

## Intraspecific Molecular Phylogeny, Genetic Variation and Phylogeography of *Reticulitermes speratus* (Isoptera: Rhinotermitidae)

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Population structure was investigated in *Reticulitermes speratus* populations in the Korean Peninsula and the Japanese Archipelago. All trees derived from analyses of the combined sequence dataset of two mitochondrial genes, COII and COIII, showed that *R. speratus* populations cluster into two major clades comprising the Korean/southern Japanese populations and the northern Japanese populations. Analysis of population genetic structure showed strong genetic partitioning between populations of the two clades. To understand historical migration routes and current distributions, the phylogeographic history of *R. speratus* was inferred from intra-/interspecific phylogeny and divergence times estimated between the clades of the phylogenetic tree. The estimated migration route and divergence time of ancestral *R. speratus* are congruent with recent paleogeographic hypotheses involving land-bridge connections between the Asian continent and the Japanese Archipelago. We suggest that ancestral *R. speratus* separated into northern and southern Japanese populations after its migration into the Japanese main islands from East China during the early Pleistocene via the East China Sea basin, which may have been exposed during that period. The Korean populations seem to have diverged recently from southern Japanese populations; this may explain the current distribution of *R. speratus* in the Japanese Archipelago, and account for why it is restricted to northern areas of the Tokara Strait.

**Keywords:** COII and COIII; Intraspecific Phylogeny;

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Isoptera; Japanese Archipelago; Phylogeography; *Reticulitermes speratus*; Rhinotermitidae.

### Introduction

Hypotheses involving dispersal across land-bridge connections and vicariance via loss of such connections play a major role in explaining the terrestrial faunal distributions of islands. These processes can also affect regional persistence and extinction of species in islands. Dispersals via earlier land-bridge connections allow islands to share their terrestrial fauna with faunal source pools in neighboring continents, and regional terrestrial faunas can diverge from source continental fauna via vicariance events.

The Japanese Archipelago is a long chain of islands located off the eastern coast of the Asiatic continent, and includes the four main islands of Hokkaido, Honshu, Shikoku and Kyushu. Recent studies suggest that the main Japanese islands were connected to the Asiatic continent several times during the Pleistocene via four possible paleogeographic land bridges (the Korea Strait/Tsushima Strait, the East China Sea basin, the Taiwan/Ryukyu Islands, and the Mamiya land bridge) (Kimura, 2000; Lin *et al.*, 2002; Ota, 1998; Ota *et al.*, 2002) and that the exchange of terrestrial fauna may have been possible between the Asiatic continent and the Japanese Archipelago via land bridge connections (Millien-Parra and Jaeger, 1999). The current distributions of the Japanese terrestrial fauna seem to have resulted mainly from colonization from the faunal source pools of Asiatic mainland groups via land-bridge connections formed during the climate fluctuations of the Quaternary (Kimura, 2000; Lin *et al.*, 2002; Millien-Parra and Jaeger, 1999). In addition, inter-island isolations/connections during the Quaternary facili-



**Fig. 1.** Collection localities for *R. speratus*, and geographic distribution of East Asian *Reticulitermes*. Numbers correspond to each locality number in Table 1. Geographic distributions of *R. speratus* subspecies (*R. s. speratus*, *R. s. kyushuensis* and *R. s. leptolabralis*) are designated by different dashed lines. Distributions of the other *Reticulitermes* species in East Asia are also shown based on the references corresponding to each species in Table 1. Localities of *R. sp.* (Austin *et al.*, 2002) and *R. speratus* (Austin *et al.*, 2002) were obtained from Austin *et al.* (2002).

tated rapid genetic differentiation and speciation, structuring biodiversity and shaping the distributions of terrestrial fauna in the Japanese islands (Goodman *et al.*, 2001; Kato and Yagi, 2004; Maekawa *et al.*, 1998; Millien-Parra and Jaeger, 1999; Ohdachi *et al.*, 2001).

Termites (Isoptera) are very important ecological components in the circulation of organic matter via decomposition of litter and dead wood (Donovan *et al.*, 2001; Holt and Lepage, 2000; Sugimoto *et al.*, 2000). They are also destructive pests causing extensive damage to wooden structures (UNEP and FAO, 2000; Su, 2002). Over 70 species of *Reticulitermes* have been recognized in East Asia, including the Korean Peninsula, the Japanese Archipelago, Taiwan and China, with most of their distribution in China (ESK and KSAE, 1994; Huang *et al.*, 2000; Kitade and Hayashi, 2002; Morimoto, 1968; Takematsu,

1999; Zhu *et al.*, 1994). In the Korean Peninsula and Japanese Archipelago, five *Reticulitermes* species are currently recognized (Kitade and Hayashi, 2002; Takematsu, 1999; Yasuda *et al.*, 2000). Japanese *Reticulitermes* (Rhinotermitidae) are believed to have historical distributions dating back to the Quaternary (Morimoto, 1968), like some other invertebrates such as the *Hodotermopsis* termites (Maekawa *et al.*, 1998) and *Parides* butterflies (Kato and Yagi, 2004).

Among the East Asian *Reticulitermes* species, *R. speratus* is especially interesting because of aspects of its distribution and taxonomic status. In contrast to the other endemic *Reticulitermes*, which have their distributions in southern parts of the Tokara Strait, *R. speratus* is distributed over the northern part of the Tokara Strait and in the Korean Peninsula (Fig. 1). In Korea, its distribution

ranges widely throughout South Korea (ESK and KSAE, 1994). In the Japanese Archipelago, it ranges from the southern part of Hokkaido and the remaining three major islands (Honshu, Shikoku and Kyushu) to outlying islands including Yakushima Island and Tanegashima Island (Kitade and Hayashi, 2002; Morimoto, 1968; Takematsu, 1999), but it has not been reported in the islands on the southern part of the Tokara Strait. Since the Tokara Strait has played an important role as a barrier to the migration of terrestrial fauna from its initial formation during the Pliocene (Kizaki and Oshiro, 1980), it may have been a factor shaping the current distribution of *R. speratus*.

