	14	Higashi-iyayama, Tokushima, Japan	AB031438
	procerulus miyakei	15	Hinokage, Miyazaki, Japan	AB031439/AB031405
	procerulus miyakei	16	Mt. Hikosan, Fukuoka, Japan	AB031440
	procerulus procerulus	17	Mutsu, Aomori, Japan	AB031441/AB031405
	procerulus procerulus	18	Maze, Gifu, Japan	AB031442
	procerulus procerulus	19	Gonbee-toge, Nagano, Japan	D50357[a]
	procerulus procerulus	20	Suzuka, Mie, Japan	AB031444
	procerulus procerulus	21	Murayama, Yamagata, Japan	AB031445
	procerulus procerulus	22	Asago, Hyogo, Japan	AB031446
	procerulus procerulus	23	Murakami, Niigata, Japan	AB031447
	kumagaii	24	Takayama, Gifu, Japan	AB031448
	kumagaii	25	Hirakata, Osaka, Japan	AB031449
	kumagaii	26	Minobu, Yamanashi, Japan	AB031450
Koreanus (K)	*koreanus coreanicus*	27	Gonjiam, Kyonggi-do, Korea	AB031451/AB031406
Semiopacus (S)....	*semiopacus*	28	Jiri-san Mountains, Kyongsangnam-do, Korea	AB031452/AB031407
	semiopacus	29	Jungsanli, Kyongsangnam-do, Korea	AB031453
	semiopacus	30	Muju, Chollabuk-to, Korea	AB031454/AB031408
	semiopacus	31	Mt. Palgong-san, Kyongsangpuk-to, Korea	AB031455
	semiopacus	32	Odae-san Mountains, Kangwon-do, Korea	AB031456/AB031409
	seishinensis seishinensis	33	Gonjiam, Kyonggi-do, Korea	AB031457/AB031410
	seishinensis seishinensis	34	Odae-san Mountains, Kangwondo, Korea	AB031458
	seishinensis seunglaki	35	Jiri-san Mountains, Kyongsangnam-do, Korea	AB031459/AB031411
	seishinensis seunglaki	36	Jungsanli, Kyongsangnam-do, Korea	AB031460
	arboreus arboreus	37	Hakodate, Hokkaido, Japan	AB031461/AB031412
	arboreus arboreus	38	Fukushima, Hokkaido, Japan	AB031462
	arboreus pararboreus	39	Kitami, Hokkaido, Japan	AB031463
	arboreus shimoheiensis	40	Morioka, Iwate, Japan	AB031464
	arboreus parexilis	41	Mt. Kurikoma-yama, Miyagi, Japan	AB031465
	arboreus fujisanus	42	Yamanaka-ko, Yamanashi, Japan	AB031466
	arboreus nepta	43	Mutsu, Aomori, Japan	AB031467
	arboreus shinanensis	44	Chino, Nagano, Japan	AB031468/AB031413
	arboreus gracillimus	45	Mt. On-take, Gifu, Japan	AB031469/AB031414
Canaliculatus (C) ..	*kurilensis rausuanus*	46	Tokachi, Hokkaido, Japan	D50341[a]
	canaliculatus canaliculatus	47	Terelji, Mongolia	AB031471/AB031415
	canaliculatus canaliculatus	48	Academia Obrucheva Mts., Russia	AB031472/AB031415
	canaliculatus sichotensis	49	Vysokogornyi, Amur, Russia	AB031473
	canaliculatus canaliculatus	50	Beijing, China	AB031416
	canaliculatus jankowskiellus	51	Dandong, Liaoning, China	AB031474/AB031415
Truncaticollis (T) ..	*truncaticollis*	52	Ural, Russia	AB031475/AB031417
Outgroup	*Campalita chinense*	53	Oasa, Hiroshima, Japan	D50343[a]/AB031418
	Calosoma inquisitor	54	Tokachi, Hokkaido, Japan	D50342[a]/AB031419

[a] Taken from Su et al. (1996*a*).

