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Geographical Divergence in the Japanese Land Snail *Euhadra herklotsi* Inferred from Its Molecular Phylogeny and Genital Characters

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We studied genetic variation within the Japanese land snail *Euhadra herklotsi*, which occurs on Kyushu and the surrounding islands, using partial sequences of the mitochondrial COI gene and nuclear ITS2 genes. The phylogenetic analysis revealed the existence of two major clades: clade N in the north and clade S in the south. These clades were parapatric and overlapped in southern Kyushu. Genetic divergence was high in clade N, whereas it was much lower in clade S. In addition, isolation-by-distance within each clade was implied. Since no current geographical barriers separate these clades, the genetic structure of clade S might have been influenced by historical events, such as volcanic activity, and a resulting population bottleneck followed by range expansion. The genital characteristics of clade-S snails were distinct from those of clade-N snails, and snails in both clades were sympatric at one locality. The shells of clade-N snails were generally larger than those of clade-S snails, but the shell-size variation within each clade could not be explained simply by environmental variables. Our study suggests that *E. herklotsi* likely consists of two sibling species. The taxonomic status of the previously proposed subspecies of *E. herklotsi* and related species requires reassessment.

Key words: mitochondrial DNA, land snail, biogeography, *Euhadra herklotsi*, nuclear DNA, bottleneck effect

INTRODUCTION

Land snails have low dispersal ability, and gene flow between populations appears to be limited. Therefore, land snail populations have been studied to investigate population genetic structure and phylogeography in order to understand geographic differentiation and historical biogeography (*e.g.*, Gittenberger *et al*., 2004; Rundell *et al*., 2004; review in Backeljau *et al*., 2001). In addition, land snails show marked variation in shell morphology between and within populations, including shell size, shape, coiling, coloring, and pigment patterns, and these polymorphisms have attracted the attention of many evolutionary biologists (review in Jones, 1973; Goodfriend, 1986; Backeljau *et al*., 2001). The diversity in shell morphology is influenced not only by local adaptation, but also by phylogenetic constraints, genetic drift, and phylogeographic factors. To resolve the confounding effects of ecological, phylogenetic, and phylogeographic factors on the phenotypic differentiation of land snails, a phylogeographic approach utilizing molecular markers provides a powerful tool (Avise, 2000).

The Japanese land snail genus *Euhadra* is a highly divergent group that has proven to be an excellent model organism for molecular phylogenetic studies, which have focused on the phylogeography of two species in central Japan (Hayashi and Chiba, 2000; Shimizu and Ueshima, 2000; Watanabe and Chiba, 2001) and speciation due to reversed chirality in northern Japan (Ueshima and Asami, 2003; Davison *et al*., 2005). The land snail *Euhadra herklotsi* (Martens) occurs throughout Kyushu in southwest Japan, including the surrounding islands, western tip of Shikoku, western tip of Honshu, and Cheju Island. It inhabits lucido-phyllous forests and thickets, and exhibits marked variation in shell size, shape, banding pattern, and coloring. It is divided into several subspecies based on shell shape and distribution, but its classification differs among researchers (see Minato, 1985, 1988; Azuma, 1995).

Volcanic activity is one of the historical events that can affect intraspecific phylogeography (Emerson *et al*., 2006). On Kyushu, where *E. herklotsi* occurs, volcanic activity has been frequent over the past 100,000 years, and has likely influenced the flora and fauna (Matsushita, 2002; Sugiyama, 2002). Although its effects on the fauna have not been studied in detail, land snails are terrestrial animals with a low power of dispersal, and their populations were probably influenced greatly by this past volcanic activity.

This paper investigates geographic differentiation within the *E. herklotsi* lineage using mitochondrial and nuclear gene sequences. In addition, we analyze genital and shell morphology and examine the relationship between lineages distinguished by molecular markers and morphological types.

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Table 1. Specimens used in the phylogenetic and morphological analyses.