Three subspecies of *R. speratus* are proposed based on geographic distribution and morphological variation (Takematsu, 1999). As shown in some recent studies (Morimoto, 1968; Takematsu, 1999; Takematsu and Yamaoka, 1999), however, the species status of *R. speratus* is under discussion. Our study was conducted to investigate the extent of genetic differentiation among the geographic subspecies proposed previously on morphological/chemical grounds, as well as to infer the migration route of ancestral *R. speratus* with respect to the paleogeographic land-bridge hypotheses proposed above, to help understand why the species is currently present in only northern areas of the Tokara Strait, and finally to infer ecological factors that might have influenced the well separated distribution of the subspecies.

In termites like *Reticulitermes* that have a cryptobiotic social structure and variable morphological characters it is difficult to investigate gene flow, population structure, taxonomy and dispersal by traditional methods. Thus, studies of termites of *Reticulitermes* with variable morphological characters have been making increasing use of molecular markers (Austin *et al.*, 2002; Jenkins *et al.*, 2001; Marini and Mantovani, 2002). In the present study, we used two mitochondrial gene sequences to examine the above issues. The extent of genetic variation among the subspecies of *R. speratus* proved to be more intelligible when interpreted within a phylogenetic framework including closely related *Reticulitermes* species.

## Materials and Methods

**Sample collection** *Reticulitermes speratus* populations were collected from most regions where the species occurs. Three colonies were collected from three locations in South Korea and twenty four colonies from ten localities in the three main Japanese islands, with the exception of Hokkaido. The localities are shown in Table 1 and Fig. 1. Previously published sequences of *Reticulitermes* in East Asia were also used to infer migration routes of *R. speratus*. Accession numbers of the sequences used in this study are shown in Table 1.

**DNA extraction** For extraction of genomic DNA, one to ten

individuals were chosen randomly from each colony. Head tissues were severed from bodies of samples preserved in 100% ethanol. After vortexing for 1 min, they were washed in a 1.5 ml microcentrifuge tube containing 500 µl of cold (4°C) 5% citric acid. After centrifugation at 8000 rpm for 5 min, the tissues were homogenized with a homogenizer in 1.5 ml microcentrifuge tube containing 500 µl of lysis buffer (100 mM Tris-HCl, 5 mM EDTA, 0.2% SDS, 200 mM NaCl) and 50 µl of proteinase K, then digested overnight at 56°C. After the addition of 10 µg of RNase A (Boehringer Mannheim), the mixture was incubated at 37°C for 20 min. Total DNA were extracted with an equal volume of chloroform after digestion with proteinase K, isopropanol-precipitated, and dissolved in 40 µl distilled water.

**DNA amplification, purification, and sequencing** The fragments of COII and COIII gene sequences were amplified by polymerase chain reaction (PCR). Primer sequences for the amplifications of COII gene were according to Park *et al.* (2004). Primers for the amplification of COIII gene were newly designed in a conserved insect region. The forward/reverse primer sequences were 5'-tagt(a/g/t)gattatag(a/t/c)ccatgacc-3' and 5'-tgtca(a/g)tatcatgc(a/t)gc(t/a)gcttc-3'. Amplification was carried out on a Gene<sup>®</sup>Amp PCR system 9700 (Perkin-Elmer). PCR was carried out in 20 µl volumes, using 1 µl of a 1:20 dilution of genomic DNA as template. The reactions included 10 pmol of each primer, dNTPs at 250 µM, 2.5 mM MgCl<sub>2</sub>, and 1 units of *Taq* Polymerase (Promega). The temperature profile for amplifying COII gene sequences was modified from Park *et al.* (2004): denaturation at 94°C for 1 min, and 35 cycles of 94°C for 30 s, 52°C for 40 s, and 72°C for 40 s followed by a final extension of 72°C for 2 min. For amplification of COIII gene sequences, the PCR profile was as follows; denaturation at 94°C for 1 min, and 35 cycles of 94°C for 30 s, 52°C for 40 s, and 72°C for 2 min followed by one cycle of 72°C for 2 min. PCR products were electrophoresed on a 1% agarose gel and purified with a Cleanmix DNA Purification Kit (TA 200 CLN). Purified products were used as templates for sequencing. Sequencing reactions were performed using a ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer), according to the manufacturer's instructions, and a Gene<sup>®</sup>Amp PCR system 9700 (Perkin-Elmer). Sequences were determined by automatic sequencing on a 3730 DNA Sequencer (ABI) on 8% polyacrylamide gels. Both complementary strands were sequenced for all individuals.

