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Phylogeny of Japanese Stag Beetles (Coleoptera: Lucanidae) Inferred from 16S mtrRNA Gene Sequences, with Reference to the Evolution of Sexual Dimorphism of Mandibles

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ABSTRACT—As a first step in reconstructing the phylogeny of world stag beetles (Coleoptera: Lucanidae), phylogenetic relationships among the major members of Japanese stag beetles were explored by analyzing a sequence of 1030 nucleotides from the mitochondrial 16S ribosomal RNA (16S rRNA) gene. A total of 20 species and three additional subspecies representing 13 genera were examined to provide basic information on the phylogeny of world Lucanidae. The resultant phylogenetic tree indicates that the family Lucanidae is monophyletic, and contains two major lineages: one consists of the genera *Platycerus*, *Aesalus*, *Ceruchus*, and *Nicagus*, and the other includes *Dorcus*, *Rhaetulus*, *Prosopocoilus*, *Aegus*, *Neolucanus*, *Prismognathus*, *Lucanus*, *Figulus*, and *Nigidius*. Generic members of the latter lineage are further divided into the following four sublineages: i) *Figulus* and *Nigidius*; ii) *Prismognathus* and *Lucanus*; iii) *Aegus* and *Neolucanus*; and iv) *Dorcus*, *Rhaetulus*, and *Prosopocoilus*. These molecular phylogenetic relationships are used as a basis for a preliminary exploration of the evolution of sexual dimorphism in the shape of the mandible. The results of this investigation suggest that strong sexual dimorphism with well-developed mandibles in males evolved independently at least twice, once in the genus *Aegus* and once in the ancestor of the *Lucanus–Prismognathus* and *Dorcus–Rhaetulus–Prosopocoilus* clades. Alternatively, it is possible that sexual dimorphism of mandibles has undergone secondary loss in the genera *Figulus* and *Nigidius*.

Key words: Lucanidae, Coleoptera, molecular phylogeny, 16S rRNA, mitochondrial DNA, evolution, sexual dimorphism, mandibles

INTRODUCTION

The family Lucanidae (stag beetles), consisting of about 1000 species, is widely distributed around the world and is particularly abundant in the Oriental region (Didier and Séguy, 1953; Benesh, 1960; Maes, 1992; Mizunuma and Nagai, 1994; Krajcik, 2001). Stag beetles have received wide attention from scientists because of their remarkable sexual dimorphism and the individual variation exhibited by males, especially in the shape of the mandibles (*e.g.*, Inukai, 1924; Huxley, 1931; Arrow, 1937, 1950; Otte and Stayman, 1979; Sakaino, 1987, 1988; Kawano, 1997, 2000; Tatsuta *et al.*, 2001).

Systematically, however, controversy has surrounded the considerable changes affecting the taxonomic status of many taxa in this family and the higher classification levels, such as tribes and subfamilies (Didier and Séguy, 1953; Benesh, 1960; Kikuta, 1986; Maes, 1992; Mizunuma and Nagai, 1994; Krajcik, 2001), as shown in Table 1. Additionally, only a few comparative studies, such as Sharp and Muir (1912) and Houlbert (1915), based on adult morphology have demonstrated hypothetical affinities of several subfamilies in the family Lucanidae, but the results of these studies did not agree with one another. This suggests a difficulty in selecting morphological characters for a phylogenetic analysis of the family Lucanidae.

A phylogenetic analysis based on molecular data is expected to significantly improve the resolution of the phylogeny of the family Lucanidae. Several studies have utilized various types of molecular data to infer phylogenetic relationships of lucanid taxa, including allozyme electrophoresis (Igarashi *et al.*, 1994; Matsuoka *et al.*, 1998; Matsuoka and Hosoya, 2003), random amplified polymorphic DNA (Hosoya *et al.*, 2002), and nucleotide sequences of the mitochondrial cytochrome c oxidase subunit I (COI) (Trewick, 2000;

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Table 1. List of lucanid beetles classification according to different authors used in this study.

Species and subspecies (Mizunuma and Nagai, 1994)	Didier and Séguy (1953)	Benesh (1960) Krajcik (2001)	Kikuta (1986)	Maes (1992)
Dorcus curvidens binodulosus *	Dorcinae	Dorcinae	Lucaninae	Dorcinae
Dorcus titanus pilifer				
Dorcus titanus okinawanus				
Dorcus rectus rectus				
Rhaetulus recticornis	Lucaninae			
Prosopocoilus inclinatus inclinatus	Cladognathinae			
Prosopocoilus pseudodissimilis				
Prismognathus angularis angularis				
Prismognathus dauricus				
Aegus laevicollis subnitidus	Dorcinae			
Lucanus maculifemoratus maculifemoratus	Lucaninae	Lucaninae		Lucaninae
Lucanus ferriei				
Neolucanus protogenetivus protogenetivus	Chalcodinae			Odontolabinae
Neolucanus protogenetivus hamaii				
Platycerus delicatulus delicatulus	Dorcinae	Aesalinae		Platycerinae
Platycerus acuticollis acuticollis				
Figulus binodulus	Figulinae	Figulinae		Figulinae
Figulus boninensis				
Nigidius lewisi				
Ceruchus lignarius lignarius	Aesalinae	Aesalinae	Ceruchinae	Syndesinae
Ceruchus lignarius monticola				
Aesalus asiaticus asiaticus			Aesalinae	Aesalinae
Nicagus japonicus			Trogidae**	

* We treated *Dorcus curvidens binodulus* in the catalogue of Mizunuma and Nagai (1994) as followed by *Dorcus curvidens binodulosus* followed by Ikeda and Nishimura (1995).

** Kikuta (1986) treated that *Nicagus* did not include into the family Lucanidae, including into the family Trogidae.

Hosoya *et al.*, 2003) and 16S ribosomal RNA (rRNA) genes (Hosoya *et al.*, 2001). However, these studies at most dealt with several taxa within a single genus or a few closely related genera, but did not examine phylogenetic relationships among the higher groups of the family Lucanidae, such as tribes and subfamilies.

In the present study, we investigated the phylogenetic relationships among Japanese stag beetles based on nucleotide sequences of the mitochondrial 16S rRNA gene as a first step in reconstructing the phylogeny of world lucanid beetles. This gene was chosen because it has provided phylogenetic resolution among genera, tribes, and/or subfamilies of other insects (*e.g.*, Fang *et al.*, 1993; Dowton and Austin, 1994; Kambhampati *et al.*, 1996; Han and McPheron, 1997; Kambhampati *et al.*, 2000). Although the higher classification of the family Lucanidae has varied considerably among different studies, the Japanese lucanid beetles include most of the major lineages of the family outlined by any of the competing classification schemes. The Japanese species represent at least half the number of subfamilies proposed thus far, and six of the ten subfamilies proposed by Didier and Séguy (1953), four of the eight suggested by Benesh (1960), three of the six outlined by Kikuta (1986), seven of the nine put forward by Maes (1992), and four of the eight recognized by Krajcik (2001).

We also discuss evolutionary aspects of sexual dimorphism of the shape of the mandible within this beetle family on the basis of the phylogenetic trees we obtained.

MATERIALS AND METHODS

Insects

The taxa used in this study are listed in Table 1, which also shows differences among higher classification systems of the family Lucanidae. We mainly followed the taxonomic treatment of Mizunuma and Nagai (1994). A total of 20 species and three additional subspecies representing 13 genera were collected from Japan and subjected to DNA analysis (Table 2; Fig. 1). For some genera, such as *Dorcus*, which include many Japanese species, we selected a few representative species from each genus, since our main purpose here was to reconstruct phylogenetic relationships among the genera of Japanese stag beetles. As for the three additional subspecies, we selected two different subspecies pairs to represent most of the major zoogeographic distribution patterns.

Table 2. List of taxa, sampling places and sequence accession numbers of lucanid beetles used in this study.