Locality[a]	Latitude	Longitude	Altitude	Estimated mean temperature	Estimated mean rainfall	Haplotype (n)		Number of individuals used for morphological analysis (genital type)
						mtDNA	ITS2[b]	
1 Uchiyama, Izuhara, Nagasaki, Japan	34.63	129.39	unknown	N/A	N/A	N361(1)		0
2 Mitsushima, Nagasaki, Japan	34.28	129.36	20	15.0	1901.6	N358(2)		1(N)
3 Shuhoh, Yamaguchi, Japan	34.22	131.31	100	14.0	1980.2	N278(1)		1(N)
4 Mine, Yamaguchi, Japan	34.17	131.19	90	13.8	1892.2	N280(1)		1(N)
5 Kamiagata, Nagasaki, Japan	34.16	129.23	125	14.2	2207.5	N30(1)		0
6 Tsutsu, Izuhara, Nagasaki, Japan	34.12	129.19	40	15.2	2051.4	N362(1)	L(1)	1(N)
7 Onoda, Yamaguchi, Japan	34.02	131.21	40	15.0	1617.5	N216(1), N281(1), N424(1)		2(N)
8 Genkai, Fukuoka, Japan	33.90	130.49	40	15.8	1647.3	N216(2)		2(N)
9 Tsuyazaki, Fukuoka, Japan	33.78	130.49	60	15.1	1661.6	N198(1), N216(1), N907(1), N908(1)		4(N)
10 Gonoura, Nagasaki, Japan	33.74	129.71	200	15.4	1833.0	N933(1)	L(1)	0
11 Dazaifu, Fukuoka, Japan	33.52	130.54	60	15.0	1832.6	N1142(1)		1(N)
12 Setouchi, Ehime, Japan	33.45	132.29	110	15.0	1412.0	N389(2)		1(N)
13 Higashisefuri, Saga, Japan	33.34	130.41	30	15.5	2140.0	N1142(1), N1154(2)		2(N)
14 Cheju, Korea	33.33	126.55	unknown	N/A	N/A	N32(2), N702(9)	F(1)	6(N)
15 Kitashigeyasu, Saga, Japan	33.33	130.48	60	15.7	1935.2	N713(1), N714(1)		2(N)
16 Mitagawa, Saga, Japan	33.32	130.41	10	15.8	1997.4	N1157(2)		2(N)
17 Beppu, Oita, Japan	33.31	131.51	5	15.1	1731.6	N37(1)		0
18 Eda, Kikusui, Kumamoto, Japan	32.97	130.61	25	15.5	1955.7	N1159(1)		0
19 Motegi, Kikusui, Kumamoto, Japan	32.95	130.66	50	15.3	1943.9	N715(4), N717(1), N1142(1)		6(N)
20 Aso, Kumamoto, Japan	32.94	131.09	520	12.8	2841.9	N285(4)		0
21 Ueki, Kumamoto, Japan	32.94	130.73	40	15.5	1918.1	N392(5), N395(1), N766(2)	L(1)	7(N)
22 Ikeda, Kumamoto, Japan	32.81	130.71	30	16.6	1992.8	N252(1), N934(1)		0
23 Furukyo, Kumamoto, Japan	32.80	130.71	50	16.4	1978.4	N116(2)		1(N)
24 Nagasaki, Japan	32.75	129.89	160	16.4	2022.6	N289(1), N290(1)		2(N)
25 Kitaura, Miyazaki, Japan	32.72	131.86	10	16.2	2254.9	N964(1), N965(1)		2(N)
26 Kamitogawa, Hinokage, Miyzaki, Japan	32.70	131.41	240	14.0	2568.2	N743(1)	J-F(1)	1(N)
27 Shironita, Hinokage, Miyazaki, Japan	32.70	131.41	300	14.0	2568.2	N271(1)		1(N)
28 Shiidani, Hinokage, Miyazaki, Japan	32.69	131.33	400	13.7	2352.3	N270(1), N271(1)	G(1)	0
29 Tamanoura, Nagasaki, Japan	32.65	128.69	300	15.1	2260.7	N755(1)		1(N)
30 Shiranui, Kumamoto, Japan	32.64	130.64	10	16.3	1920.2	N116(1)		0
31 Oyano, Kumamoto, Japan	32.60	130.43	40	16.5	1812.2	N116(1)		1(N)
32 Miyahara, Kumamoto, Japan	32.55	130.71	40	15.9	2034.5	N1171(1)		1(N)
33 Shikizakimisaki, Reihoku, Kumamoto, Japan	32.53	130.02	50	16.6	1839.0	N816(5), N818(1), N1147(1), N1150(1), N1151(2)	L(1)	6(N)
34 Matsushima, Kumamoto, Japan	32.51	130.43	10	16.1	1965.0	N1046(1)		1(N)
35 Suko, Ariake, Kumamoto, Japan	32.51	130.34	10	16.2	1968.3	N303(2), N816(1), N1046(1)		2(N)
36 Sendan, Yatsushiro, Kumamoto, Japan	32.50	130.61	5	16.7	1950.8	N1045(1)		1(N)
37 Shimoge, Ariake, Kumamoto, Japan	32.50	130.37	10	15.9	2037.7	N1064(1)		1(N)
38 Sozo, Yatsushiro, Kumamoto, Japan	32.48	130.57	10	16.4	1934.9	N284(5)	G-H(1)	5(N)
39 Oshimago, Ariake, Kumamoto, Japan	32.47	130.26	20	16.0	2076.7	N1072(1), N1073(2)	L(1)	3(N)
40 Shiiba, Miyazaki, Japan	32.46	131.03	1300	7.7	2960.3	N48(1)		0
41 Kadogawa, Miyazaki, Japan	32.45	131.56	200	15.0	2475.4	N115(1)		1(N)
42 Hichiya, Hyuga, Miyazaki, Japan	32.43	131.67	10	16.6	2215.4	S919(1)		1(S)
43 Sumoto, Kumamoto, Japan	32.42	130.28	20	16.0	2132.4	N816(2)		2(N)
44 Haki, Sakamoto, Kumamoto, Japan	32.41	130.64	30	15.6	2334.1	N297(1), N1046(1)		2(N)
45 Arase, Sakamoto, Kumamoto, Japan	32.40	130.64	30	15.2	2354.7	N1046(2)		2(N)
46 Sakamoto, Hyuga, Miyazaki, Japan	32.36	131.63	5	16.6	2314.2	S947(1)		1(S)
47 Saiwaki, Hyuga, Miyazaki, Japan	32.34	131.62	100	16.6	2362.5	S105(4)		4(S)
48 Kitaganjouji, Hitoyoshi, Japan	32.21	130.78	160	15.1	2346.0	S105(1), S1043(1)		2(S)
49 Ishikawauchi, Kijo, Miyazaki, Japan	32.20	131.41	100	14.9	2881.6	S120(1)		0
50 Saito, Miyazaki, Japan	32.20	131.32	100	14.4	2872.3	N26(2), N93(1), S27(1), S105(1), S345(2)		3(N), 4(S)
51 Koshinoo, Nishimera, Miyazaki, Japan	32.20	131.23	350	13.5	2804.5	S733(1)		0
52 Fumoto, Hitoyoshi, Kumamoto, Japan	32.20	130.77	140	15.2	2412.2	S38(3), S105(3), S906(1), S1036(1)		8(S)
53 Taniuchi, Kijo, Miyazaki, Japan	32.20	131.44	200	14.9	2804.0	N121(1)		0
54 Kobaru, Nishimera, Miyazaki, Japan	32.19	131.24	210	14.3	2800.7	S105(1), S728(1)		1(S)
55 Kuranomoto, Nagashima, Kagoshima, Japan	32.19	130.11	10	17.1	2068.9	N314(1)		0
56 Kawara, Kijo, Miyazaki, Japan	32.18	131.42	60	15.7	2755.8	S105(2), S363(3), S367(1)		5(S)
57 Omata, Nishimera, Miyazaki, Japan	32.15	131.13	470	12.6	2965.3	N734(1)		0

To be continued.

Table 1. Continued.