## Data analysis

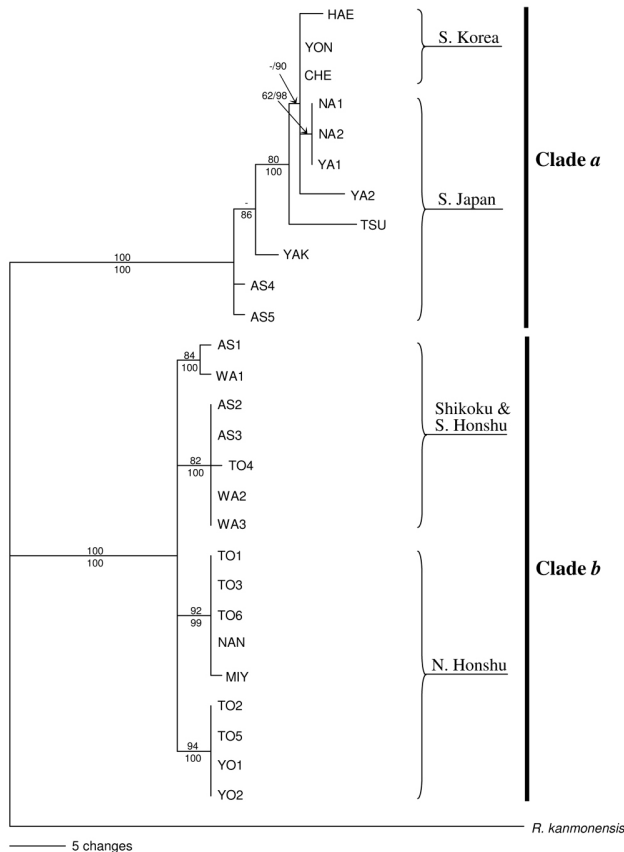
**Phylogenetic analysis** Sequences were aligned using the Clustal X program package (Thompson *et al.*, 1997). Two data sets were analyzed: (i) a fragment of COII used to investigate divergence for a range of *Reticulitermes* from Korea, Japan and China; and (ii) fragments from COII and COIII used to investigate divergences among a series of populations of *R. speratus* sampled from Korea and Japan.

Data were analyzed using maximum parsimony (MP) and Bayesian inference (BI) procedures implemented in PAUP

**Table 1.** List and sampling localities of the termites used in this study.

Species	No. of sampling Localities	Sampling localities or distributional localities of samples	Abbreviation of colony or references	Genbank accession number	
				COII	COIII
<i>R. speratus</i>	1	Mt. Cheonggye, Gwacheon City Kyeonggi province, South Korea	CHE	DQ270489	DQ270517
<i>R. speratus</i>	2	Yongmun-sa(temple), Mt. Youngmun, Kyeonggi Province, South Korea	YON	DQ270490	DQ270518
<i>R. speratus</i>	3	Haein-sa (temple), Mt. Gaya, Kyeongnam Province, South Korea	HAE	DQ270491	DQ270519
<i>R. sp.</i>	–	Daejeon city, South Korea	DAE (Austin <i>et al.</i> , 2002)	AF525354*	–
<i>R. speratus</i>	4	Yokohama, Kamikita district, Aomori prefecture, Japan	YO1	DQ270492	DQ270520
<i>R. speratus</i>	4	Yokohama, Kamikita district, Aomori prefecture, Japan	YO2	DQ270493	DQ270521
<i>R. speratus</i>	5	Nanatsumori, Shizukuishi, Iwate district, Iwate prefecture, Japan	NAN	DQ270494	DQ270522
<i>R. speratus</i>	6	Tsugaruishi, Miyako City, Iwate prefecture, Japan	MIY	DQ270495	DQ270523
<i>R. speratus</i>	7	Mt. Tatera, Tsushima Is., Nagasaki prefecture, Japan	TSU	DQ270496	DQ270524
<i>R. speratus</i>	8	Onoaida, Yakushima Is. Kagoshima prefecture, Japan	YAK	DQ270497	DQ270525
<i>R. speratus</i>	9	Cape Ashizuri, Kochi prefecture, Japan	AS1	DQ270498	DQ270526
<i>R. speratus</i>	9	Cape Ashizuri, Kochi prefecture, Japan	AS2	DQ270499	DQ270527
<i>R. speratus</i>	9	Cape Ashizuri, Kochi prefecture, Japan	AS3	DQ270500	DQ270528
<i>R. speratus</i>	9	Cape Ashizuri, Kochi prefecture, Japan	AS4	DQ270501	DQ270529
<i>R. speratus</i>	9	Cape Ashizuri, Kochi prefecture, Japan	AS5	DQ270502	DQ270530
<i>R. speratus</i>	10	Esuzaki, Susami, Wakayama prefecture, Japan	WA1	DQ270503	DQ270531
<i>R. speratus</i>	10	Esuzaki, Susami, Wakayama prefecture, Japan	WA2	DQ270504	DQ270532
<i>R. speratus</i>	10	Esuzaki, Susami, Wakayama prefecture, Japan	WA3	DQ270505	DQ270533
<i>R. speratus</i>	11	Ejio Park, Onoda, Yamaguchi prefecture, Japan	YA1	DQ270506	DQ270534
<i>R. speratus</i>	11	Ejio Park, Onoda, Yamaguchi prefecture, Japan	YA2	DQ270507	DQ270535
<i>R. speratus</i>	12	Tokai, Ibaraki prefecture, Japan	TO1	DQ270508	DQ270536
<i>R. speratus</i>	12	Tokai, Ibaraki prefecture, Japan	TO2	DQ270509	DQ270537
<i>R. speratus</i>	12	Tokai, Ibaraki prefecture, Japan	TO3	DQ270510	DQ270538
<i>R. speratus</i>	12	Tokai, Ibaraki prefecture, Japan	TO4	DQ270511	DQ270539
<i>R. speratus</i>	12	Tokai, Ibaraki prefecture, Japan	TO5	DQ270512	DQ270540
<i>R. speratus</i>	12	Tokai, Ibaraki prefecture, Japan	TO6	DQ270513	DQ270541
<i>R. speratus</i>	13	Nankan, Kumamoto prefecture, Japan	NA1	DQ270514	DQ270542
<i>R. speratus</i>	13	Nankan, Kumamoto prefecture, Japan	NA2	DQ270515	DQ270543
<i>R. speratus</i>	–	Mito City, Ibaraki prefecture, Japan	MIT (Austin <i>et al.</i> , 2002)	AF525344*	–
<i>R. kanmonensis</i>	11	Ejio Park, Onoda, Yamaguchi prefecture, Japan	YA3	DQ270516	DQ270544
<i>R. okinawanus</i>	–	Okinawa Islands, Ryukyu Archipelago, Japan	Ohkuma <i>et al.</i> , 2004	AB109534*	–
<i>R. yaeyamanus</i>	–	Yaeyama Islands (Ishigaki and Iriomote islands) Ryukyu Archipelago, Japan	Ohkuma <i>et al.</i> , 2004	AB109535*	–
<i>R. khaoyaiensis</i>	–	–	Ohkuma <i>et al.</i> , 2004	AB109531*	–
<i>R. miyatakei</i>	–	Amami-Oshima islands and Tokunoshima island, Ryukyu Archipelago, Japan	Ohkuma <i>et al.</i> , 2004	AB109533*	–
<i>R. ampliceps</i>	–	Mt. Qinling, China (around Yellow River Basin, Southeast China)	Xing <i>et al.</i> , 2001	AB050704*	–
<i>R. chinensis</i>	–	Beijing, China	Xing <i>et al.</i> , 2001	AB050705*	–
<i>R. guangzhouensis</i>	–	Guangzhou, China	Xing <i>et al.</i> , 2001	AB050709*	–
<i>R. arenicola</i>	–	USA	Austin <i>et al.</i> , 2004	AY453589*	–