which contains a 1,069-bp 3′ region of the ND5 gene, 8 bp of noncoding sequence, and a 7-bp 5′ terminus of the Phe-tRNA gene was amplified by the ver 1.04 primer (5′-GTC ATA CTC TAA ATA TAA GCT A-3′) and the ver 1.06 primer (5′-CCT GTT TCT GCT TTA GTT CA-3′) (Su et al. 1996*a*). The 852–881-bp sequence of the 5′ region of 28S rDNA was amplified by the 28S-01 primer (5′-GAC TAC CCC CTG AAT TTA AGC AT-3′) and the 28SR-01 primer (5′-GAC TCC TTG GTC CGT GTT TCA AG-3′), which were designed from the 28S rDNA sequences of *Drosophila melanogaster* (Tautz et al. 1988) and *Xenopus laevis* (Clark et al. 1984). Mostly, the two primers used for PCR were sufficient to read 1,084 bp of the ND5 sequences and

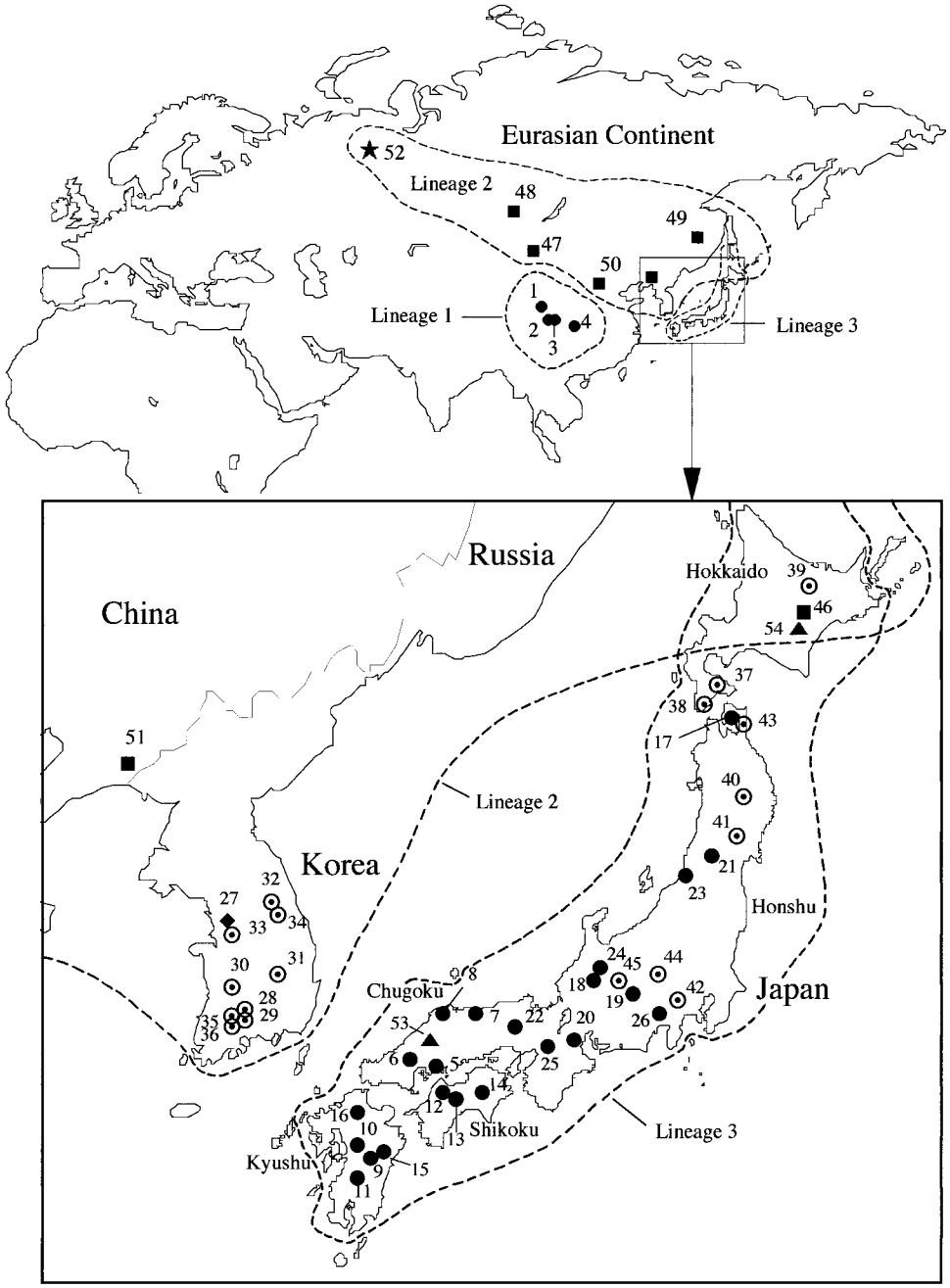

FIG. 2.—Specimen locations of the *Leptocarabus* species used in the present study. (⊙) S type species; (●) P type species; (♦) K type species; (■) C type species; (★) T type species; (▲) outgroup species. Numerals correspond to the locality numbers in table 2 and figures 3–5. Approximate distribution boundaries for respective lineages are shown by dotted lines.