Family	Species and subspecies (Mizunuma and Nagai, 1994)	Sampling place	Accession numbers
Lucanidae	*Dorcus curvidens binodulosus* *	Yamanashi	AB178292
	Dorcus titanus pilifer	Kyoto Univ., Sakyo, Kyoto, Kyoto	AB178293
	Dorcus titanus okinawanus	Kunigami, Okinawa I., Okinawa	AB178294
	Dorcus rectus rectus	Meya dam, Nishimeya, Aomori	AB178295
	Rhaetulus recticornis	Amami I., Kagoshima	AB178296
	Prosopocoilus inclinatus inclinatus	Meya dam, Nishimeya, Aomori	AB178297
	Prosopocoilus pseudodissimilis	Ishigaki I., Okinawa	AB178298
	Prismognathus angularis angularis	Mt. Hikosan, Fukuoka	AB178299
	Prismognathus dauricus	Kamiagata, Tsushima, Nagasaki	AB178300
	Aegus laevicollis subnitidus	Kiikatsuura, Wakayama	AB178301
	Lucanus maculifemoratus maculifemoratus	Meya dam, Nishimeya, Aomori	AB178302
	Lucanus ferriei	Amami I., Kagoshima	AB178303
	Neolucanus protogenetivus protogenetivus	Mt. Yui, Amami I., Kagoshima	AB178304
	Neolucanus protogenetivus hamaii	Daisen, Uke I., Kagoshima	AB178305
	Platycerus delicatulus delicatulus	Tsuta, Towadako, Aomori	AB178306
	Platycerus acuticollis acuticollis	Mt. Nanba, Joetsu, Niigata	AB178307
	Figulus binodulus	Mt. Yoshida, Sakyo, Kyoto, Kyoto	AB178308
	Figulus boninensis	Mt. Tsutsuzi, Chichizima I., Ogasawara Is., Tokyo	AB178309
	Nigidius lewisi	Kiikatsuura, Wakayama	AB178310
	Ceruchus lignarius lignarius	Mt. Ozaki, Hiraka, Aomori	AB178311
	Ceruchus lignarius monticola	Mt. Fuji, Shizuoka	AB178312
	Aesalus asiaticus asiaticus	Ashu, Miyama, Kyoto	AB178313
	Nicagus japonicus	Oirase, Fukaura, Aomori	AB178314
Passalidae	*Cylindrocaulus patalis*	Mt. Ishizuchi, Ehime	AB178315
Trogidae	*Trox uenoi*	Mt. Yuwan, Amami I., Kagoshima	AB178316
Geotrupidae	*Geotrupes laevistriatus*	Katsurazawa dam, Mikasa, Hokkaido	AB178317
Scarabaeidae	*Trypoxylus dichotomus septentrionalis*	Hirosaki Univ., Hirosaki, Aomori	AB178318
	Heptophylla picea picea	Meya dam, Nishimeya, Aomori	AB178319

* We treated *Dorcus curvidens binodulus* in the catalogue of Mizunuma and Nagai (1994) as followed by *Dorcus curvidens binodulosus* followed by Ikeda and Nishimura (1995).

Neolucanus protogenetivus is distributed on the islands of the Ryukyu archipelago, and we selected two insular subspecies from neighboring islands (*Ne. p. protogenetivus* and *Ne. protogenetivus hamaii*). *Dorcus titanus* is widely distributed in East and Southeast Asia, including many islands, and we selected a subspecies on the Japanese mainland (*D. titanus pilifer*) and an insular subspecies of the Ryukyu archipelago (*D. titanus okinawanus*). *Ceruchus lignarius* is only found in mountainous areas of the Japanese mainland, and we selected two subspecies from the mountains of Honshu Island (*Ce. l. lignarius* and *Ce. lignarius monticola*). In some species of stag beetles, morphological differentiation has been reported between conspecific subspecies and/or populations (*e.g.*, Benesh, 1960; Maes, 1992; Mizunuma and Nagai, 1994; Krajcik, 2001). However, in the present study, intraspecific and/or intra-subspecific samples were designated as a single operational taxonomic unit (OTU). This was because a previous study (Hosoya *et al.*, 2001) showed that sequence divergence within a single species and/or subspecies was very low, with the relevant taxa exclusively forming the same clades with high bootstrap values. The nucleotide sequences of *Dorcus rectus rectus*, *D. titanus pilifer*, *Ce. l. lignarius*, and *Ce. lignarius monticola* were available from the published data of Hosoya *et al.* (2001).

The outgroups selected for the present analysis are given in Table 2 and include *Cylindrocaulus patalis* of the family Passalidae, *Trox uenoi* of the family Trogidae, *Geotrupes laevistriatus* of the family Geotrupidae, and *Trypoxylus dichotomus septentrionalis* and *Heptophylla picea picea* of the family Scarabaeidae, all of which belong to the superfamily Scarabaeoidea, as does the family Lucanidae (Browne and Scholtz, 1999).

DNA extraction, amplification and sequencing

Detailed methods concerning DNA extraction, amplification, and sequencing are described in Hosoya *et al.* (2001). We used specimens of adults, pupae, or larvae stored in 99.5% ethanol or at –80°C. A part of the mitochondrial 16S rRNA gene was amplified by polymerase chain reaction (PCR) (Saiki *et al.*, 1988) using seven primers: 16SA, 16SH, 16SB, 16SC, 16SD, 16SK (Hosoya *et al.*, 2001) and 16SL (Fig. 2). 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