\* These accession numbers indicate the sequences previously published in each reference designated in the Table and the sequences can be obtained from GenBank under each Accession numbers.



**Fig. 2.** Bayesian tree showing the phylogenetic relationship between *R. speratus* populations derived from the combined COII+COIII dataset. Populations of *R. speratus* were separated into two major clades. Korean and southern Japanese populations clustered into Clade *a*, and northern Japanese populations clustered into Clade *b*. Populations of *R. speratus* are indicated by the abbreviations of the localities where they were collected (refer to Table 1). The numbers above/below branches indicate bootstrap and PP values, respectively.

4.0b10 (Swofford, 2001) and MrBayes 3.0 (Huelsenbeck and Ronquist, 2001), respectively. MP analyses weighted all sites and transitions and transversions equally, and topological space was explored using a heuristic search, with 10 random sequence additions holding 5 trees at each step, and with TBR branch swapping. Bootstrap support was calculated using 500 pseudoreplicates with the same searching protocol as for the heuristic search.

Recent Bayesian software (MrBayes 3.0) (Huelsenbeck and Ronquist, 2001) allows separate models to be fitted to gene partitions, and in cases where partitions are likely to show very different substitutional dynamics this can help avoid problems arising from long branch effects (Schwarz *et al.*, 2004). While one of our data sets (*R. speratus*) comprised populations that are likely to have very recent divergences, the data (see **Results**) indicated a mixture of very short and very long branches. The second data set, comprising the East Asian *Reticulitermes*, con-

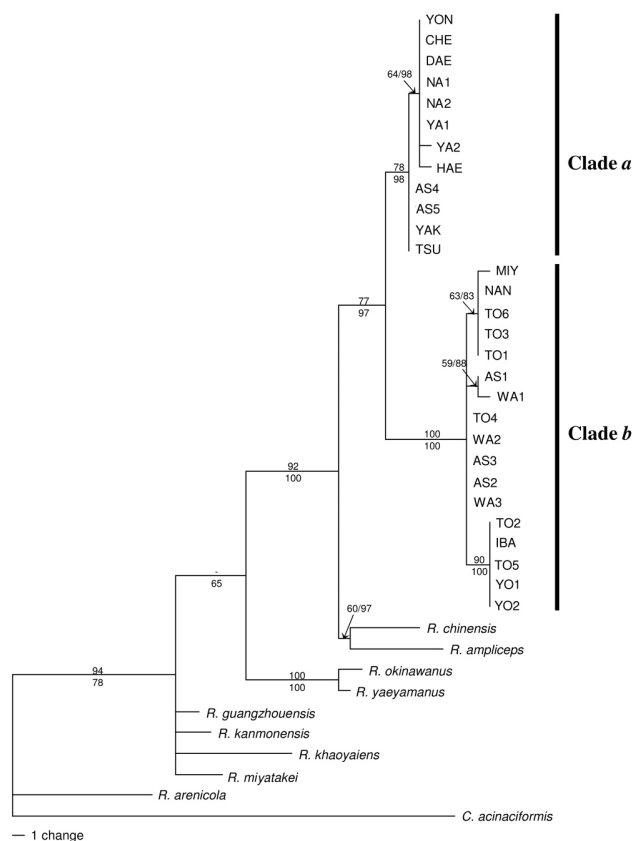
tained some species with more ancient divergences where the likelihood of homoplastic changes allows the possibility of long branch effects. We therefore also used Bayesian analysis with separate partitions for 1st, 2nd and 3rd codon positions. For the data set comprising COII and COIII, the two gene fragments were combined because base pair composition and substitution matrices were similar for both, and fitting separate models to codon positions for each gene separately runs the risk of overparameterization (eg. Huelsenbeck and Ronquist, 2001), given the relatively small number of nucleotide differences among taxa in this set. For the codon partitions we unlinked base composition, transition matrices, proportions of invariant sites, and gamma shapes. We used flat priors and one cold and three hot Monte Carlo Markov Chains. We ran simulations for one million generations, sampling every 500th generation. Stationarity was checked by plotting log likelihood values against generation number. The previously published COII sequence of *Coptotermes acinaciformis* was used as outgroup of the COII data set, and the combined sequence of the COII and COIII genes obtained newly from *R. kanmonensis* was used as outgroup of the combined COII and COIII sequence data set.