852–881 bp of the 28S rDNA sequences. In some cases, four internal primers were used for the ND5 gene: forward primers Ezo-2 (5′-TTC ATC TTT TAA CTC ATG CA-3′) and RC4-4 (5′-GAT CAA GGT TGA AAT GAA T-3′), and reverse primers AO-3 (5′-ATA TTC ATT TCA ACC TTG ATC-3′) and RCE-2 (5′-TGC ATG AGT TAA AAG ATG AA-3′) (Su et al. 1998). PCR amplifications were carried out in a 100-μl mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 0.2 mM of each dNTP, 100 pM of each primer, and 2.5 U of *Taq* polymerase (TaKaRa EX Taq, Takara). Each reaction mixture was overlaid by one drop of mineral oil. PCR was performed for 35 cycles of denaturation at 94°C for 1 min, primer annealing at 50°C for 1 min, and extension at 70°C for 2 min. The final single cycle was performed under the same conditions but with an extension step at 70°C for 7 min using a DNA Thermal Cycler 480 (Perkin Elmer). The PCR product was purified with QIAquick PCR purification kit (QIAGEN GmbH).

Direct sequencing was performed with an automated ABI PRISM 377 DNA sequencer using the dideoxy chain termination method (Sanger, Nicklen, and Coulson 1977). The reaction mixture for cycle sequenc-

ing consisted of 6 μl of dRhodamine terminator cycle sequencing ready reaction with AmpliTaq DNA Polymerase, FS (Applied Biosystems, Foster City, Calif.), 0.1–0.3 pmol/μl of DNA, 2.4 μl (1 pmol/μl) of sequencing primer, and distilled water to a total volume of 15 μl. The cycle-sequencing conditions were 25 cycles at 96°C for 10 s, 50°C for 5 s, and 60°C for 4 min, followed by an indefinite hold at 4°C using a GeneAmp PCR system 9600 (Perkin Elmer). The DNA product was cleaned with Centri-Sep spin columns (Applied Biosystems) and vacuum-dried before applying. The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL, and GenBank nucleotide sequence databases with the accession numbers shown in table 2.

Phylogenetic Analysis

The ND5 and the 28S rDNA sequences were aligned and compared using the multiple-alignment program CLUSTAL W (Thompson, Higgins, and Gibson 1994) and DNASIS, version 3.7 (Hitachi Software Engineering, Japan). The evolutionary distances (*D*) were computed by Kimura's (1980) two-parameter method, and the phylogenetic trees were constructed by the neighbor-joining (NJ) method (Saitou and Nei 1987) and the unweighted pair grouping method with arithmetic means (UPGMA). All of these processes were performed with the DNA sequence analysis package SINCA, version 3.0 (Fujitsu System Engineering, Japan). A maximum-parsimony (MP) tree was also constructed with PHYLIP, version 3.5 (Felsenstein 1993). Bootstrap analysis was performed for all the trees (Felsenstein 1985) based on 500 resamplings. The gene sequences of two Calosomina species were used as an outgroup.

Dating

For setting the timescale, a 0.01*D* unit corresponding to 3.6 Myr was used (Su et al. 1998; Osawa et al. 1999; revised by Su et al. [unpublished data]).

Results and Discussion

Sequence Divergence and G+C Content of DNA

The maximum sequence divergences of the ND5 gene were 3.81% for the Japanese species and 11.24% for the species on the Eurasian continent, indicating that the Japanese species are much more closely related to each other than are the species on the continent. The maximum sequence divergences of the 28S rDNA were 0.81% for the Japanese species and 2.76% for the continental species (table 3). The 28S rDNA evolved about one fourth as fast as the ND5 gene, so a meaningful resolution between allied species was not possible on the 28S rDNA phylogenetic tree. There were no insertions/deletions and no length variations for the ND5 gene throughout the species examined. A few deletions were found in some 28S rDNA sequences. The G+C content was nearly constant (21 ± 0.5%) for the ND5 gene, and it was 58 ± 0.8% for 28S rDNA in the *Leptocarabus* species that were analyzed.