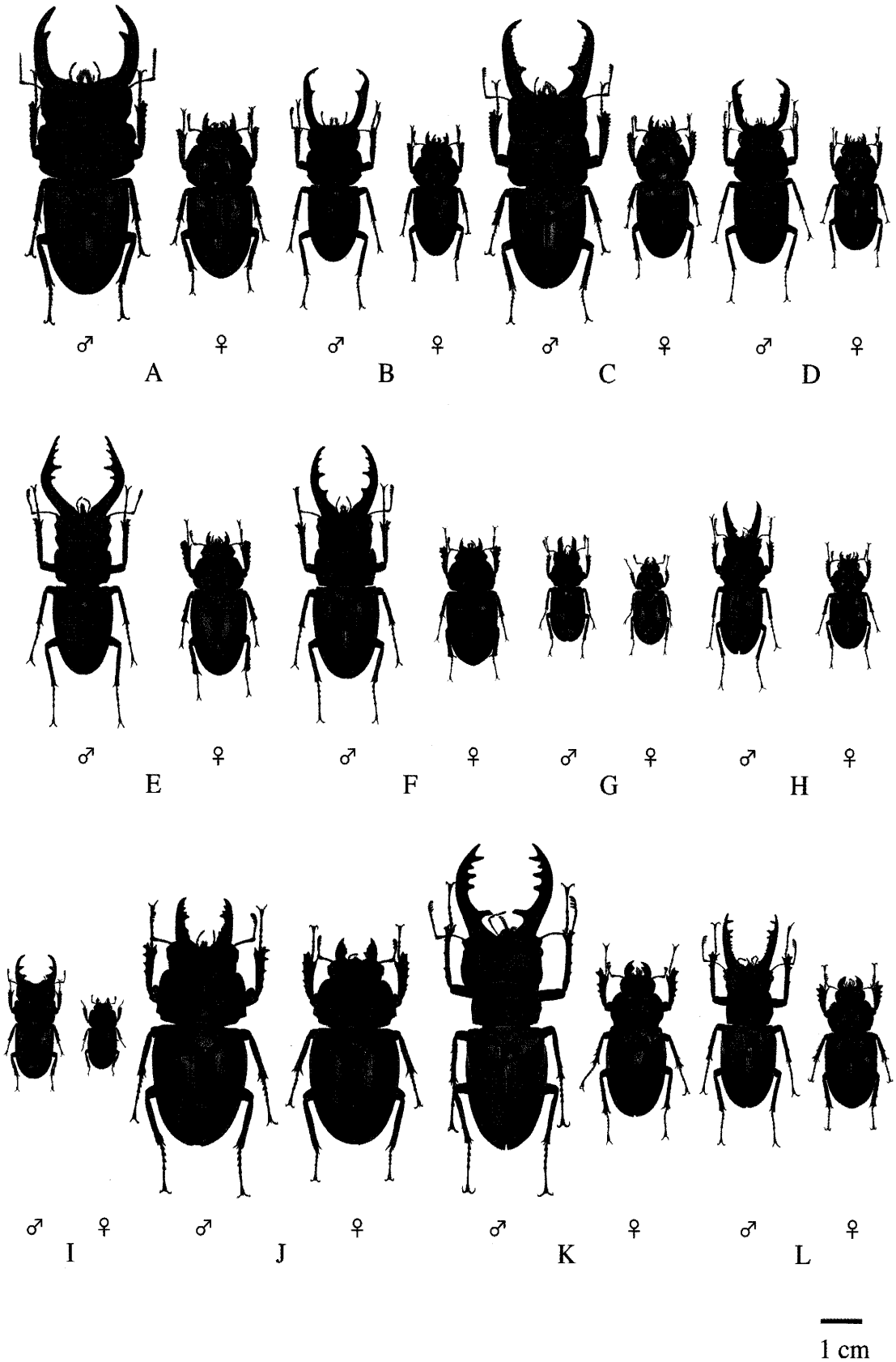


Fig. 1. To be continued.

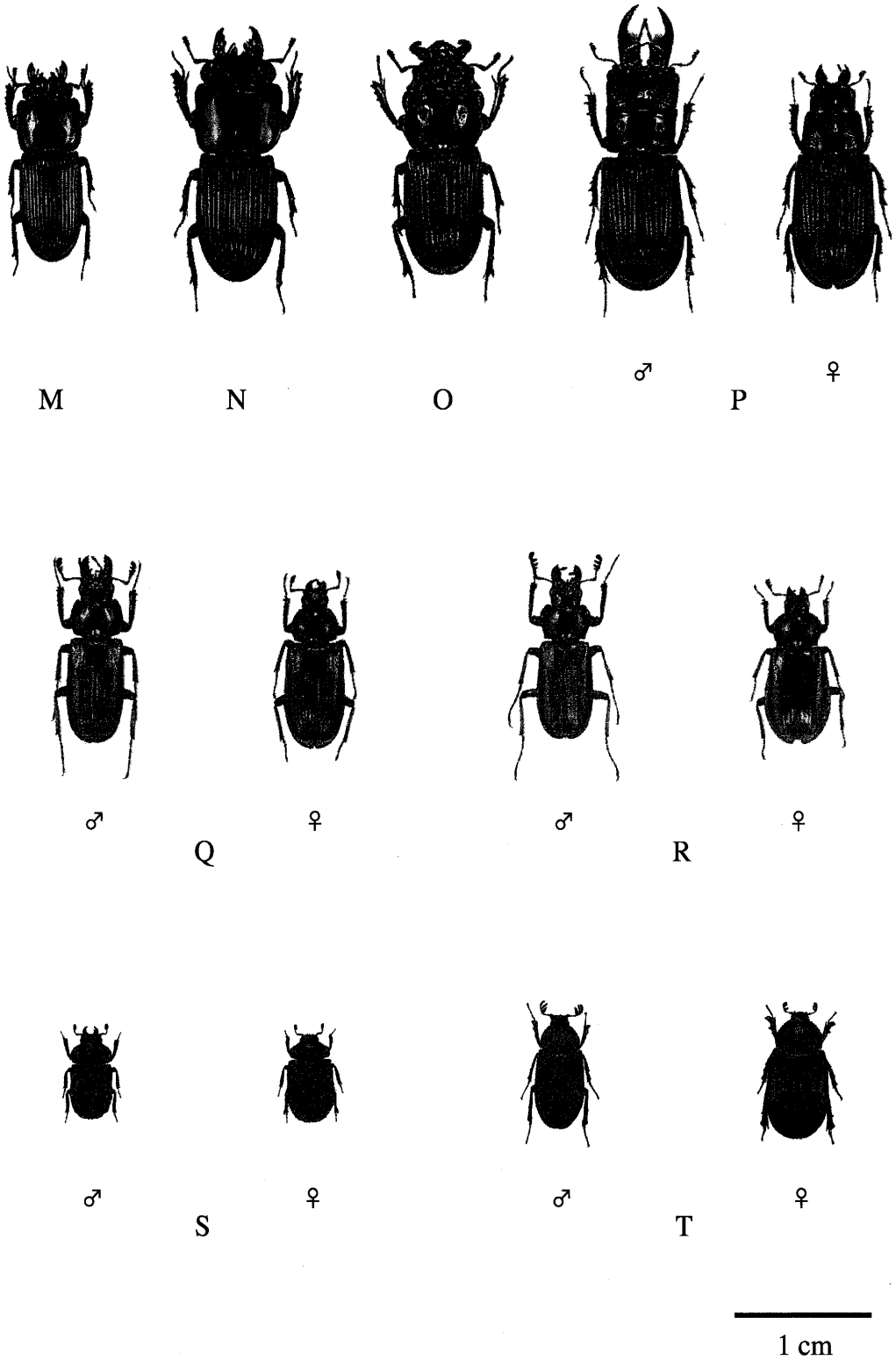
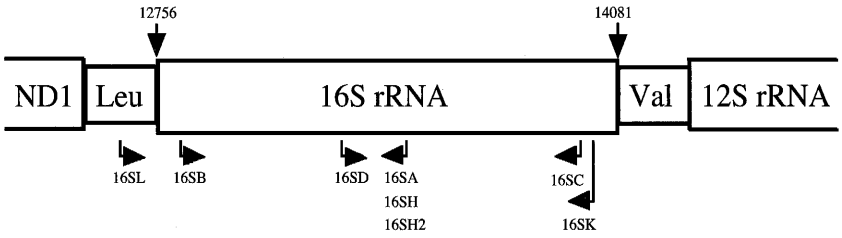


Fig. 1. Japanese lucanid species examined with respect to sexual dimorphism of mandible shape, grades of which are given in parentheses. A, *Dorcus curvidens binodulosus* (2); B, *Dorcus rectus rectus* (2); C, *Dorcus titanus pilifer* (2); D, *Rhaetulus recticornis* (2); E, *Prosopocoilus inclinatus inclinatus* (2); F, *Prosopocoilus pseudodissimilis* (2); G, *Prismognathus angularis angularis* (1); H, *Prismognathus dauricus* (2); I, *Aegus laevicollis subnitidus* (2); J, *Neolucanus protogenetivus protogenetivus* (1); K, *Lucanus maculifemoratus maculifemoratus* (2); L, *Lucanus ferriei* (2); M, *Figulus binodulus* (0); N, *Figulus boninensis* (0); O, *Nigidius lewisi* (0); P, *Ceruchus lignarius lignarius* (1); Q, *Platycerus delicatulus delicatulus* (1); R, *Platycerus acticollis acticollis* (1); S, *Aesalus asiaticus asiaticus* (1); T, *Nicagus japonicus* (0). Scale bars, 1 cm.

Sequencer (LI–COR, Lincoln, USA) using 16SA, 16SH2, 16SB, 16SC, and 16SD as sequencing primers (Fig. 2).

Phylogenetic analysis

Sequence data were aligned with web-based CLUSTAL W 1.8 (Thompson *et al.*, 1994) software available through the DNA Data Bank of Japan (DDBJ), using default gap penalties. Gap sites were



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'
16SL (30bp : 12721-12750) 5' ATT CTA AAT (C/T)(C/T)A (A/T)(A/C/G/T)G CAC TA(A/T) TCT GCC AAA 3'

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

Table 3. Uncorrected p-distance (%) for the 16S rRNA gene sequences.