No. Locality	Lat	Long				Haplotypes		
58 Sasue, Nagashima, Kagoshima, Japan	32.15	130.12	20	17.0	2064.0	N311(1), N312(1)		1(N)
59 Takanabe, Miyazaki, Japan	32.13	131.52	10	17.1	2327.8	S916(1), S917(1), S1060(1)		3(S)
60 Mt. Nishimata, Suki, Miyazaki, Japan	32.12	131.08	800	11.8	2909.6	N336(1)		1(N)
61 Nakahara, Suki, Miyazaki, Japan	32.12	131.07	540	12.2	2894.7	S105(1), S740(1)		2(S)
62 Karakuma, Nagashima, Kagoshima, Japan	32.11	130.12	10	17.2	2038.7	N306(2)		1(N)
63 Kiuragi, Kobayashi, Miyzaki, Japan	32.10	130.99	450	12.9	2985.7	S79(1)		0
64 Akune, Kagoshima, Japan	32.08	130.19	20	17.1	2074.2	N298(1)		1(N)
65 Izumi, Kagoshima, Japan	32.07	130.36	30	16.4	2150.3	N298(1)		1(N)
66 Baba, Ebino, Miyazaki, Japan	32.05	130.77	220	14.9	2849.9	S38(1)		1(S)
67 Kawanaka, Aya, Miyazaki, Japan	32.05	131.16	75	14.9	2773.4	S906(1)		1(S)
68 Uchiyama, Suki, Miyazaki, Japan	32.05	131.11	500	13.1	2768.6	S105(1)		1(S)
69 Kyomachi, Ebino, Miyazaki, Japan	32.05	130.77	220	15.0	2827.3	S911(1), S912(2), S913(1)		4(S)
70 Teruhaohashi, Aya, Miyazaki, Japan	32.02	131.21	75	15.1	2750.9	S105(2)		2(S)
71 Kitakobayashibaru, Kobayashi, Miyazaki, Japan	32.00	130.99	200	15.2	2937.7	N107(1), N119(6)	L-M(1)	6(N)
72 Mitsutani, Ebino, Miyazaki, Japan	31.98	130.84	600	11.5	3986.3	S56(1), S79(1)		1(S)
73 Sendan, Kobayashi, Miyazaki, Japan	31.97	130.98	200	15.2	3064.8	S79(1)		1(S)
74 Hosono, Kobayashi, Miyazaki, Japan	31.96	130.96	240	14.7	3338.4	S58(1)		0
75 Konan, Miyazaki, Japan	31.90	131.39	10	17.1	2445.3	S105(1)		1(S)
76 Miike, Takaharu, Miyazaki, Japan	31.89	130.97	360	14.4	3159.8	S38(1)		1(S)
77 Makizono, Kagoshima, Japan	31.89	130.83	560	13.1	3735.8	S79(3)	B(1)	3(S)
78 Miikekaitaku, Miyakonojo, Miyazaki, Japan	31.86	130.94	530	13.3	3117.8	S79(1)		1(S)
79 Tano, Miyazaki, Japan	31.85	131.33	110	16.2	2592.6	S105(6)	B(1)	6(S)
80 Miike, Miyakonojo, Miyazaki, Japan	31.85	130.93	580	13.6	3076.4	S25(1)		0
81 Sato, Kagoshima, Japan	31.85	129.92	5	17.6	2261.1	N298(2), N544(1)		3(N)
82 Gumisaki, Sendai, Kagoshima, Japan	31.84	130.21	10	17.0	2293.0	N34(1)		0
83 Takae, Sendai, Kagoshima, Japan	31.82	130.27	20	16.9	2349.6	N34(1)	J-L(1)	0
84 Kaeda, Miyazaki, Japan	31.81	131.46	10	17.3	2766.2	S118(1)		1(S)
85 Miyazato, Sendai, Kagoshima, Japan	31.81	130.28	20	16.8	2290.3	N34(1), N403(1), N404(1), N406(1), N1146(1)		4(N)
86 Shirahama, Miyzaki, Japan	31.79	131.48	10	17.3	2720.7	S735(1), S736(1)		1(S)
87 Oiwata, Miyakonojo, Miyazaki, Japan	31.70	131.04	160	15.8	2538.0	N213(1)		1(S)
88 Kuroyama, Kitago, Miyazaki, Japan	31.69	131.29	220	15.1	3137.9	S99(1)		0
89 Komatsu, Kitago, Miyzaaki, Japan	31.68	131.29	300	14.7	3158.8	S99(1)		1(S)
90 Higashiichiki, Kagoshima, Japan	31.65	130.33	10	16.8	2152.2	N298(1), N961(1)		0
91 Nichinan, Miyazaki, Japan	31.62	131.33	30	16.9	2856.9	S99(1)		0
92 Iso, Kagoshima, Japan	31.60	130.58	5	17.3	2278.4	S334(1)	B(1)	1(S)
93 Shiroyama, Kagoshima, Japan	31.60	130.56	100	17.7	2228.1	S439(1)		1(S)
94 Osumi, Kagoshima, Japan	31.55	131.01	100	16.2	2418.0	N213(2), S726(2)	E(1)	4(S)
95 Usuki, Kagoshima, Japan	31.55	130.51	10	17.6	2388.3	N34(5), N338(2)	I-K(1)	7(S)
96 Sakuragaoka, Kagoshima, Japan	31.54	130.53	60	18.0	2362.9	N34(1), N97(1)		2(S)
97 Fukiage, Kagoshima, Japan	31.51	130.36	50	17.3	2341.9	S79(1)		0
98 Osaki, Kagoshima, Japan	31.50	130.93	220	15.8	2494.5	S319(2)		0
99 Kushira, Kagoshima, Japan	31.44	130.92	115	16.4	2399.7	S319(2), S726(1)		3(S)
100 Kaseda, Kagoshima, Japan	31.42	130.31	20	17.4	2197.4	S79(1), S1167(1)		2(S)
101 Kasasa, Kagoshima, Japan	31.41	130.13	20	17.3	2164.8	S1088(1)		1(S)
102 Makurazaki, Kagoshima, Japan	31.25	130.28	20	17.7	2123.3	S1163(2)		2(S)
103 Sata, Sata, Kagoshima, Japan	31.09	130.69	10	17.8	2200.5	S215(1)		0
104 Satamisaki, Sata, Kagoshima, Japan	31.00	130.67	50	18.1	2154.9	S197(5)		5(S)
105 Iseki, Nishinoomote, Kagoshima, Japan	30.76	131.07	10	19.2	2315.9	S228(1)		0
106 Okigahamada, Nishinoomote, Kagoshima, Japan	30.75	131.07	10	19.1	2461.0	S228(1)		1(S)
107 Ishidoh, Nishinoomote, Kagoshima, Japan	30.72	131.04	50	19.0	2456.1	S225(1)		0
108 Kumano, Nakatane, Kagoshima, Japan	30.47	130.97	60	19.3	2929.8	S935(1)		0
109 Shioya, Nakatane, Kagoshima, Japan	30.45	130.96	1	19.2	2959.2	S226(1)		0
110 Shitoko, Kamiyaku, Kagoshima, Japan	30.45	130.52	5	19.2	3235.1	S197(2)	C(1), C-D(1)	2(S)
111 Miyanoura, Kamiyaku, Kagoshima, Japan	30.42	130.57	20	19.3	3673.9	S145(1), S369(1)	B-C(1)	1(S)
112 Nakanokami, Minamitane, Kagoshima, Japan	30.40	130.91	150	18.5	2946.1	S227(3)		3(S)
113 Matsubara, Minamitane, Kagoshima, Japan	30.38	130.93	20	19.3	2951.6	S231(2), S935(1)	A(2)	2(S)
114 Mt. Kankake, Kamiyaku, Kagoshima, Japan	30.37	131.38	160	18.7	3584.9	S234(1)	B(1)	1(S)

[a] Locality numbers are those used in Fig. 1
[b] Haplotypes connected by hyphen are of same individuals.

We show the existence of two distinct lineages within *E. herklotsi*, which are also discriminated by genital morphology.

## MATERIALS AND METHODS

Study organisms and sampling

This study examined *E. herklotsi* as defined by Azuma (1995), who recognized six subspecies: *E. h. herklotsi*, *E. h. yakushimana*, *E. h. nesiotica*, *E. h. tsushimana*, *E. h. hyugana*, and *E. h. kirishimensis*. Of these subspecies, Minato (1985, 1988) treated *yakushimana* as an independent species. We examined 256 individuals belonging to *E. herklotsi* (sensu Azuma, 1995) from 114 localities in western Japan and Cheju Island, Korea, which covered its entire range (Table 1, Fig. 1). We collected one to 11 individuals per sampling site. *Euhadra idzumonis* from Hiroshima, Japan, was used as the outgroup for the molecular phylogenetic analysis, based on the results of a previous molecular phylogenetic study (Ueshima and Asami, 2003). Specimens collected in the field were boiled to separate the bodies and shells, and the tips of the feet were preserved in 99% ethanol for use in the DNA analysis.

DNA extraction, PCR, and sequencing

Total genomic DNA was isolated using a modification of Chiba's (1999) procedure. Muscle tissues were homogenized in 500 ml of 2X CTAB solution with 10 mg/ml proteinase K and incubated at 55°C for more than 1 h. Standard phenol/chloroform extraction and ethanol precipitation were used to extract DNA, which was dissolved in 30 to 60 μl of TE buffer.