Relation Between Phylogeny and Morphology

Figures 3 and 4 show the NJ phylogenetic trees of the ND5 gene and the nuclear 28S rDNA, respectively.

Table 3
Pairwise Sequence Divergence (by Kimura's [1980] Two-Parameter Method) for ND5 DNA and 28S rDNA of the Representative *Leptocarabus* Species that Were Examined in this Study

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1. *marcilhaci* (1)		11.24	9.51	8.55	9.51	8.54	9.40	9.20	9.82	10.15	10.36	9.72	9.72	10.14	9.92	13.62
2. *koreanus coreanicus* (27)	2.27		9.43	8.07	8.79	7.54	8.37	7.65	10.86	10.99	11.42	10.32	10.43	10.76	10.54	14.70
3. *truncaticollis* (52)	2.03	2.25		6.81	7.53	6.10	7.02	6.61	10.01	10.79	11.00	10.12	10.23	10.55	10.55	13.35
4. *seishinensis seishinensis* (33/34)	1.79	1.89	1.53		5.49	5.21	5.30	4.81	9.48	9.48	9.93	8.96	9.17	9.38	9.38	13.02
5. *semiopacus* (28)	2.76	1.53	2.25	1.76		6.12	6.41	5.40	9.90	10.68	10.89	9.80	9.91	10.23	10.01	13.79
6. *canaliculatus canaliculatus* (47)	1.43	1.65	1.05	0.94	1.88		2.35	2.55	9.59	9.83	10.14	9.18	9.49	9.60	9.39	13.02
7. *kurilensis rausuanus* (46)	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.		3.03	10.01	10.36	10.68	9.17	9.69	9.59	9.38	14.14
8. *canaliculatus jankowskiellus* (51)	1.43	1.65	1.05	0.94	1.88	0.00	n.e.		8.95	9.72	10.03	8.64	8.96	9.07	8.85	12.57
9. *kyushuensis kyushuensis* (10)	2.28	2.61	2.26	2.25	2.73	1.65	n.e.	1.65		3.81	3.71	3.12	2.83	3.32	3.12	13.11
10. *hiurai* (12)	2.28	2.61	2.26	2.25	2.73	1.65	n.e.	1.65	0.80		1.97	1.87	1.78	3.03	2.06	13.79
11. *procerulus miyakei* (15)	2.65	2.61	2.50	2.25	2.97	1.89	n.e.	1.89	0.80	0.80		1.97	1.87	2.06	2.12	13.59
12. *procerulus procerulus* (17)	2.65	2.61	2.50	2.50	2.97	1.89	n.e.	1.89	0.80	0.80	0.80		0.64	0.65	0.37	12.90
13. *arboreus arboreus* (37)	2.41	2.13	2.27	2.02	2.62	1.66	n.e.	1.66	0.57	0.57	0.57	0.34		1.11	1.02	12.90
14. *arboreus shinanensis* (44)	2.16	2.49	2.38	2.14	2.74	1.78	n.e.	1.78	0.68	0.68	0.68	0.80	0.81		0.83	13.68
15. *kumagaii* (25)	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.		12.90
16. *Campalita chinense* (53)	3.72	4.99	4.87	4.47	4.86	4.48	n.e.	4.48	4.58	4.58	4.83	4.83	4.72	4.33	n.e.	

NOTE.—The numbers above the diagonal are for the ND5 gene, and those below the diagonal are for 28S rDNA. Numbers in parentheses are locality numbers. n.e. = not examined.

The UPGMA and MP trees gave essentially the same topology (not shown). Three major lineages are recognized in both the ND5 and 28S rDNA trees. Lineage 1 is composed of three Chinese species, *L. marcilhaci*, *L. yokoae*, and *Leptocarabus* sp., all belonging to the *Procerulus* (P) type (for classification of the type = species group, see *Introduction*). Lineage 2 includes six species from the northeastern Eurasian continent including the Korean Peninsula and Hokkaido, Japan: *L. koreanus* (*Koreanus* [K] type), *L. truncaticollis* (*Truncaticollis* [T] type), *L. seishinensis* and *L. semiopacus* (both *Semiopacus* [S] type), and *L. canaliculatus* and *L. kurilensis* (both *Canaliculatus* [C] type). All the Japanese species belong to lineage 3, which contains *L. kyushuensis*, *L. hiurai*, *L. procerulus*, and *L. kumagaii* (all P type) and *L. arboreus* (S type). Of special interest is that *L. truncaticollis* is very different from other *Leptocarabus* species in appearance (see fig. 1), and yet it belongs to lineage 2 together with the S and C type species. Thus, clustering of the species examined is closely related to their geographic distribution and does not correlate with morphologically defined type. The members of the P type appear in both lineage 1 and lineage 3; those of the S type appear in lineages 2 and 3, which are, respectively, related to the species belonging to the other types in the same lineage on the phylogenetic trees.