**Population structure** Nucleotide diversity ( $\pi$  per nucleotide site) (Nei, 1987) and other information on nucleotide polymorphism were obtained using the ARLEQUIN 2.0 package (Schneider *et al.*, 2000). Population pairwise  $F_{ST}$  estimates according to Tajima and Nei's  $D$  (Tajima and Nei, 1984) were also obtained from the same software package. A hierarchical analysis of population subdivision (AMOVA) (Excoffier *et al.*, 1992) was conducted to assess genetic variation according to geographical distribution, using Tajima & Nei's  $D$  between haplotypes in the ARLEQUIN 2.0 package. The significance of the resulting  $F$  statistics and variance components were tested with 10,000 permutations. The allowed level of missing data was 0 in all analyses of population structure. A minimum spanning network (MSN) was constructed from absolute numbers of substitution differences between all pairs of haplotypes.

Pairwise genetic distances ( $p$ -distances) between haplotypes were estimated using the Kimura two-parameter method (Kimura, 1980) implemented using PAUP 4.0b10 (Swofford 2001). Significance levels of  $p$ -distance values between/among haplotypes were tested using ANOVA,  $t$ -tests, and Mann-Whitney  $U$ -tests implemented in SPSS 8.0 (SPSS Inc., USA). Normality tests for the  $t$ -tests employed the Shapiro-Wilk test (Shapiro and Wilk, 1965).

## Results

**Intra-/interspecific phylogeny of *Reticulitermes speratus*** MP and BI analyses gave congruent topologies, the only differences being the levels of support for nodes and the collapse of some nodes into polytomies under a 50% majority rule consensus. The data set, comprising a total of 997 nucleotides (356 from COII and 641 from COIII),



**Fig. 3.** Bayesian tree showing the phylogenetic relationships among of East Asian *Reticulitermes* species derived from the COII dataset. Clades *a* and *b* indicate Korean/southern Japanese and northern Japanese populations, respectively. The numbers above/below branches indicate bootstrap and PP values, respectively.

contained 103 variable sites of which 51 were parsimony-informative. The MP analysis returned 432 equally most parsimonious trees and a strict consensus tree of these trees was substantially identical to the BI tree except that TSU formed a monophyletic group with Korean populations (HAE, YON and CHE) and some southern Japanese populations (NA1, NA2, YA1 and YA2) (Clade *a* in Fig. 2; see Table 1 for abbreviations). PP support from the Bayesian analysis was consistently higher for all nodes than the MP bootstrap values. All analyses provided very strong support for the Korean species being a monophyletic clade that also contained the Japanese samples of NA1, NA2, YA1 and YA2, but bifurcation order among the samples was not well supported. The other southern Japanese populations (TSU and YAK, AS4 and AS5) comprised a sister group to the monophyletic clade. The populations of northern Japanese *R. speratus* formed four distinct clades (Clade *b*; Fig. 2), with the collapse of the nodes into polytomies. Samples of the four clades were separated geographically into two groups, present in the Shikoku/southern Honshu (S. Honshu) and northern Hon-

shu (N. Honshu) regions, respectively (Clade *b*; Fig. 2).

In order to examine whether divergences were consistent with species-level differentiation, we examined the phylogeny of a subset of East Asian *Reticulitermes* species using a 356 bp fragment of COII with the same analytic protocols as for the previous analyses. The data set for MP analysis contained 112 variable characters of which 55 were parsimony informative and the MP analysis of the dataset reproduced one single most parsimonious tree (tree length = 166; CI = 0.753; RI = 0.783; RC = 0.590; HI = 0.247). As in the analyses above, MP and BI analyses gave congruent topologies with the only differences being the levels of support for nodes and the collapse of some nodes into polytomies under a 50% majority rule consensus (Fig. 3). The analyses of *R. speratus* with East Asian *Reticulitermes* at species level also suggested that the populations of *R. speratus* formed two distinct clades, each with many distal branches. Interestingly, *R. speratus* populations were sisters to Chinese *Reticulitermes* species (*R. chinensis* and *R. ampliceps*) rather than to Japanese *Reticulitermes* (*R. okinawanus* and *R. yaeyamanus*), and the relationship was supported by high bootstrap and PP values (92% in bootstrap and 100% in PP, respectively). The clade of *R. okinawanus* and *R. yaeyamanus*, supported by high supporting values (100% in both bootstrap and PP), formed a sister to the clade [*R. speratus* (*R. chinensis*, *R. ampliceps*)], but with low supporting levels for the node (below 50% in bootstrap and 65% in PP).