A mitochondrial DNA fragment encoding the cytochrome oxidase subunit I gene (COI) was amplified with the polymerase chain reaction (PCR) using primers CO1DF (5'-TTTTGRTTTTTTTG-GKCAYCCNGA-3') and 16Scs1r (5'-CCATTATGCAAAAGGTAT-3'; a modification of 16Scs1 in Chiba, 1999) for all 256 specimens. We also PCR-amplified a nuclear DNA region including the internal transcribed spacer 2 (ITS2) gene using primers 18d ("fruitfly") 5'-CA-CACCGCCCGTCGCTACTACCGATTG-3' (Hillis and Dixon, 1991) and ITS-4 5'-TCCTCCGCTTATTGATATGC-3' (White *et al.*, 1990) for 21 specimens (Table 1). The specimens used for the ITS2 analyses were selected randomly from the major clades on the COI tree (see below). Primer 18d is located in the 18S rRNA gene region, and ITS-4 is in the 28S rRNA gene region. PCR amplifications were performed with a GeneAmp PCR System 9700 Thermal Cycler (PE Applied Biosystems, Foster City, CA), using an Ex *Taq* Polymerase Kit (Takara Shuzo, Otsu, Japan). The thermocycling regime consisted of an initial 2 min at 94°C followed by 30 cycles of 20 sec
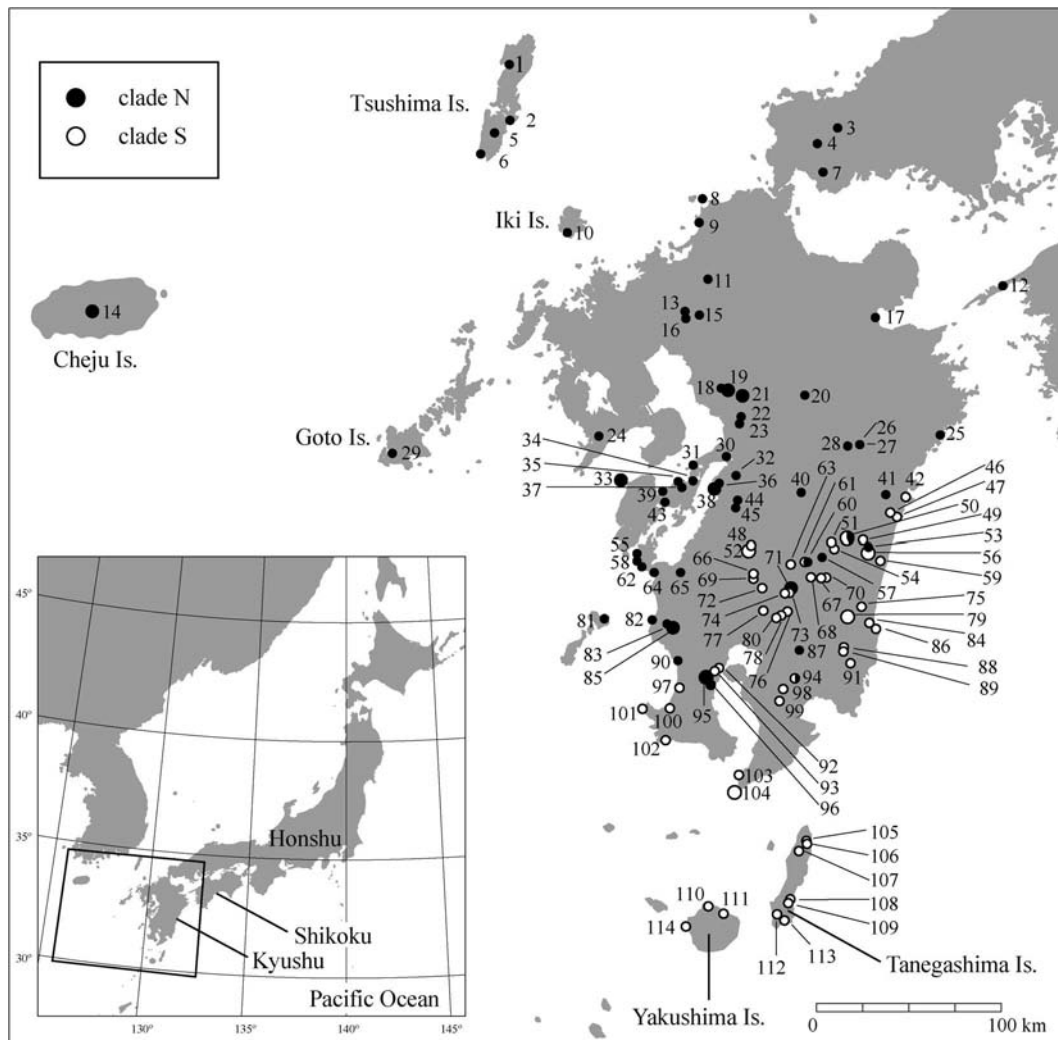
Fig. 1. Sampling localities of the snails and the distribution of clades N and S of mitochondrial COI haplotypes. Large circles indicate localities with five or more samples, and small circles those with one to four samples. See Table 1 for the localities indicated by numbers beside the circles. The proportions of clade-N and -S haplotypes at locality 50 are represented in a pie graph.

at 94°C, 20 sec at 50°C, and 2 min at 72°C, with a final 10 min at 72°C.

The amplified fragment was purified with silica gel (Boom *et al*., 1990) for subsequent direct sequencing by the dideoxy chain-termination method using an ABI Prism BigDye Terminator Cycles Sequencing Ready Reaction Kit (PE Applied Biosystems). In the cycle sequencing reaction for ITS2, primer ls5.8f (5'-CATTGAA-CATCGACATCTTGAACGC-3'; Y. Kameda and M. Kato, personal communication) was used instead of 18d. Nucleotide sequences of 752 bp (COI) and 498–511 bp (ITS2) were determined for both strands by electrophoresis on an ABI 377 automated sequencer (PE Applied Biosystems). Although the sequenced region included 19 bp of the 5.8S ribosomal RNA gene and 6 bp of the 28S ribosomal RNA gene in addition to 473–486 bp of the ITS2 gene, we call it "ITS2" for convenience. The nucleotide sequences reported here have been deposited in GenBank (accession numbers AB267490–AB267630).

Phylogenetic analysis

The COI sequences were unambiguously aligned manually. Only a 1-bp gap was required at the end of the sequences. This gap was treated as missing data in the analyses. Neighbor-joining, maximum parsimony, maximum likelihood, and Bayesian analyses were used to reconstruct the phylogenetic relationships among the taxa. The neighbor-joining and parsimony analyses were performed using PAUP* version 4.0b10 (Swofford, 2002). The TVM+I+G substitution model (transversional model with the gamma shape parameter and proportion of invariable sites) was selected as the best-fit model by both the Akaike information criterion and hierarchical likelihood ratio tests implemented in Modeltest 3.07 (Posada and Crandall, 1998). TVM is a variation of the general time reversible (GTR) model, with equal substitution rates for transitions. Estimates of genetic divergence used this substitution model. In the parsimony analysis, a heuristic search was conducted using 100 random-addition analyses with tree bisection–reconnection (TBR) branch swapping (MulTrees option activated). The confidence in each node was assessed using 1,000 bootstrap pseudo-replicates. The GTR+I+G substitution model was used for the maximum likelihood (ML) analysis performed with the June 2005 version of TREEFINDER (Jobb, 2005) because this was the closest model to TVM+I+G available in this program. Bayesian analysis was performed using MrBayes version 3.1.2 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003). A Bayesian Markov Chain Monte Carlo (MCMC) analysis was performed using the GTR+I+G model. The MCMC analysis was run for 2 million generations sampling every 100th tree. The first 2,000 trees were discarded as burn-in.