Interpretations of the Results

There may be several ways to interpret the phylogenetic trees obtained in this study. One is that the P type is the ancestral form of the genus, and the appearance of the P type in lineages 1 and 3 is simply explained by shared ancestral characters. This is possible, but the ND5 tree suggests an almost simultaneous diversification of the three lineages. On the 28S rDNA tree, lineage 1 forms the most basal branch of the genus. Since the base substitution rate of 28S rDNA is much slower than that of the ND5 gene, the molecular clock by the ND5 gene may be more accurate than that by 28S rDNA. The primary purpose of the use of 28S rDNA is simply to show the existence of the three lineages, each containing the same members as the ND5 tree. Thus, we tentatively explain the parallel appearance of the P and S types in lineages 1 and 3.

The possibility of lineage sorting of ancestral polymorphism is now considered. If the ancestral species or population is assumed to contain both P and S (and the other) types, the current character distribution may be explained by random lineage sorting. For the reason mentioned above, there is at present no way to specify the ancestral form. Suppose that the P type is the ancestral form. The distribution range of the P type in lineage 1 is exclusively distributed in central China, where no other types have been discovered in reasonably extensive expeditions by a number of entomologists. The distribution range of lineage 1 does not overlap with that of the other types (see fig. 2). There is no evidence showing the presence of polymorphism in a single population. Of course, this does not exclude its absence in the past. The ancestral polymorphism–random lineage sorting possibility may be theoretically pleasing, but it cannot be verified.

One might argue that the ND5 phylogeny would be brought about by introgression of the mitochondrial gene via hybridization. This possibility can be largely ruled out by the overall congruence of the 28S rDNA and the ND5 phylogenies. Furthermore, the distributional isolation of the three lineages, which would have occurred long time ago, is not consistent with the introgression hypothesis.

One plausible interpretation for the appearance of the same type in different lineages would be that parallel evolution took place for the P type in lineages 1 and 3 and for the S type in lineages 2 and 3. If the P type is the ancestral form, parallelism should be considered only for the S type. In addition, the intermingled occurrence of different types in lineages 2 and 3 suggests the morphological transformation from one type to another within the respective lineages. The situation resembles that of *Ohomopterus* (Su et al. 1996*b*), in which taxonomically the ''same species'' or the members belonging to the same species group (=type) appear in more than two different places on the ND5 tree. Su et al. (1996*b*) proposed that parallel evolution took place in different lineages, possibly through discontinuous morphological transformation called ''type-switching.''

Diversification Within the Respective Lineages

The branching of the three lineages mentioned above seems to have started within a short time, as judged from the ND5 tree. No ancestral lineage can be defined (see above). Within lineage 2, to which the northern and eastern Eurasian species belong, initiation of diversification into various species is much older than that in lineage 3, to which all of the Japanese species belong. *Leptocarabus koreanus*, *L. truncaticollis*, *L. seishinensis*, and *L. semiopacus*, respectively, form a well-defined cluster. The *L. canaliculatus* specimens from various localities are clustered together, and *L. kurilensis* from Hokkaido is included in this cluster, although *L. canaliculatus* and *L. kurilensis* are usually regarded as two distinct species by most morphologists. This suggests that *L. kurilensis* branched off from *L. canaliculatus*, with accompanying morphological changes. Thus, taxonomy based on morphology at the species level is consistent with the ND5 and 28S rDNA phylogeny in lineage 2, except that *L. kurilensis* was not separated from *L. canaliculatus*. In lineage 3 (fig. 5), two sublineages are recognized: the *L. kyushuensis* sublineage (sublineage 1), which is further divided into two clades containing, respectively, the habitants in Kyushu and those in Honshu (the Chugoku district), and sublineage 2, which consists of the habitants in Hokkaido, Honshu, Shikoku, and Kyushu. Within sublineage 2, four clades are recognized: clade a (*L. procerulus miyakei* in Kyushu), clade b (*L. hiurai* in Shikoku), clade c (*L. arboreus* in Hokkaido), and clade d (the habitants in Honshu [*L. procerulus procerulus*, *L. kumagaii*, and *L. arboreus*]). Thus, clustering is more or less linked to geographical distribution. Especially noteworthy are the inhabitants of Honshu (sublineage 2, clade d). As noted