	1	2	3	4	5	6	7	8	9	10	11	12	13
1. *Dorcus curvidens binodulosus*	–												
2. *D. titanus pilifer*	15.6	–											
3. *D. titanus okinawanus*	16.4	1.8	–										
4. *D. r. rectus*	6.7	15.2	15.9	–									
5. *Rhaetulus recticornis*	19.9	20.0	20.0	18.8	–								
6. *Prosopocoilus i. inclinatus*	18.5	19.6	19.7	17.9	14.1	–							
7. *Pro. pseudodissimilis*	17.7	19.9	19.9	17.4	14.9	6.6	–						
8. *Prismognathus a. angularis*	21.5	21.6	21.7	21.9	23.3	21.7	21.6	–					
9. *Pri. dauricus*	23.0	22.4	22.5	22.9	23.8	22.6	22.4	8.6	–				
10. *Lucanus m. maculifemoratus*	23.2	23.1	23.2	22.7	25.3	22.2	22.7	18.8	20.3	–			
11. *L. ferriei*	23.4	23.3	23.0	22.2	24.2	22.0	22.0	20.2	21.2	11.5	–		
12. *Neolucanus p. protogenetivus*	22.2	21.8	22.0	22.7	22.2	22.9	23.6	21.8	23.3	25.8	24.2	–	
13. *Ne. protogenetivus hamaii*	22.2	21.7	21.9	22.9	22.2	22.6	23.4	22.0	23.7	26.0	24.4	0.6	–
14. *Aegus laevicollis subnitidus*	20.5	20.9	20.8	20.3	19.9	19.9	20.9	20.6	21.8	23.4	23.3	19.5	19.5
15. *Platycerus d. delicatulus*	23.6	23.7	23.8	23.4	26.0	24.9	24.9	26.4	25.6	26.5	27.3	26.1	26.1
16. *Pl. a. acticollis*	23.6	23.7	23.8	24.2	25.8	24.9	25.0	26.0	25.3	26.4	26.4	23.9	23.9
17. *Figulus binodulus*	24.3	25.2	25.7	24.0	24.4	21.6	23.2	24.6	25.7	24.5	27.0	25.0	24.7
18. *F. boninensis*	24.7	24.6	25.2	24.4	24.3	23.1	23.8	25.8	26.3	26.0	27.8	25.0	24.7
19. *Nigidius lewisi*	22.0	23.0	23.2	21.5	24.2	22.3	22.6	21.6	22.2	22.5	23.0	24.0	24.0
20. *Ceruchus l. lignarius*	23.9	23.9	24.2	24.3	25.0	23.1	24.2	24.7	24.6	26.8	26.0	25.3	25.6
21. *Ce. l. monticola*	23.7	22.7	22.9	23.7	24.9	22.5	23.6	25.1	24.9	26.7	25.9	24.5	24.7
22. *Aesalus a. asiaticus*	25.8	24.4	24.5	26.0	28.7	27.6	27.7	26.6	26.7	28.8	28.6	27.8	27.8
23. *Nicagus japonicus*	25.7	25.4	25.8	25.3	26.4	25.2	25.9	25.8	27.0	28.7	28.7	26.6	26.6
24. *Cylindrocaulus patalis*	28.6	27.6	27.4	27.9	29.7	27.0	26.6	26.0	26.7	28.5	27.8	27.4	27.7
25. *Trypoxylus dichotomus septentrionalis*	22.2	23.3	23.6	21.1	24.6	23.0	23.8	23.3	22.9	26.3	25.1	22.4	22.6
26. *Heptophylla p. picea*	23.7	24.9	24.9	23.6	26.1	24.4	24.5	24.0	25.2	26.7	26.7	24.3	24.5
27. *Trox uenoi*	23.6	23.4	24.0	23.3	24.3	21.5	23.1	23.0	22.9	25.1	25.6	23.7	24.2
28. *Geotrupes laevistriatus*	25.1	25.2	25.2	24.2	26.4	24.4	24.9	27.1	26.0	27.1	25.9	25.8	26.0

excluded in all analyses. The data were also tested to determine if the resultant phylogenetic structure was significantly different from random by using the permutation tail probability (PTP) test (Faith and Cranston, 1991) with 10000 random matrices whilst randomizing all taxa by means of PAUP* 4.0b10 (Swofford, 2002).

We examined the number of transitions (TI) and transversions (TV) against uncorrected p-distance to explore the possibility of saturated base substitutions. Saturation was judged to have taken place if the resulting scatter-plots were non-linear in shape (Maekawa *et al.*, 1999; Maekawa and Matsumoto, 2000).

Sequence data were analyzed with the program MODELTEST (3.06) (Posada and Crandall, 1998), which uses hierarchical likelihood-ratio tests to determine the best-fit substitution model for the data. The optimal model defined by MODELTEST was then used to estimate maximum-likelihood (ML) distances for a neighbour-joining (NJ) analysis and to define parameters for a ML analysis. The NJ method was implemented in PAUP* to infer the relationships among taxa based on the ML distance. For the ML and maximum-parsimony (MP) analyses, heuristic searches were performed with PAUP*. Heuristic searches were performed using the tree-bisection-reconnection (TBR) branch-swapping algorithm, and 10 and 100 replicates of a random-addition sequence for ML and MP, respectively. MP analyses were conducted with characters weighted in several ways (*e.g.*, Maekawa *et al.*, 1999): by equal weighing; by weighting TV two, four, and eight times TI; and by excluding TI.

The confidence levels of each branch were estimated by 1000 bootstrap replications (Felsenstein, 1985) for the NJ analysis, 1000 bootstrap replications using heuristic searches with nearest-neighbor interchange (NNI) branch-swapping for the ML analysis, and 1000 bootstrap replications using heuristic searches with simple addition sequence and TBR branch swapping for the MP analysis. Decay indices (Bremer, 1988, 1994) were also estimated for the MP analysis.

Baysian phylogenetic analyses were conducted with MrBayes 3.0 (Huelsenbeck and Ronquist, 2001) under the general time reversible (GTR) model with the proportion of invariable sites and the gamma shape parameter (GTR + I + Γ). Each Markov chain was started from a random tree and run for 5×10^5 generations, sampling the chains every 100th cycle. All sample points prior to reaching stationary (1000 trees) were discarded as burn-in samples. Data remaining after discarding burn-in samples were used to generate a majority-rule consensus tree, where the percentage of samples recovering any particular clade represented the clade's

Table 3. continued.

	14	15	16	17	18	19	20	21	22	23	24	25	26	27
1. *Dorcus curvidens binodulosus*														
2. *D. titanus pilifer*														
3. *D. titanus okinawanus*														
4. *D. r. rectus*														
5. *Rhaetulus recticornis*														
6. *Prosopocoilus i. inclinatus*														
7. *Pro. pseudodissimilis*														
8. *Prismognathus a. angularis*														
9. *Pri. dauricus*														
10. *Lucanus m. maculifemoratus*														
11. *L. ferriei*														
12. *Neolucanus p. protogenetivus*														
13. *Ne. protogenetivus hamaii*														
14. *Aegus laevicollis subnitidus*	–													
15. *Platycerus d. delicatulus*	23.4	–												
16. *Pl. a. acticollis*	24.0	6.4	–											
17. *Figulus binodulus*	21.5	27.4	27.6	–										
18. *F. boninensis*	23.6	28.5	27.9	7.9	–									
19. *Nigidius lewisi*	20.4	24.4	24.4	21.8	22.3	–								
20. *Ceruchus l. lignarius*	23.3	23.0	21.5	26.6	27.8	26.0	–							
21. *Ce. l. monticola*	22.3	22.9	21.5	26.1	27.7	25.4	2.7	–						
22. *Aesalus a. asiaticus*	26.5	22.5	22.9	28.8	29.5	25.1	23.4	23.1	–					
23. *Nicagus japonicus*	24.0	23.3	23.9	26.6	27.4	24.6	20.6	21.0	25.8	–				
24. *Cylindrocaulus patalis*	27.0	27.2	26.6	27.9	29.5	26.1	26.3	26.3	28.4	25.8	–			
25. *Trypoxylus dichotomus septentrionalis*	22.6	21.3	19.8	23.8	24.5	22.6	22.7	22.7	26.1	23.4	21.8	–		
26. *Heptophylla p. picea*	24.9	25.3	24.5	25.0	25.9	25.0	23.4	23.7	26.6	24.0	24.3	14.1	–	
27. *Trox uenoi*	23.2	21.2	21.2	24.4	25.8	22.4	21.9	21.7	23.6	19.8	21.1	14.8	17.4	–
28. *Geotrupes laevistriatus*	24.2	21.9	22.6	25.7	27.1	22.0	22.0	21.8	25.3	21.6	22.3	15.5	17.7	13.8

posterior probability.