To detect the effect of isolation by distance, we conducted the Mantel test (Mantel, 1967) using the computer software R Package (Casgrain and Legendre, 2000). We calculated Fu's F_S (Fu, 1997) as an indicator of the effects of past demography using ARLEQUIN 3.01 (Excoffier *et al*., 2005). The F_S statistic is said to be sensitive to population demographic expansion, which generally leads to a large negative F_S (Fu, 1997). A mismatch analysis was also performed to study the demographic history. The expected pairwise mismatch distribution under the model of population expansion (Rogers and Harpending, 1992; Rogers, 1995) was calculated, and the goodness of fit was tested using ARLEQUIN.

The alignment of the ITS2 data required gaps in two regions, but no alignment-ambiguous region occurred. In the parsimony analysis, gaps were treated as missing data and coded separately; each gap represented one character with the states absent (0) or present (1). Gaps spanning more than one nucleotide position were scored as a single character. A haplotype network was constructed using the computer program TCS version 1.21 (Clement *et al*., 2000) based on the algorithm given in Templeton *et al*. (1992).

Morphological analysis

Euhadra land snails are hermaphrodites. Genital morphology was studied for adult specimens because they provide the most reliable taxonomic characters for this genus (Minato, 1985). In addition, the shell width and height of adult specimens were measured using vernier calipers to the nearest 0.01 mm. The shell shape was compared between clades using analysis of covariance (ANCOVA) based on the width and height data. To examine the effects of environmental variables on shell size and shell shape within each clade, we used general measurements of shell size and shape based on shell width and height data, the PC1 and PC2 scores obtained by a principal component analysis. Greater PC1 scores indicate larger shell sizes, and greater PC2 scores denote flatter shell shapes. These PC1 and PC2 scores were subject to multiple linear regression analysis with five environmental factors as explanatory variables. The environmental variables comprised three geographical (latitude, longitude, and altitude) and two climatic (annual mean temperature and mean annual rainfall) variables. The climatic data were obtained from Mesh Climatic Data 2000 (Japan Meteorological Agency, 2002), which compiled 30-year averages of climatic data at a 1-km mesh scale. In the multiple regression, variables were selected by a stepwise procedure using the Akaike information criterion.
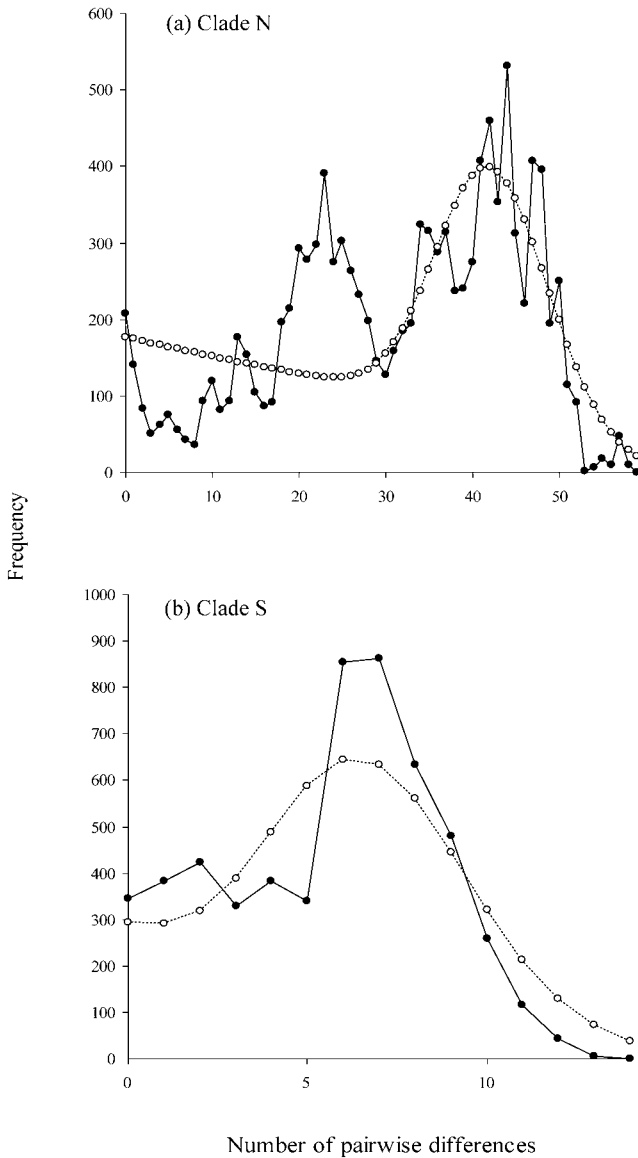
RESULTS

Mitochondrial gene genealogy

We observed marked variation in the COI sequences within *E. herklotsi* and obtained 126 unique COI haplotypes from 256 individuals (Table 1). The topologies resulting from different tree search methods were congruent, except for some nodes with low support. Therefore, only the maximum-likelihood tree is shown in Fig. 2. Two major clades, named clade N and clade S, were distinguished. The genetic diversity within clade S was lower than that within clade N, which consisted of two well differentiated clades: L1 and L2 (Fig. 2). The mean pairwise sequence divergence (±SE) was 0.008±0.002 (*n*=105) for clade S and 0.044±0.004 (*n*=151) for clade N. These clades occurred largely in different regions (clade N in the northwest, clade S in the southeast; Fig. 2). We collected haplotypes from both clades at locality 50.