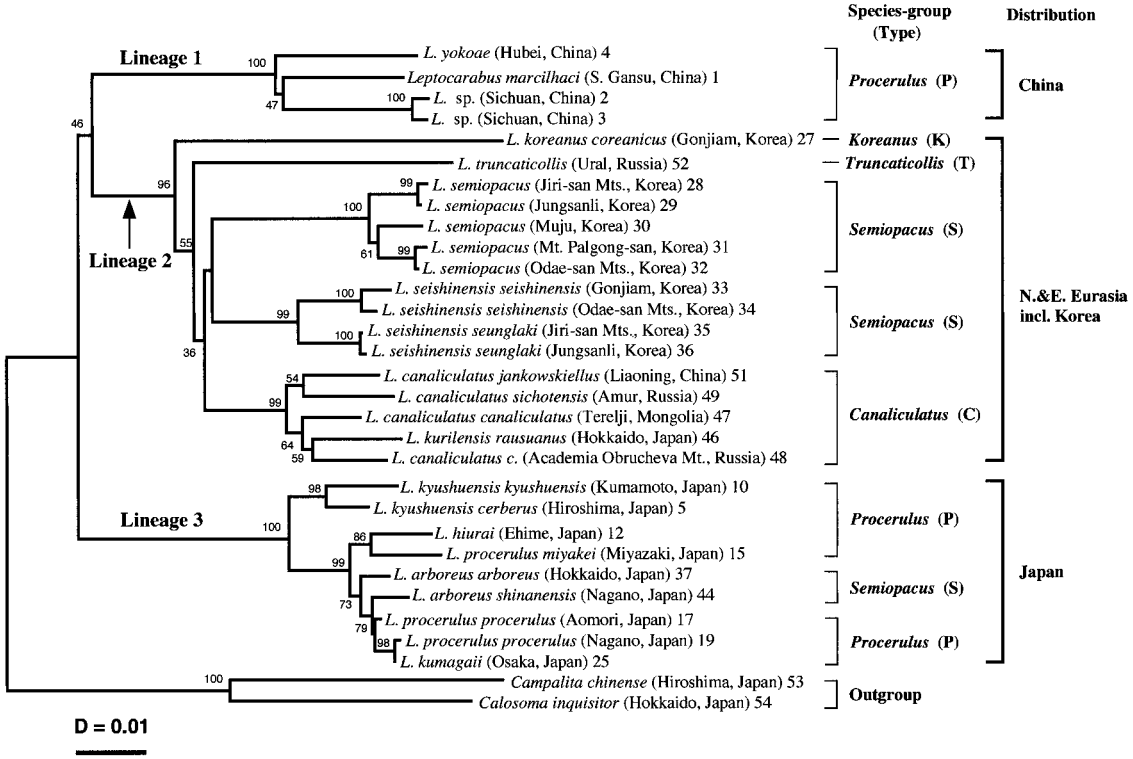

FIG. 3.—Neighbor-joining phylogenetic tree of the ND5 gene of the genus *Leptocarabus*. Throughout figures 3–5, distance (*D*) denotes Kimura's (1980) two-parameter evolutionary distance. The value at the node represents the percentage bootstrap confidence level based on 500 resamplings. The tree was outgroup-rooted using the ND5 gene sequences of two species of the Calosomina, *Calosoma inquisitor* and *Campalita chinense*. The symbol of type (=species group) by morphology (Imura and Mizusawa 1996) is indicated on the right. For explanation of the types, see the text.