Analysis of evolution of sexual dimorphism of mandibles

To evaluate the degree of sexual dimorphism of mandibles in stag beetles, we used a scoring system with values of 0: no sexual dimorphism; 1: weak sexual dimorphism; and 2: strong sexual dimorphism (Fig. 1). These states were treated as ordered characters and mapped by parsimony optimization under accelerated transformation (ACCTRAN) and delayed transformation (DELTRAN) (Swofford and Maddison, 1987; Swofford and Olsen, 1990) using MacClade 4.0 (Maddison and Maddison, 2000).

RESULTS

Sequence variation

All sequences are deposited in DDBJ, EMBL and GenBank nucleotide sequence databases under accession numbers AB178292–AB178319 (Table 2). All sequences in the present study showed a substantial bias for adenine (A) and thymine (T) (average=74.0%). Several observations have demonstrated the existence of a strong AT bias in the insect mitochondrial genome (Simon *et al.*, 1994).

Of the 1030 total aligned sites of the mitochondrial 16S rRNA gene fragment, 177 sites involved an insertion or deletion and were subsequently excluded from the analysis. In

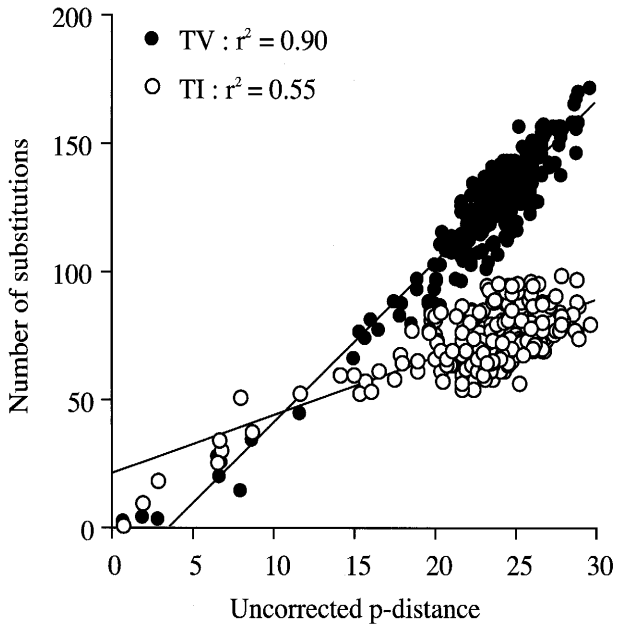
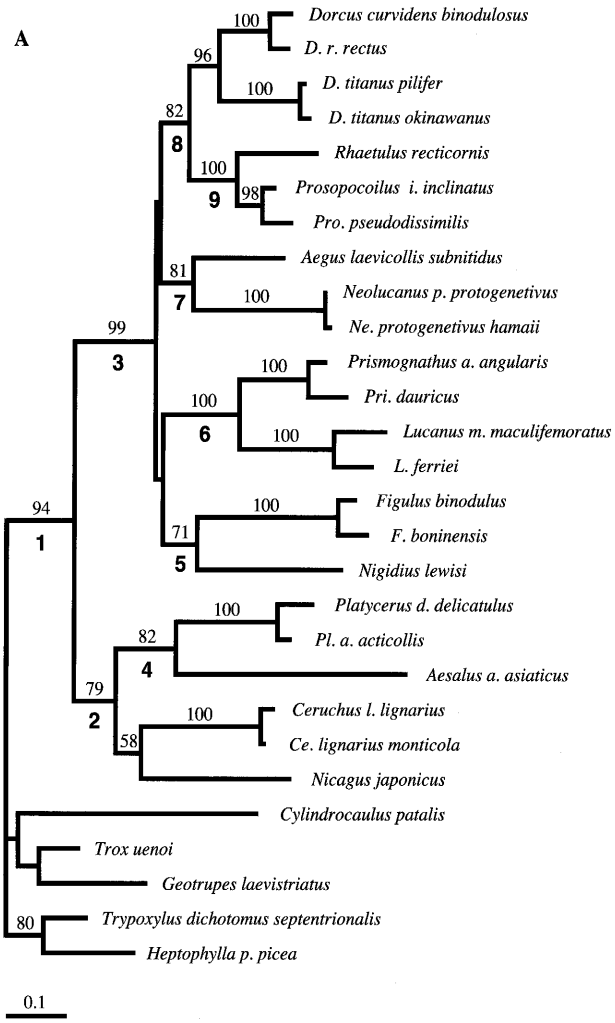


Fig. 3. Scatter plots showing the number of substitutions (TI and TV) vs. uncorrected p distance in the 16S rRNA gene sequence for pairwise comparisons among lucanid taxa. Closed and open circles represent TV and TI, respectively. Squared regression coefficients (r) for each scatter-plot are also shown.



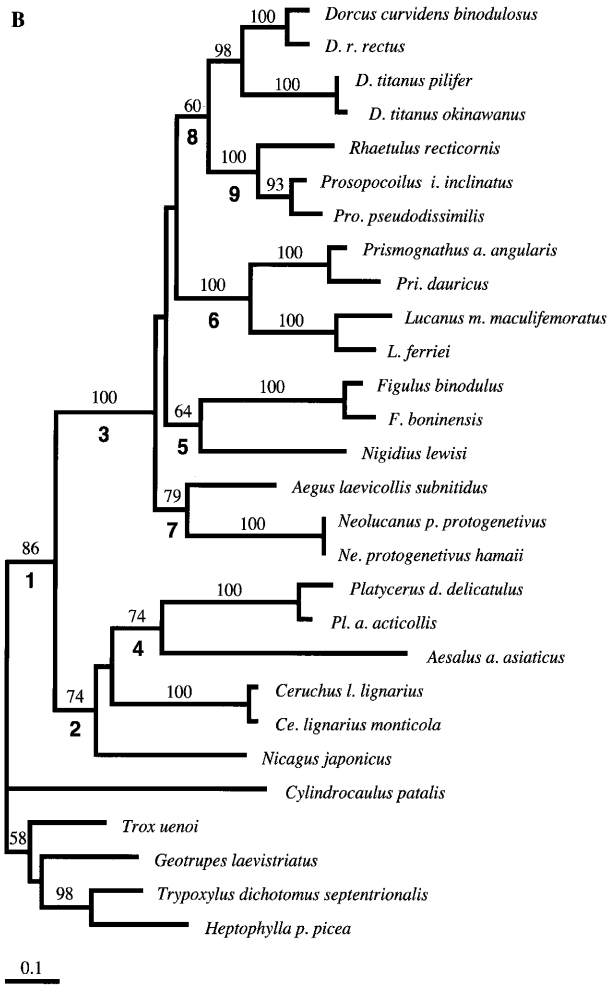


Fig. 4. To be continued.