We tested the hypothesis that the low genetic diversity in clade S resulted from a bottleneck followed by a recent demographic expansion. The simulated value of Fu's F_S (Fu, 1997) was significantly smaller than the observed F_S in clade S (F_S=−25.27, *P*<0.001), while it was not significantly different in clade N (F_S=−10.13, *P*=0.09). Therefore, a bottleneck likely occurred in clade S. A mismatch analysis was performed to compare the demographic history of the two clades. The mismatch distribution for clade N was multimodal (Fig. 3a), reflecting the high genetic diversity (see Fig. 2), whereas it was close to unimodal for clade S (Fig. 3b). For both clades, however, the observed mismatch distribution fit the expected distribution under a model of population expansion (clade N: sum of the squared deviation *SSD*=0.0039, *P*=0.68 and raggedness index *r*=0.0026, *P*=0.52; clade S: *SSD*=0.0076, *P*=0.31 and *r*=0.015, *P*=0.43). Mantel tests revealed a significant positive correlation between genetic and geographic distance within clades N and S (Fig. 4; clade N: *n*=153, Mantel's *r*=0.44, *P*<0.01; clade S: *n*=105, Mantel's *r*=0.58, *P*<0.01), indicating the effect of isolation by distance.
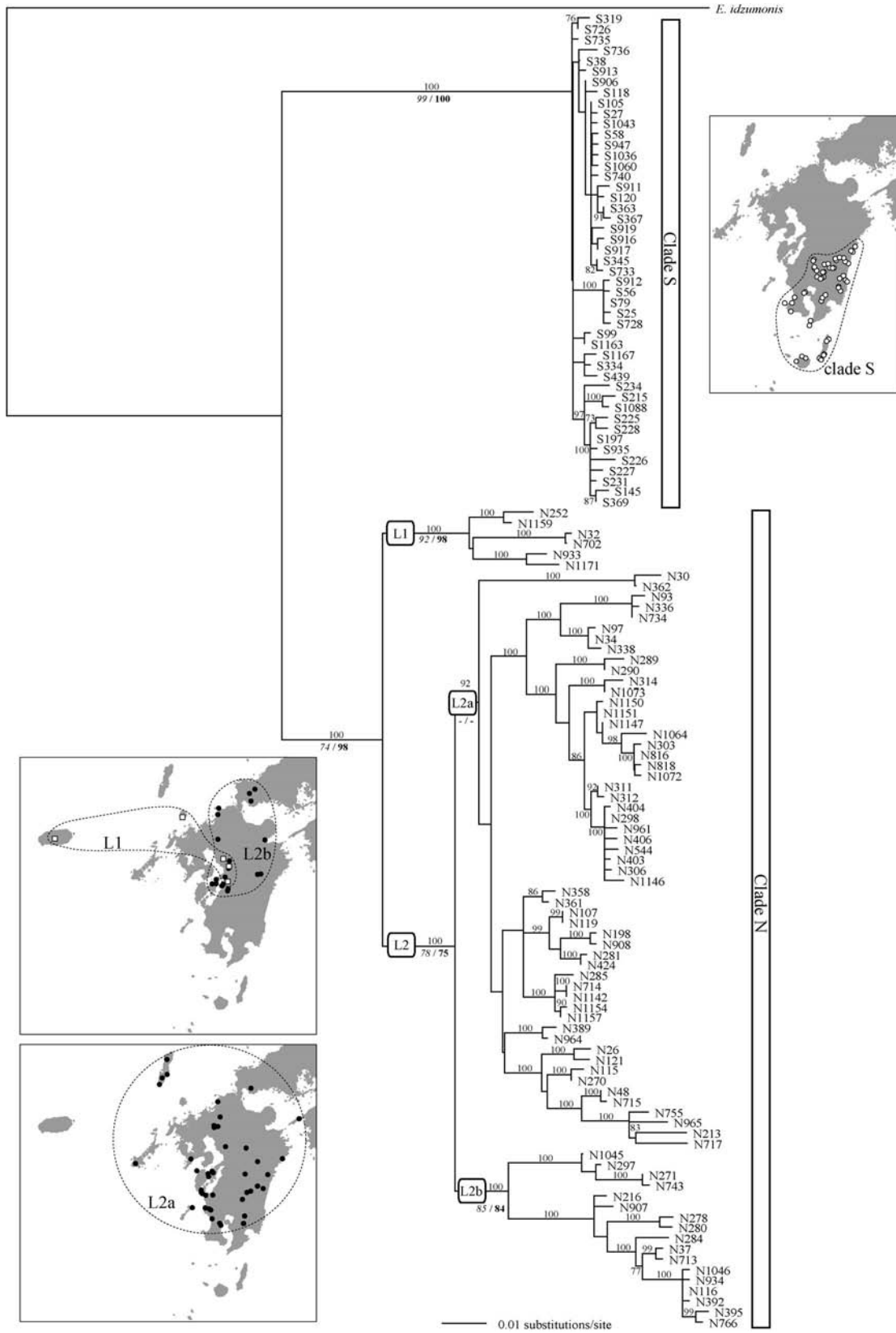
Fig. 2. Maximum-likelihood tree of the mitochondrial COI gene sequences. The posterior probability values of nodes resulting from the Bayesian analysis are shown above branches when >70%. Bootstrap values derived from 1,000 replicates in the NJ and parsimony analyses are shown in italics and bold font, respectively, below branches for major clades (when >70%). Maps show the geographical distributions of the major clades.
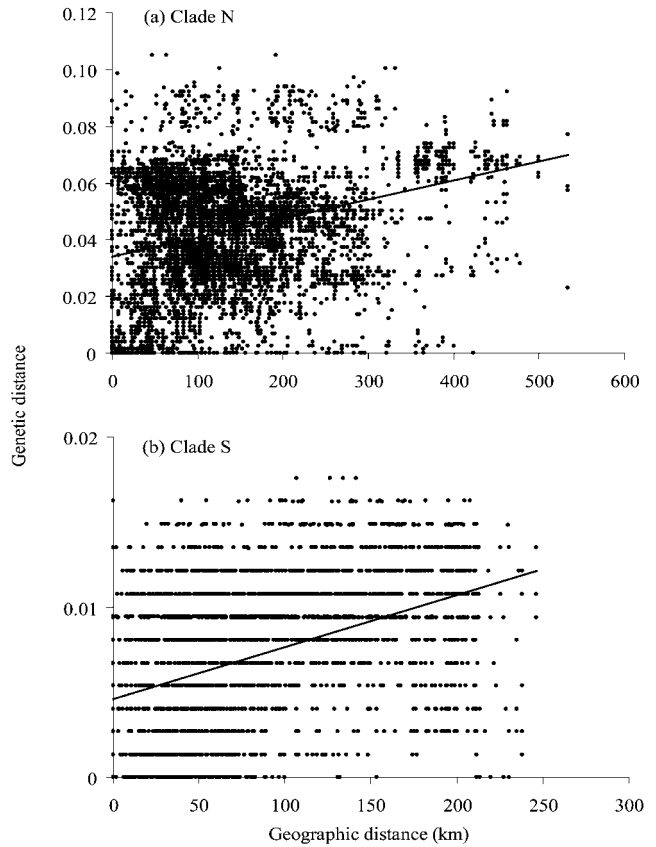
Fig. 4. Relationship between the genetic and geographic distances between populations of clades N and S. Linear regression lines are drawn for illustrative purposes.

Fig. 3. Mismatch distributions of the mitochondrial COI haplotypes for clades N and S. Closed circles show the observed distributions, and open circles the expected distribution under the sudden demographic expansion model.

Nuclear gene genealogy

For ITS2, 13 haplotypes were obtained from 21 specimens. The direct sequencing results for six of the 21 specimens revealed duplicated signals, probably caused by a difference among the multiple copies of ITS2. These were resolved manually based on the two sequences from the forward and reverse primers. Variation within the ITS2 region was low, and all the haplotypes were connected with each other without missing haplotypes in the statistical parsimony tree (Fig. 5). In the ITS2 tree, four ITS2 haplotypes from clade S snails formed a monophyletic group, and eight ITS2 haplotypes formed another group.