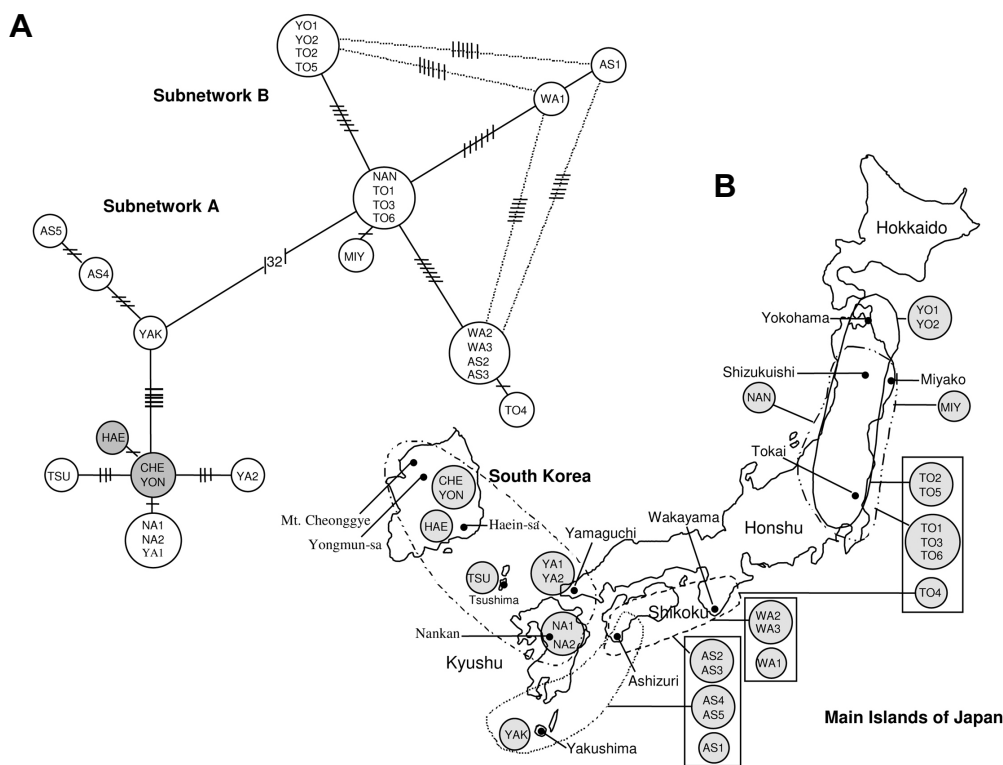
**Population structure** Nucleotide bases for 61 positions of the combined COII and COIII dataset could not be reliably inferred among the 997 nucleotide positions that were sequenced. Thus, only 936 nucleotide positions were included from 27 sequences. A total of 53 polymorphic sites were detected in the sequences (Table 2). No deletion or insertion of bases was observed. There were 15 different haplotypes in the combined COII and COIII dataset of 27 sequences. They were classified into two major classes of Haplotype I (haplotype No. 1–8) and Haplotype II (haplotype No. 9–15) (Table 2), reflecting two well-separated clades (Clade *a* and *b*) shown in the phylogenetic tree (Fig. 2). Both classes of Haplotype I and II were present in samples from Ashizuri (haplotype No. 7, 9/No. 1, 2, 4), as shown in the clades of the phylogenetic trees (Fig. 2). The average number of pairwise differences, calculated according to Tajima & Nei's *D*, was 4.36 within Haplotype I, and 4.92 within Haplotype II, but the average pairwise difference of 37.36 was between Haplotype I and II ( $P < 0.001$ ). Nucleotide diversity of haplotypes was similar between populations of Clade *b* and Clade *a* in the phylogenetic tree (Table 3). The nucleotide diversity within Clade *a* was considerably higher in Japanese populations than Korean populations. In Clade *a*, however, pairwise  $F_{ST}$  estimates did not show



**Table 3.** Summary statistics of population variation at the combined COII and COIII gene sequences.

	<i>n</i>	No. of haplotypes	Transition/ Transversion	No. of polymorphic site	$\pi$ (Average $\pm$ SD)
Korean populations	3	2	0/2	2	0.001363 $\pm$ 0.001401
Southern Japanese populations	8	6	14/4	18	0.006510 $\pm$ 0.003935
Korean/southern Japanese populations	11	8	14/6	20	0.005436 $\pm$ 0.003209
Northern Japanese populations	16	7	14/1	15	0.005247 $\pm$ 0.003021
Total	27	15	43/10	53	0.022542 $\pm$ 0.011434

*n* is the number of individuals included in the calculation.  $\pi$  is the estimation of nucleotide diversity (Nei 1987). Korean/southern Japanese and northern Japanese populations correspond to those of Clade *a* and *b* in the phylogenetic tree (Fig. 2), respectively.



**Fig. 4.** Minimum spanning network (A) showing the relationship between *R. speratus* mtDNA haplotypes, and haplotype geographical distributions (B) according to the patterns of haplotype grouping in the MSN. In the MSN (A), the area of the circle is roughly proportional to the haplotype frequency, and the length of connecting lines is roughly proportional to the number of substitution. Labels inside each circle indicate individual colonies bearing each haplotype. The bars on the connecting lines indicate the number of substitutions. Grey circles are haplotypes occurring in South Korea. The dotted lines indicate alternative connections. Korean/southern Japanese and northern Japanese haplotypes are separated into two networks, subnetworks A and B, by 32 substitutions; the substitution number is given on the connecting line between the two subnetworks. In the Korean/southern Japanese populations (subnetwork A), there are two major groupings of haplotypes, indicating that the haplotypes of Yakushima (YAK) and Ashizuri (AS4/5) are separated from all the other haplotypes in the Korean/southern Japanese populations by 5 substitutions. With the exception of haplotypes WA1 and AS1, three major groupings of haplotypes separated by 6 substitutions occur in northern populations (subnetwork B). Haplotypes WA1 and AS1 are connected to all three groups by the alternate connections. In (B), haplotypes collected from each locality are re-grouped according to the haplotype grouping patterns in MSN, resulting in five geographical groupings surrounded by different dotted lines. Each haplotype in the circles is designated near its collection locality. Each circle containing haplotypes is located within its own geographical group, or is connected to its own geographical group by a line stemming from it. Haplotypes from Tokai, Wakayama, and Ashizuri are boxed according to locality. With the exception of haplotypes WA1 and AS1, haplotype groupings in MSN were quite well geographically grouped. Both northern and southern haplotypes of Japan coexist in Ashizuri. In contrast with their separation in MSN, two haplotype groups of northern Japan (Yokohama, Shizukuishi, Tsugarushi and Tokai) overlap in their geographical distributions.