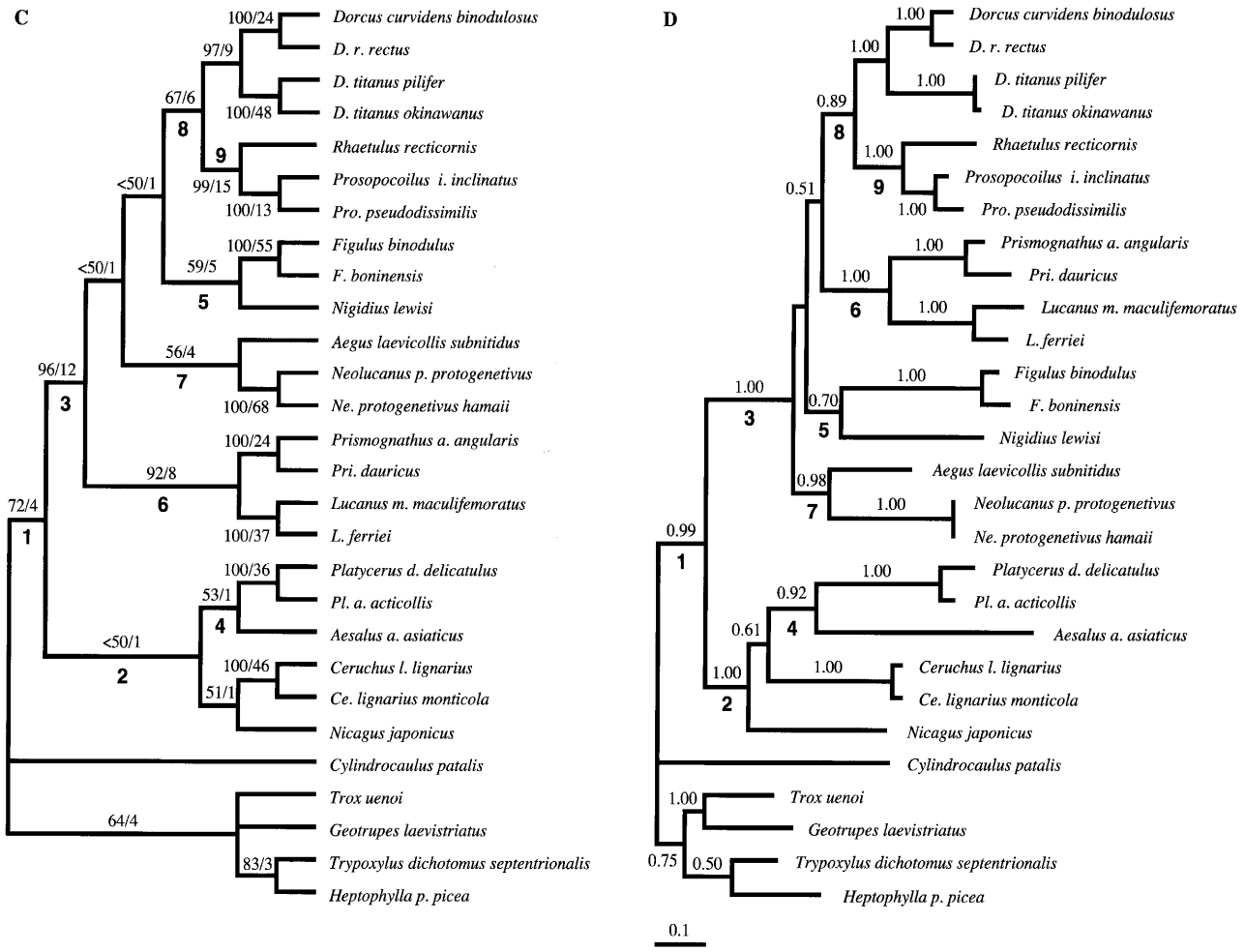


Fig. 4. A, Neighbor-joining (NJ) dendrogram derived from a distance matrix from the mitochondrial 16S rRNA gene sequence. The numbers above internal branches represent the percentage of 1000 bootstrap replications that included the nodes. Nodes with bold numbers are identical with those in the ML and MP dendrograms below. Bar equals 0.1 substitutions/site. B, Maximum-likelihood (ML) dendrogram (ln likelihood=–10234.21213). The numbers above internal branches represent the percentage of 1000 bootstrap replicates. Bar equals 0.1 substitutions/site. C, Strict consensus tree of two most parsimonious dendrograms resulting from non-weighted parsimony (MP) analysis (length=2330, consistency index=0.39, retention index=0.52, rescaled consistency index=0.20). Numbers above or below internal branches represent the percentage of 1000 bootstrap replicates and decay indices, respectively. D, Consensus tree inferred by Bayesian analysis. The numbers above or below internal branches represent the Bayesian posterior probabilities. Bar equals 0.1 substitutions/site.

the analyzed sites, 503 (59.0%) were variable. Sequence divergence (uncorrected p-distance) is summarized in Table 3. Inter-subspecific nucleotide substitutions ranged from 0.6% (*Ne. protogenetivus*, insular subspecies collected on neighboring islands) to 2.7% (*Ce. lignarius*, mountainous subspecies collected on two mountains of the mainland of Japan). Inter-specific substitutions within the same genus ranged from 6.4% (*Platycerus d. delicatulus* vs. *Pl. a. acuticollis*) to 16.4% (*D. curvidens binodulosus* vs. *D. titanus okinawanus*). Nucleotide substitutions between genera within the family Lucanidae varied from 14.1% (*Rhaetulus recticornis* vs. *Prosopocoilus i. inclinatus*) to 29.5% (*Figulus boninensis* vs. *Aesalus a. asiaticus*), and those between Lucanidae and outgroups varied from 19.8% (*Pl. a. acuticollis* vs. *Trypoxylus dichotomus septentrionalis*, and *Nicagus japonicus* vs. *Trox uenoi*) to 29.7% (*R. recticornis* vs. *Cylindrocaulus patalis*). The PTP test indicated a significant phylogenetic structure in the data set (P=0.0001).

In a saturation analysis of the 16S rRNA gene of lucanid taxa (Fig. 3), the plot of TV vs. uncorrected p-distance was essentially linear (r^2=0.90). However, the plot of TI suggests that slight saturation of TI may have occurred between certain pairs of taxa. Since multiple substitutions at a site can potentially obscure phylogenetic associations, the MP phylogenetic analysis was performed with four weighting schemes (weighting TV two, four, and eight times TI, and excluding TI).

Phylogenetic relationships

Analysis of the 16S rRNA gene using MODELTEST supported two best-fit models of DNA substitution. The best-fit model for log-likelihood ratio tests was the general time reversible model (GTR) using five substitution types (Posada and Crandall [1998] called this the transversional model, or TVM), including the proportion of invariable sites (I) and the gamma distribution for rate variation among sites

(Γ) (TVM + I + Γ). The best fit model by the Akaike information criterion (Akaike, 1974) was the Tamura-Nei model with invariant sites and the gamma shape parameter (TrN + I + Γ). However, the two best-fit models were not congruent with each other. Of these, the more parameter-rich, GTR + I + Γ, was used to estimate ML distances for a NJ analysis and to define parameters for a ML analysis. The base frequencies were approximately 0.3327 for A, 0.0368 for C, 0.1434 for G, and 0.4871 for T. Estimates of substitution rates were 0.7616 for A–C, 4.7787 for A–G, 0.9542 for A–T, 0.1983 for C–G, 6.6954 for C–T, and 1.0000 for G–T. The proportion of invariable sites and the gamma shape-distribution parameter were approximately 0.3453 and 0.8548, respectively.