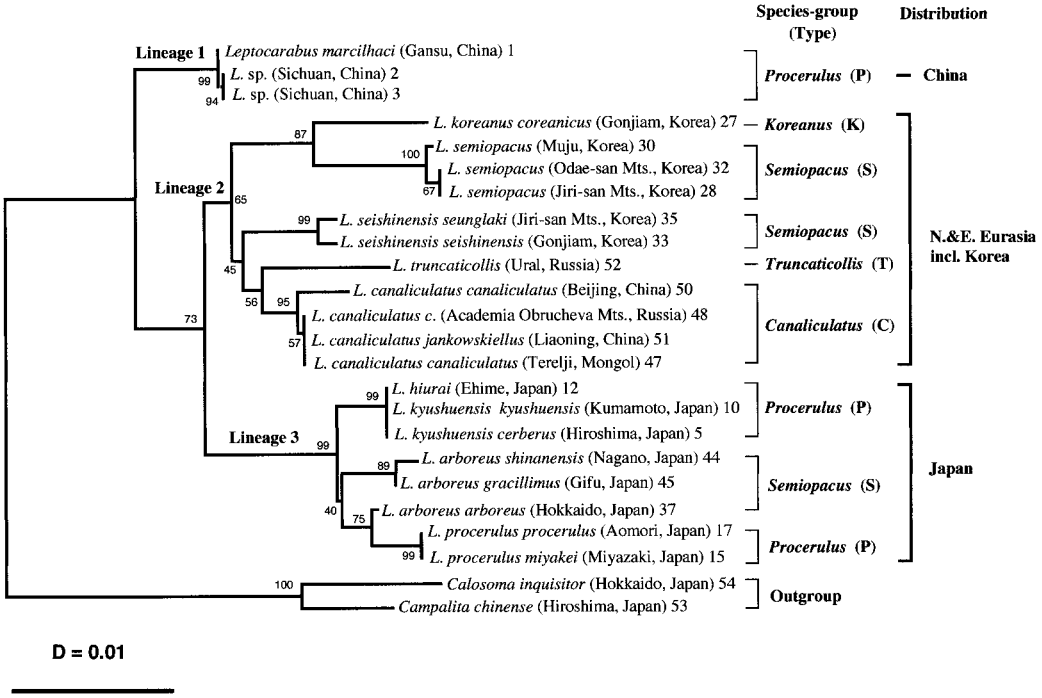

FIG. 4.—Neighbor-joining phylogenetic tree of nuclear 28S rDNA of the genus *Leptocarabus*.

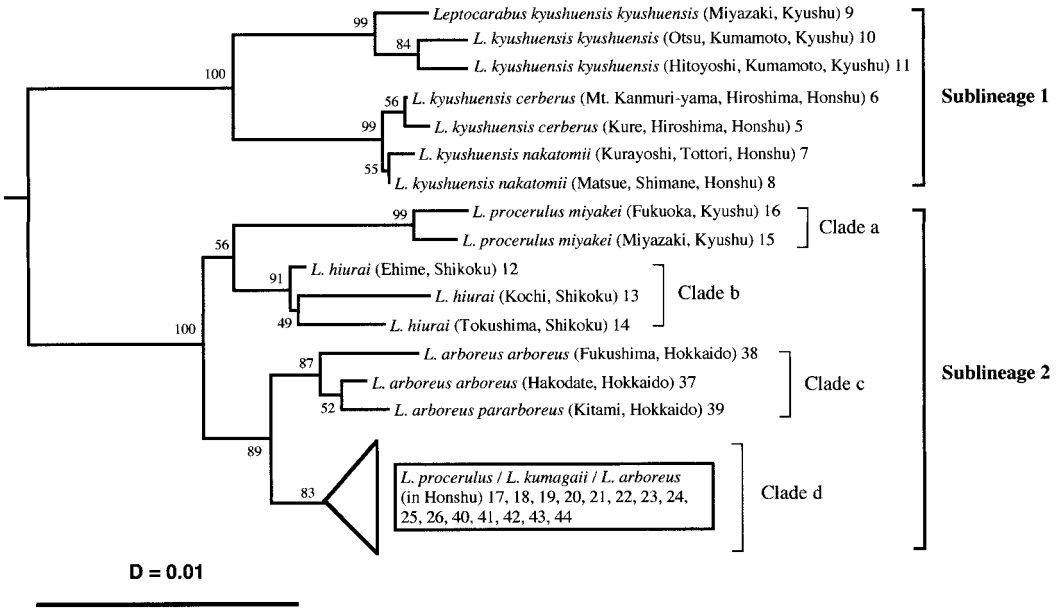

FIG. 5.—Neighbor-joining phylogenetic tree of the ND5 gene of the Japanese *Leptocarabus.*