**Table 4.** Hierarchical analyses of mtDNA diversity in *R. speratus* populations in eastern Asian regions.

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation
Among groups	1	215.60	16.26 Va	86.90
Among populations within groups	6	21.23	0.48 Vb	2.58
Within populations	19	37.42	1.97 Vc	10.53
Total	26	274.25	18.71	

Eight groupings of *R. speratus* populations for AMOVA were made according to their distributions and the phylogenetic analysis (Fig. 2) and then the eight populations were grouped into two geographic groups of Korean/southern Japanese and northern Japanese group as follows: Korean/southern Japanese group comprising four populations of Korea (CHE/YON/HAE), Tsushima (TSU)/Kyushu (NA1/2)/Yakushima (YAK), Yamaguchi (YA1/2) and Ashizuri (AS4/5), and northern Japanese group comprising four populations of Ashizuri (AS1/2/3), Wakayama (WA1/2/3), Tokai (TO1/2/3/4/5/6) and Yokohama (YO1/2)/Shizukuishi (NAN)/Miayko (MIY).

**Table 5.** Pairwise genetic distance between COII gene sequences of eastern Asian *Reticulitermes* species.

	1	2	3	4	5	6	7	8	9	10	11
1. Clade <i>a</i>	0.0028 (0.0025, n = 66)										
2. Clade <i>b</i>	0.0317 (0.0035, n = 204)	0.0051 (0.0035, n = 136)									
3. <i>R. chinensis</i>	0.0408 (0.0027, n = 12)	0.0471 (0.0026, n = 17)	—								
4. <i>R. ampliceps</i>	0.0468 (0.0027, n = 12)	0.0564 (0.0026, n = 17)	0.0407	—							
5. <i>R. okinawanus</i>	0.0648 (0.0029, n = 12)	0.0806 (0.0057, n = 17)	0.0685	0.0653	—						
6. <i>R. yaeyamanus</i>	0.0618 (0.0029, n = 12)	0.0836 (0.0066, n = 17)	0.0718	0.0623	0.0085	—					
7. <i>R. guangzhouensis</i>	0.0588 (0.0027, n = 12)	0.0753 (0.0042, n = 17)	0.0624	0.0752	0.0468	0.0501	—				
8. <i>R. kamonensis</i>	0.0558 (0.0027, n = 12)	0.0781 (0.0043, n = 17)	0.0656	0.0720	0.0499	0.0470	0.0143	—			
9. <i>R. khaoyaiensis</i>	0.0848 (0.0027, n = 12)	0.0952 (0.0032, n = 17)	0.0949	0.0882	0.0750	0.0720	0.0348	0.0378	—		
10. <i>R. miyatakei</i>	0.0528 (0.0027, n = 12)	0.0748 (0.0043, n = 17)	0.0624	0.0688	0.0468	0.0439	0.0171	0.0201	0.0408	—	
11. <i>R. arenicola</i>	0.0858 (0.0019, n = 12)	0.1046 (0.0037, n = 17)	0.0946	0.1014	0.0879	0.0849	0.0818	0.0656	0.0949	0.0752	—

Pairwise genetic distances were calculated according to Kimura two-parameter. Clade *a* and *b* indicate populations of the clades in the phylogenetic tree (Fig. 3). The *p*-distance values between populations in Clade *a/b* and the other species are indicated by average. The *p*-distance values among populations within each Clade, as well as the *p*-distance value between Clade *a* and *b*, are indicated by average values. The numbers in parentheses indicate standard deviation and the numbers of pairwise comparisons.

a significant difference between Korean and Japanese populations ( $F_{ST} = 0.06146$ ,  $P = 0.28477$ ). Pairwise  $F_{ST}$  estimates revealed a significant difference between the populations of Clade *a* (Korean and southern Japanese populations) and Clade *b* (northern Japanese populations) ( $F_{ST} = 0.87856$ ,  $P = 0.0000$ ).

A geographical grouping of 8 populations was designed for AMOVA, based on the phylogenetic tree and their geographic distributions. The eight populations were grouped into the two major geographical groups of Korean/southern Japanese populations (Clade *a*; haplotype No. 1–8) and northern Japanese populations (Clade *b*; haplotype No. 9–15). Hierarchical analyses of molecular variance indicated a high degree of substructure, with a geographical grouping showing variation of 86.90% ( $P < 0.001$ ) considering the two geographical groups as different population groups (Table 4). This is not surprising because the two populations do not share any mtDNA haplotypes (Table 2), and because the two groups were clearly clustered into two major clades of the phyloge-

netic tree (Fig. 2).

A Minimum Spanning Network analysis (MSN) (Fig. 4A), indicated that two sub-networks of haplotypes could be discerned, with 32 substitutions between the two connecting haplotypes. Sub-network A had two major groups, discriminated by five substitutions, and three groupings separated by six substitutions are shown in Sub-network B. Five geographic groupings were produced by combining haplotype grouping in the MSN with geographical distributions of the haplotypes (Fig. 4B).