The NJ dendrogram using ML distance is shown in Fig. 4A. Following Hillis and Bull (1993), we considered a bootstrap proportion (BP) value ≥70% as a significant indicator of monophyly. In the NJ tree, conspecific and congeneric taxa clustered monophyletically, with high BP values (96–100%). The monophyly of the family Lucanidae was supported by a BP of 94% (node 1). The lucanid ingroup in the NJ dendrogram comprised two clusters, one consisting of the genera *Platycerus*, *Aesalus*, *Ceruchus*, and *Nicagus*, supported by a BP of 79% (node 2), and the other including *Dorcus*, *Rhaetulus*, *Prosopocoilus*, *Aegus*, *Neolucanus*, *Prismognathus*, *Lucanus*, *Figulus*, and *Nigidius*, supported by a BP of 99% (node 3). In the former cluster, *Platycerus* and *Aesalus* form one cluster (node 4: BP 82%). The latter cluster was split into four clusters. The first (node 5) consists of *Figulus* and *Nigidius* (BP 71%); the second (node 6) includes *Prismognathus* and *Lucanus* (BP 100%); the third (node 7) encompasses *Aegus* and *Neolucanus* (BP 81%); and the fourth (node 8) contains *Dorcus*, *Rhaetulus*, and *Prosopocoilus* (BP 82%). Node 8 was further split into a monotypic cluster of the genus *Dorcus* (BP 96%) and a *Rhaetulus–Prosopocoilus* cluster (node 9, BP 100%).

The topology of the ML dendrogram (Fig. 4B) was congruent with the NJ dendrogram with respect to nodes 1–9 at a BP level ≥70%. Most nodes of the ML dendrogram were supported with BP values ≥70%, with the only exception being a lower support level (BP 60–64%) for nodes 5 and 8.

The topology of the strict consensus dendrogram of two non-weighted MP trees (Fig. 4C) was congruent with the NJ dendrogram with respect to nodes 1–9 at a BP level ≥70%. Most nodes of the MP dendrogram were supported with BP values ≥70% (nodes 1, 3, 6 and 9) or 50–69% (nodes 4, 5, 7 and 8), although a lower support level (BP <50%) was observed for node 2.

MP trees resulting from four weighting schemes were all identical to one another in topology (data not shown). The dendrogram common to all the weighted MP analyses was congruent with the strict consensus dendrogram from the non-weighted MP analysis, except for the absence of node 2 in the former. Most nodes were supported with BP values ≥50% for both the non-weighted (Fig. 4C: nodes 1 and 3–9) and weighted MP dendrograms, although there was lower support (BP<50%) for node 8 in weighted MP trees that excluded TI.

The topology of the consensus dendrogram from the Bayesian analysis (Fig. 4D) was congruent with the NJ dendrogram with respect to nodes 1–9 at a BP level ≥70%. Most nodes of the Bayesian dendrogram were supported with posterior probability (PP) values ≥0.90, except for a lower support level (PP 0.70–0.89) for nodes 5 and 8.

Reconstructing ancestral character states of sexual dimorphism of mandible shape

The Bayesian tree topology (Fig. 4D) was used in MacClade to trace minimum sexual dimorphism grade shifts. This is identical to the ML ingroup topology (Fig. 4B). Character tracing with parsimony optimization is shown in Fig. 5. These reconstructions required six steps.

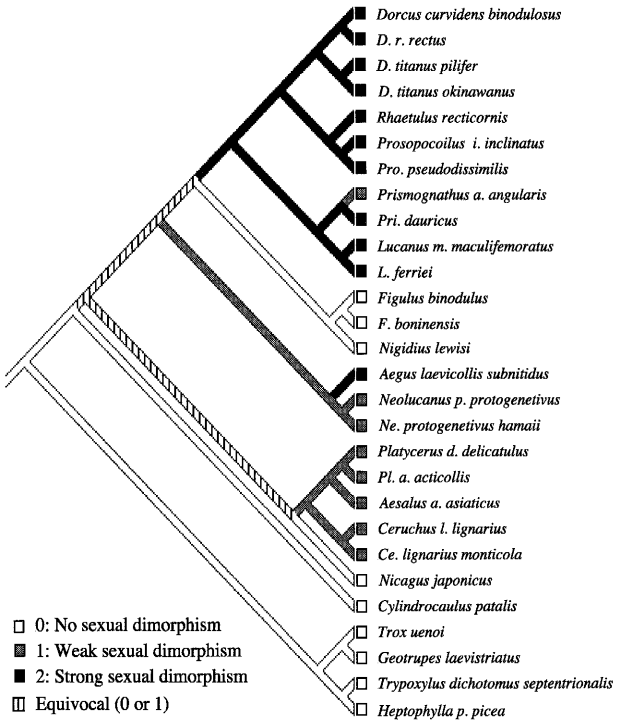


Fig. 5. Parsimony optimization of sexual dimorphism in mandible shape on a phylogenetic estimation based on the tree inferred by Bayesian analysis.

DISCUSSION

Sequence divergence of Lucanidae

Among the three species selected with two subspecies pairs each, inter-subspecific nucleotide substitution of *Ce. lignarius* (2.7%) collected from mountains of the mainland of Japan was roughly twice than that of *D. titanus* (1.8%) collected from mainland Japan and the Ryukyu archipelago. The relatively larger inter-subspecific nucleotide substitution of *Ce. lignarius* suggests that this species strongly reflects the geohistory of the Japanese islands, because its distribution is restricted to mountainous areas.

In this study, sequence differentiation increased in the order of inter-subspecies (0.6–2.7%), interspecies (6.4–16.4%), and inter-genera (14.1–29.5%). Additionally, nodes containing all taxa included for a species or for a genus were supported with high BP values (96–100% in NJ; 93–100% in ML; 97–100% in non-weighted MP) and Bayesian PP values (1.00). The results of the PTP test also indicate that the 16S rRNA data set has significant phylogenetic structure. All of the weighted MP analyses were congruent with the strict consensus tree from the non-weighted MP analysis. These results suggest that saturation of TI was slight, and that the 16S rRNA gene was a good molecule with which to infer the phylogeny of the lucanids used in this study.

Phylogenetic relationships of Lucanidae

The molecular phylogeny presented herein provides a robust framework in which to evaluate previous taxonomic schemes relating to the family Lucanidae. Our study suggests that the family Lucanidae occurring in Japan is monophyletic (node 1). This result contradicts placement of the genus *Nicagus* in the family Trogidae (Nomura, 1960; Kurosawa, 1976; Kikuta, 1986, see Table 1). On the basis of adult and larval morphological characters, Tabana and Okuda (1992) suggested that *Nicagus* belongs to Lucanidae, rather than Trogidae. Browne and Scholtz (1999) also indicated the monophyly of Lucanidae and inclusion of *Nicagus*, on the basis of nine synapomorphies inferred from a cladistic analysis of mainly adult characters.

Our phylogenetic tree shows that the family Lucanidae contains two lineages: an "ancestral cluster" (node 2) consisting of morphologically ancestral genera (Arrow, 1950), and a "true lucanid cluster" (node 3) that includes species with well developed mandibles in males. The former lineage indicates the monophyly of the subfamily Aesalinae sensu Benesh (1960) and Krajcik (2001), including *Platycerus*, *Aesalus*, *Ceruchus*, and *Nicagus*. The "ancestral cluster" also indicates non-monophyly of *Aesalus* and *Nicagus*, which contradicts the treatment of Maes (1992) that suggested *Aesalus* and *Nicagus* are closely related.

Only two lucanid fossils are known from the Mesozoic era: *Paralucanus mesozoicus*, the older one, from the upper Jurassic of Mongolia (Nikolajev, 2000), and *Cretaesalus ponomarenkoi* from the upper Cretaceous of Kazakhstan (Nikolajev, 1993). Of these, *Cretaesalus ponomarenkoi* is allied to the genus *Aesalus* of the "ancestral cluster" discussed above. The oldest lucanid fossils considered as belonging to the "true lucanid cluster" are from the Eocene (Krell, 2000). The fossil data suggest that the "ancestral cluster" contains both morphologically and paleontologically ancestral genera that may have already diverged in the Mesozoic or early Tertiary.