above, in clade d there occur three morphological species, *L. procerulus* (P type), *L. kumagaii* (P type), and *L. arboreus* (S type). These three species are clearly separable morphologically, and yet the evolutionary distances among them are either null or very small (less than 0.5% difference), such that they are unresolved on the ND5 phylogenetic tree. This would represent ''evolution in action''; i.e., these (sub)species recently started their speciation, in which ''type-switching'' would be involved. Thus, besides intermingled occurrence of the species of different types in one sublineage, taxonomically the ''same species'' appear in different sublineages or clades. For example, *L. procerulus* appears in clade d (subspecies *procerulus*) and clade a (subspecies *miyakei*), and *L. arboreus* appears in clade c (subspecies *arboreus* and *pararboreus*) and clade d (many subspecies). The ''species'' mentioned above are most probably paraphyletic as judged from the ND5 phylogenetic tree; i.e., each ''species'' arose in parallel in the different pyhlogenetic lines with minor morphological differences and can be recognized as subspecies or local races. Although the 28S rDNA tree is mostly consistent with the ND5 tree, some discrepancies between them do exist, especially in lineage 3. In the 28S rDNA phylogeny, *L. hiurai* form a clade with *L. kyushuensis* (Sublineage 1), while in the ND5 phylogeny, *L. hiurai* clusters with the other members of sublineage 2. In the 28S rDNA tree, *L. procerulus procerulus* of Honshu forms a clade with *L. procerulus miyakei* of Kyushu, whereas in the ND5 tree, *L. procerulus procerulus* of Honshu clusters with the other Honshu members, *L. kumagaii* and *L. arboreus.* As was noted above, the maximum *D* among the Japanese species or races in the 28S rDNA tree is less than 0.005 (0.81%), in contrast to that in the ND5 gene (maximum $D = 11.42\%$). Therefore, it is not possible to decide whether the apparent discrepancies between the ND5 and the 28S rDNA phylogenies are really meaningful or whether or not this situation can be explained by the introgression hypothesis.

Origin

The origin of *Leptocarabus* may be traced back on a phylogenetic tree of the genus *Carabus* (s. lat.) covering the representative species from all over the world (Imura et al. 1998). According to Imura et al. (1998), *Leptocarabus* started to radiate slightly after the ''big bang,'' i.e., a large-scale radiation into the major subgenera or divisions of *Carabus* (s. lat.). The ''big bang'' and the radiation of *Leptocarabus,* which presumably occurred somewhere in the eastern Eurasian continent, were estimated to have taken place 45–40 and 35–30 MYA, respectively. The phylogenetic tree in figure 3 suggests that the ancestral form of *Leptocarabus* was divided, presumably by geographic barriers, into three lineages, followed by species diversification in each lineage.

The origin of the Japanese *Leptocarabus* (lineage 3) is now considered. As noted above, the Japanese species started to diversify much later (ca. 12–10 MYA) than the continental species (ca. 28–25 MYA). We tentatively assume that the ancestor of the Japanese *Leptocarabus* inhabited the ancient Japanese area in the eastern periphery of the continent. Upon its split from the continent ca. 15 MYA, followed by the archipelago formation by an extensive submergence, the Japanese *Leptocarabus* ancestor was isolated on some island(s). Upon subsequent upheaval of the Japanese Islands (9–6 MYA), the ancestor spread all over Japan, resulting in various species (even different types) and subspecies upon isolation by various barriers such as straits, tectonic lines, rivers, mountains, etc. (For geohistory of the Japanese Islands, see Su et al. [1998]). It has been generally believed that all the Japanese *Leptocarabus* immigrated from the Korean Peninsula via land-bridges in

the glacial era (<2 MYA) (Ishikawa 1989). Indeed, morphologies including genitalia of the S type Japanese *Leptocarabus* are very similar to those of *L. seishinensis* or *L. semiopacus* from the Korean Peninsula, and the P type Japanese *Leptocarabus* species resemble *L. koreanus* from Korea, or *L. yokoae*, *L. marchilhaci*, and *Leptocarabus* sp. from central China. In particular, *L. kyushuensis* from Japan is morphologically quite similar to *L. yokoae*. However, lineage 3 includes solely the Japanese species and none from the Korean Peninsula or China, suggesting that there exist no direct sister relationships between the Japanese and the Korean (and Chinese) species. Moreover, the emergence of the Japanese species is much older (12–10 MYA) than previously thought (<2 MYA). Thus, the present phylogenetic analyses and dating would reject the Korean origin hypothesis, although it is possible, but not very likely, that such a Korean species, if they existed, became extinct or have not been discovered.

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