Morphological analysis

Individuals of the mitochondrial clades N and S had dif-

Fig. 5. Statistical parsimony network for the nuclear ITS2 gene. Open circles represent haplotypes; closed circle indicates a haplotype that did not appear in the samples. Haplotypes separated by a single line are one step apart. The number in each haplotype circle indicates the number of individuals with that haplotype.
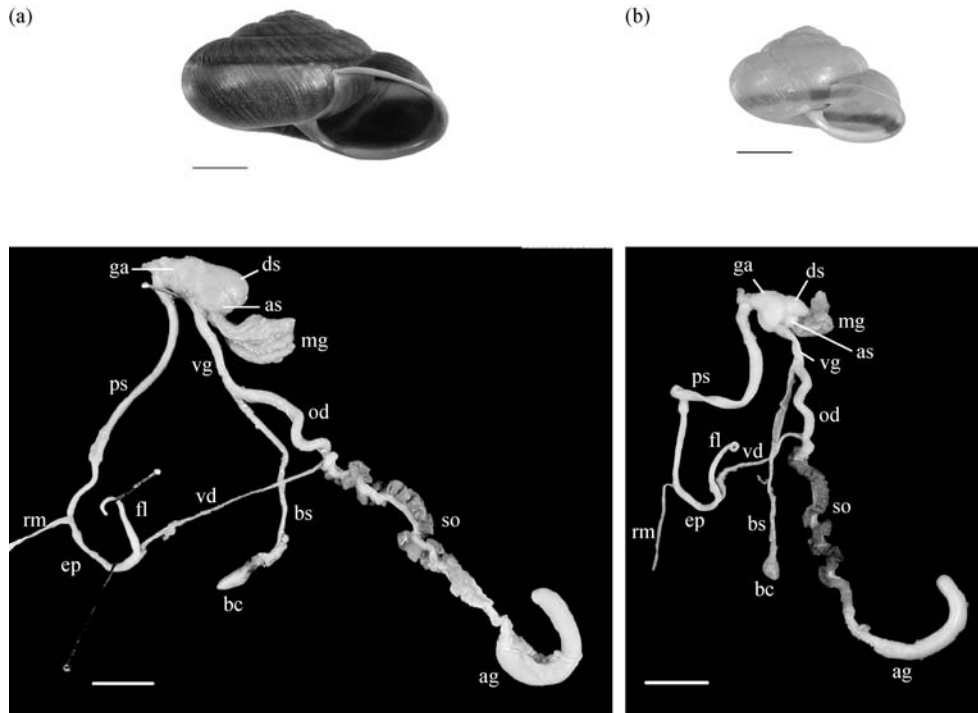
Fig. 6. Shells (upper) and reproductive organs (lower) of snails of clade N with type-N genitalia (a, locality 50) and of clade S with type-S genitalia (b, locality 50). Scale bars indicate 10 mm. Abbreviations: ag, albumen gland; as, accessory sac; bc, bursa copulatrix; bs, bursa copulatrix stalk; ds, dart sac; ep, epiphallus; fl, epiphallial flagellum; ga, genital atrium; mg, mucous glands; od, oviduct; ps, penial sheath; rm, penis retractor muscle; so, spermoviduct; vd, vas deferens; vg, vagina.

ferent genital morphologies (Fig. 6). In clade N snails, the basal part of the vagina was attached to the genital atrium vertically, *i.e.*, in almost the same direction as the penial sheath (called the N type hereafter). In contrast, the basal part of the vagina of clade-S snails was almost parallel to the genital atrium, and the genital atrium was swollen (S type). However, samples from localities 95, 96, and two of four samples from locality 94 had S-type genitalia, although they belonged to mitochondrial clade N, suggesting introgressive hybridization between the two lineages. These localities were at the geographic boundary between the two mitochondrial clades.

In the analyses of shell morphology, we excluded clade-N snails with genital type S (n=11) to remove the effect of inconsistency between mitochondrial lineage and morphological type that may have been due to hybridization. The mean shell width and height (Fig. 7) were greater for clade N than clade S (width (mean±SD): clade N, 35.2±3.33 mm; clade S, 28.0±3.15 mm; df=172, t=14.64, P<0.001; height: clade N, 21.5±1.96 mm; clade S, 18.1±1.80 mm; df=172, t=11.82, P<0.001). However, ANCOVA showed no significant difference in shell height between these clades after controlling for the effect of shell width (ANCOVA, shell width effect: df=1, F=219.6, P<0.0001; clade effect: df=1, F=0.70, P=0.40), indicating no difference in shell shape between the clades.

In the stepwise multiple linear-regression analyses of the determinants of shell size and shape (Table 2), the selected model for shell size (PC1) included altitude for both clades, and longitude and climatic variables (mean annual
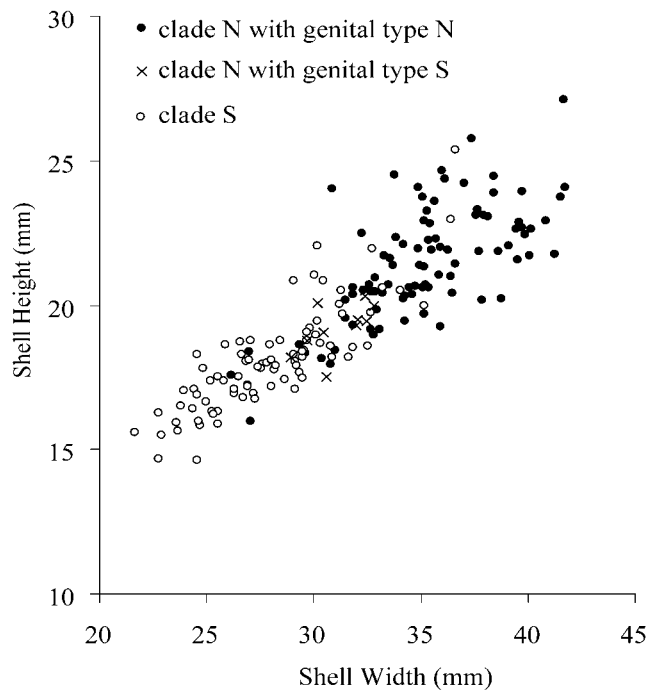
Fig. 7. Variation in shell width and shell height for each clade.

temperature, annual precipitation) for clade S only (clade N: model R^2_{adj}=0.047, P=0.022; clade S: model R^2_{adj}=0.180, P<0.001). For shell shape (PC2), the selected model

Table 2. Stepwise multiple regression analyses for factors affecting the shell size (PC1) and shell shape (PC2) of *E. herklotsi*. PC1 explained 93.4% of the variance.

	Clade / genital type	Explanatory variable	Standard regression coefficient	t	P
PC1	N	Altitude (m)	0.241	2.33	0.022
PC1	S	Longitude (°E)	−0.479	−4.40	<0.001
		Altitude (m)	−0.578	−2.81	0.006
		Annual rainfall (mm)	0.313	2.36	0.021
		Mean temperature (°C)	−0.330	−1.90	0.061
PC2	N	Longitude (°E)	0.591	5.82	<0.001
		Mean temperature (°C)	0.488	3.14	0.002
		Annual rainfall (mm)	0.492	2.91	0.005
		Latitude (°N)	0.323	1.80	0.075
PC2	S	Latitude (°N)	0.556	5.16	<0.001
		Longitude (°E)	−0.183	−1.70	0.093

included longitude, mean annual temperature, annual precipitation, and latitude for clade N (model R^2_{adj}=0.326, P<0.001), and latitude and longitude for clade S (model R^2_{adj}=0.232, P<0.001). Therefore, the factors affecting shell size and shape were not consistent between the two clades, as indicated by the difference in model composition and the regression coefficients.