Hayashi (1992) suggested on the basis of morphology that *Figulus* and *Nigidius*, which are relatively small in body size and non-sexually dimorphic, are the most ancestral genera. In our results, however, these genera are included in the "true lucanid cluster" (node 3), as is the tribe Lucanini sensu Kikuta (1986) that includes genera such as *Dorcus*, *Rhaetulus*, *Prosopocoilus*, *Aegus*, *Neolucanus*, *Prismognathus*, and *Lucanus*. Gokan *et al.* (1998) also suggested a close affinity of *Figulus* to genera within the "true lucanid cluster" on the basis of the structural organization of the compound eyes; *Figulus* shares a similar eye structure with *Lucanus*, but not with *Platycerus* or *Aesalus*. According to Kikuta (1986), a "true lucanid cluster", equivalent to that revealed in the present study, is defined by the following adult characters: second antennal segment more or less long and cylindrical, jointed at the apical upper angle of the first segment, antennae geniculate; labrum fused with clypeus and indistinct; eyes with canthus; and metepisternum distant from the mesocoxal cavity.

Unfortunately, our results cannot shed light on the phylogenetic relationships between the tribes Figulini and Lucanini sensu Kikuta (1986), nor do they clearly support the monophyly of the tribe Lucanini sensu Kikuta (1986). In our study, the tribe Lucanini sensu Kikuta (1986) is split into three subclusters: a *Lucanus-Prismognathus* cluster (node 6), an *Aegus-Neolucanus* cluster (node 7), and a *Dorcus-Rhaetulus-Prosopocoilus* cluster (node 8). Our results do not agree with previous higher classification systems such as those proposed by Didier and Séguy (1953), Benesh (1960), Maes (1992) and Krajcik (2001), or with the hypothetical phylogram of Houlbert (1915). Further study is needed to clarify these phylogenetic associations.

To our surprise, in our results *Neolucanus* and *Aegus* form one cluster (node 7), which contradicts previous taxonomic systems (Table 1). On the other hand, our results do not support close phylogenetic affinities of *Neolucanus* and *Lucanus*, and of the *Aegus* with other genera of Dorcinae, as suggested by Benesh (1960), Maes (1992), and Krajcik (2001). An analysis of ecological characteristics (Araya, 1994) also suggests a closer relationship between *Neolucanus* and *Aegus*. The larvae of both *Neolucanus* and *Aegus* are found in detritus or humus, indicating an association with the activity of termites, whereas other genera of the "true lucanid cluster" are mainly found in dead wood with white rot.

Arrow (1950) lumped many genera into the single genus *Dorcus*, including *Prosopocoilus*, *Prismognathus*, and *Rhaetulus* and its allied genera. Our previous study based on nucleotide sequences of the mitochondrial COI gene (Hosoya *et al.*, 2003) did not clearly resolve the phylogenetic relationships among *Dorcus*, *Prosopocoilus*, and *Prismognathus*. Our present results indicate the monophyly of *Dorcus*, with the exception that *Prismognathus* (node 8) shows greater affinity with *Lucanus* (node 6). These results agree with the affinities that Hayashi (1956) suggested on the basis of similarity between the larval characters *Prismognathus* and *Lucanus*, and that Kikuta (1986) suggested on the basis of adult characters.

Our study indicates that *Rhaetulus* is more closely related to *Prosopocoilus* than to *Lucanus* or *Dorcus* (node

9). This result contradicts the previously proposed taxonomic position of *Rhaetulus*, which had been suggested to have a closer relationship with *Lucanus* (Didier and Séguy, 1953) or *Dorcus* (Kikuta, 1986). Our result instead supports the suggestion of Maes (1992) that *Rhaetulus* belongs to the tribe Cladgnathini, which also includes *Prosopocoilus*.

Evolution of the sexual dimorphism of mandible shape

Stag beetles show remarkable sexual dimorphism, especially in the shape of the mandibles, and display variation in the degree of development of this dimorphism. The exaggerated mandible is found only in the males (Arrow, 1937), with mandibles being used mainly for fighting between conspecific males (Mathieu, 1969; Hayashi, 1987). Males having well-developed mandibles are at a selective advantage during male-male combat because such mandibles help them to secure beneficial habitats and/or females for mating (Morimoto, 1986; Ichikawa, 1986; Hayashi, 1987; Shiokawa and Iwahashi, 2000).

Our result (Fig. 5) indicates that strong sexual dimorphism with well-developed mandibles in males evolved independently at least twice, once in the genus *Aegus* and once in the ancestor of the *Lucanus–Prismognathus* and the *Dorcus–Rhaetulus–Prosopocoilus* clusters, and furthermore that this sexual dimorphism was secondarily weakened in *Prismognathus a. angularis*. Males of species with strong sexual dimorphism of the mandibles engage in combat with conspecific males, mainly in open habitats such as the sap on trees, by holding and throwing competitors using their mandibles (Ichikawa, 1986; Hayashi, 1987; Okajima and Yamaguchi, 1988; Shiokawa and Iwahashi, 2000). Therefore, well-developed mandibles in males of these species should evolve if the advantages of winning a fight confer greater reproductive success.

Weak sexual dimorphism of mandibles in males is found in *Prismognathus a. angularis* and the genera *Neolucanus*, *Platycerus*, *Aesalus*, and *Ceruchus*. Males of *Prismognathus a. angularis* and *Neolucanus* do not inhabit the sap regions of trees (Okajima and Yamaguchi, 1988), but wait and guard females for mating at oviposition sites, such as in hollows of trees (Okajima and Yamaguchi, 1988; Araya and Ôbuchi, 1992). Males of *Platycerus*, *Aesalus*, and *Ceruchus* also guard females at oviposition sites, such as narrow tunnels dug into decayed wood or under logs (*e.g.*, Araya, 1989). In summary, species that retain weak sexual dimorphism of mandibles in males, or as in *Prismognathus a. angularis* show a shift from strong to weak sexual dimorphism in the mandibles, do not engage in combat in open habitats, but rather in relatively narrow spaces.

Our results suggest that either the lucanid ancestor possessed weak sexual dimorphism of the mandibles, or that absence of sexual dimorphism of the mandibles is the ancestral character state. The result of our ACCTRAN character optimization, which places character transformations closer to the root of the cladogram, indicated the possibility that sexual dimorphism in mandible shape was secondarily lost at least twice, once in the genus *Nicagus* and once in the *Figulus-Nigidius* cluster. Adults and larvae of *Figulus* and *Nigidius* live within decayed wood in colonies that are composed of male-female pairs (monogamy), just as with species of the family Passalidae, which show subsocial behavior (Araya *et al.*, 1996) and in which the two sexes are of identical form. Males of *Nicagus* do not engage in combat or guard females (Tabana and Okuda, 1992; Katovich and Kriska, 2002). Additionally, a developmental cost is inevitable in producing exaggerated and elongated mandibles (Kawano, 1997; Tatsuta *et al.*, 2001). Therefore, if the lucanid ancestor possessed weak sexual dimorphism, as indicated by our ACCTRAN result, these genera appear to have lost sexual dimorphism in the mandible shape.

The result of our DELTRAN character optimization, which places character transformations far from the root of the cladogram, indicated that the lucanid ancestor was not sexually dimorphic in the mandible. This analysis suggested that the genera *Nicagus*, *Figulus*, and *Nigidius* have retained the ancestral character state, and that sexual dimorphism of mandibles evolved independently at least three times, once among members of the *Ceruchus*, *Platycerus*, and *Aesalus* cluster, once in the *Aegus* and *Neolucanus* cluster, and once in the *Dorcus*, *Rhaetulus*, *Prosopoilus*, *Prismognathus*, and *Lucanus* cluster. If the lucanid ancestor was not sexually dimorphic, these genera appear to have evolved sexual dimorphism in the mandibles for combat with conspecific males in open habitats or in relatively narrow spaces.

Our study treated the phylogenetic relationships among the main lineages of the family Lucanidae from Japan, and the evolution of sexual dimorphism in the shape of the mandibles within the family. Unfortunately, however, we could not include taxa of several subfamilies and tribes in this study. Further analyses including representatives of these taxa will be necessary to fully reveal phylogenetic relationships and the evolution of the sexual dimorphism within the Lucanidae.

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