DISCUSSION

Two lineages within *E. herklotsi*

This study revealed the existence of two distinct lineages within *E. herklotsi* (*sensu* Azuma, 1995) as evidenced by both mitochondrial and nuclear DNA markers and genital morphology. These lineages occur in parapatry, with a boundary zone in southern Kyushu. The two lineages co-occurred at one site (locality 50), and discordance of the genital morphology with the COI and ITS2 lineages was detected at three localities (94–96) at the boundary only, where all individuals possessed type-S genitals, whereas all but two had clade-N mitochondria. All of these localities are located in southern Kyushu, the boundary between the two lineages. Therefore, while the two lineages apparently have attained allopatric differentiation, secondary contact and gene flow due to introgressive hybridization may have occurred at the boundary zone.

Although we found a difference in shell size between snails of different mitochondrial lineages and genital morphologies, the variation in shell morphology within each lineage was large and could not be explained simply and consistently by environmental variables. Therefore, different selection regimes might have acted on shell morphology during the formation of the two lineages, resulting in the present divergence in shell size.

The taxonomic treatment of the two lineages remains to be resolved in a future study. Although these lineages may be treated as two species, further analysis of reproductive isolation at their contact zone is needed. The type locality of *E. herklotsi* is described as "Japan" (Martens, 1860) and is thought to be Nagasaki in northwest Kyushu (Minato, 1988). The snails of clade N likely correspond to *E. herklotsi*, because only clade N occurs in Nagasaki. The subspecies *E. h. kirishimensis* Kuroda, 1936, distributed in southern

Kyushu, corresponds to clade S, and *E. h. nesiotica* (Pilsbry, 1902), which is described from Tanegashima Island, south of mainland Kyushu, is found where only clade S occurs. Therefore, *nesiotica* may be used as the species name of clade S because of its priority. Subspecies *yakushimana* was originally described as a separate species (Pilsbry and Hirase, 1903) and has unique characteristics that easily distinguish it from *E. herklotsi*. However, we found that *yakushimana* had the same COI and ITS2 haplotypes as clade S of *E. herklotsi*. The species status of *yakushimana* needs to be studied based on other nuclear DNA genealogies and experimental crosses with *E. herklotsi* lineages from other regions.

Historical process of divergence

We found relatively low genetic diversity in clade S compared with clade N. The result of Fu's F_S (Fu, 1997) and the mismatch analysis suggest that clade S experienced a bottleneck and successive rapid range expansion. The putative bottleneck event in clade S might be related to volcanic eruptions, which occurred frequently during the last 100,000 years on Kyushu (Fig. 8). During this period, the Aso caldera in central Kyushu erupted from 85,000 to 90,000 years ago (Machida and Arai, 2003), the Aira caldera in southern Kyushu erupted 29,000 years ago (Okuno, 2002; Machida and Arai, 2003), and the Kikai caldera in the south of mainland Kyushu erupted 7,300 years ago (Okuno, 2002; Machida and Arai, 1978). Since these large volcanic eruptions affected different regions (Fig. 8), and the boundary of clades N and S lies in southern Kyushu, we hypothesize that the Aira eruption once depleted the land snails in southern Kyushu, especially clade S, and recolonization by the two clades from the south and north resulted in the present contact zone. A bottleneck event followed by population expansion was also suggested in the Japanese macaque (*Macaca fuscata yakui*) on Yakushima Island (Hayaishi and Kawamoto, 2006), and the event that caused the bottleneck is thought to be the Kikai eruption.

Application of the molecular clock to our COI data should be useful for estimating the time of the bottleneck event in clade S and identifying the related geohistorical event. Unfortunately, no appropriate calibration method or
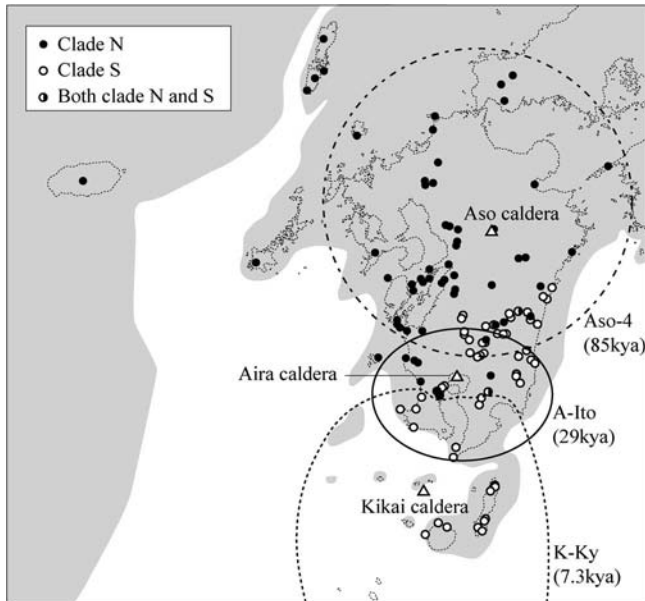
Fig. 8. Major volcanic activity in Kyushu during the last 100,000 years, land areas at the last glacial maximum, 20,000 years ago (shaded areas; after Nagaoka, 2001), and the distribution of *E. herklotsi*. Aso-4, A-Ito, and K-Ky represent the maximum range of the sediments from pyroclastic flows.

published evolutionary rate exists for the COI sequences of *Euhadra*. If we use a rate of 10% per million years estimated for the mitochondrial 16S rRNA gene of *Euhadra* (Hayashi and Chiba, 2000), the bottleneck event in clade S occurred 40,000 years ago, before the eruption of the Aira caldera, based on the mean pairwise distance between clade S haplotypes (=0.008). However, this age can be overestimated because the molecular clock is likely faster over shorter periods (Ho *et al.*, 2005). In addition, the COI clock may be very different from the 16S clock used here.

The wide distribution of clade N on islands other than Kyushu might have been facilitated by the repeated formation of land bridges during glacial periods, although rafting could be another means of dispersal. In fact, land bridges for the dispersal of clade N were available between Tsushima Island and mainland Kyushu (Keigwin and Gorbarenko, 1992; Siddall *et al.*, 2003), Shikoku and Kyushu, and Honshu and Kyushu (Kimura, 2000; Nagaoka, 2001) (Fig. 8). However, Cheju is a volcanic island that formed from 1.2 Ma through the late Pleistocene (Kim *et al.*, 1999), and no evidence exists to indicate that Cheju Island and Kyushu were connected after the formation of the island (Matsui *et al.*, 1998). The snails might have dispersed to Cheju Island by rafting, and dispersal between mainland Kyushu and the surrounding islands may have increased the genetic diversity of clade N.

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