**Genetic divergence** Pairwise genetic distances were calculated using the COII dataset of 11 Asian *Reticulitermes* species published previously, along with two clades of *R. speratus* populations (Table 5). The average *p*-distance value among populations within Clade *a* was significantly lower than the value among populations within Clade *b* ( $t_{171.395} = -5.328$ ,  $P < 0.05$ ). The average *p*-distance values among populations within each clade were significantly lower than the average value between populations

of the two clades ( $F_{2,403} = 3336.9$ ,  $P < 0.001$ ).

Analysis of the pairwise comparisons revealed that the average  $p$ -distance value between two major populations of *R. speratus* (Clade *a* and *b*) was higher than some  $p$ -distance values obtained in the species-level comparisons (between *R. okinawanus* and *R. yaeyamanus*, between *R. guangzhouensis* and *R. kanmonensis*, between *R. guangzhouensis* and *R. miyatakei*, and between *R. kanmonensis* and *R. miyatakei*) (Table 5).

The average  $p$ -distance value between Clade *a* and its sister group (*R. chinensis* and *R. ampliceps*) was  $0.043 \pm 0.004$  ( $n = 24$ ), whereas the value between Clade *b* and the sister group was  $0.052 \pm 0.005$  ( $n = 34$ ). The former value is lower than the latter one (Mann-Whitney  $U$ -test;  $Z = -5.289$ ,  $P < 0.005$ ). The pairwise comparisons of the two major populations with *R. kamonensis*, used as out-group in the phylogenetic tree (Fig. 2), also showed that the average  $p$ -distance value between Clade *a* and *R. kamonensis* was significantly lower than the value of the Clade *b* and *R. kamonensis* ( $Z = -4.5710$ ,  $P < 0.001$ ).

## Discussion

**Population genetic structure and species status** A previous study based on the morphological characters of soldiers and alates suggested five subspecies of *R. speratus*, namely *R. speratus speratus*, *R. s. leptolabralis*, *R. s. kyushuensis*, *R. s. okinawanus*, and *R. s. yaeyamanus* (Morimoto, 1968). In more recent studies, however, three distinct species of *R. speratus*, *R. okinawanus* and *R. yaeyamanus* were recognized based on morphological and chemical (cuticular hydrocarbon) characters (Takematsu, 1999; Takematsu and Yamaoka, 1999). The subspecies of *R. s. speratus*, *R. s. leptolabralis*, and *R. s. kyushuensis* were not clearly distinguished by their chemical characters, but the three subspecies could be relatively well discriminated from *R. okinawanus* and *R. yaeyamanus* by the chemical characters. These characters were also discriminated well between *R. okinawanus* and *R. yaeyamanus*. In geographical aspects, *R. okinawanus* and *R. yaeyamanus* were also clearly distinct from the other three subspecies of *R. speratus* in that their distributions are limited to only the southern regions of the Tokara Strait (Fig. 1). Thus, instead of five subspecies of *R. speratus*, it may be more justifiable to recognize only three species, namely *R. speratus* (*R. speratus speratus*, *R. s. leptolabralis*, *R. s. kyushuensis*), *R. okinawanus* and *R. yaeyamanus*.

The current study was conducted to investigate the genetic structure of *R. speratus* populations and to understand the species status of *R. speratus* populations. Samples used in the current study were collected widely over most of regions where three *R. speratus* subspecies (*R. s. speratus*, *R. s. leptolabralis*, and *R. s. kyushuensis*) have been found in previous studies (Morimoto, 1968; Take-

matsu, 1999). The combined data set of COII and COIII sequences showed enough genetic variation to reveal the genetic structure of *R. speratus* populations, which provided some insight into the species status of *R. speratus*. Since the habitats of the *R. speratus* subspecies are continuous and their chemical characters are not well discriminated (Takematsu and Yamaoka, 1999), low genetic divergence might be expected. The AMOVA analysis of population structure, however, revealed high genetic differentiation between Korean/southern Japanese populations and northern Japanese populations. According to the  $p$ -distance analyses, especially, the average  $p$ -distance value between Korean/southern Japanese and northern Japanese populations ( $0.0317 \pm 0.0035$ ) is about 6–10 times higher than the average  $p$ -distance value within each of the individual populations (0.0028 in the former and 0.0051 in the latter, respectively) (Table 5), revealing the existence of two genetically distinct lineages of *R. speratus*, with genetic partitioning intensified since their divergence. High genetic differentiation might also be expected between Korean and southern Japanese populations of Clade *a* since the East Sea and the Korean Strait have separated the main Japanese islands from the Korean Peninsula since the mid-Miocene, though land bridge connections appeared several times during the Pleistocene. In contrast to our expectation, however,  $F_{ST}$  estimate did not show significant genetic divergence between the Korean and southern Japanese populations. As shown in the MSN (Fig. 4), the patterns grouping haplotypes also indicate that some southern Japanese populations group together with Korean populations rather than with the other southern Japanese populations (Fig. 4).

The two distinct lineages of *R. speratus* recognized in this study were different from the classification of three *R. speratus* subspecies based on morphological characters (Morimoto 1968). The distributions of the two mtDNA lineages are congruent with those of *R. s. kyushuensis* and *R. s. speratus* which are well separated geographically (Fig. 1). However, the populations collected from the regions where *R. s. leptolabralis* is expected (Ashizuri and Wakayama) did not have a genetic composition distinct from its neighboring subspecies (*R. s. kyushuensis* and *R. s. speratus*). That is, some (AS4 and AS5) had a close genetic relationship to *R. s. kyushuensis*, while others (AS1, AS2 and AS3; WA1, WA2 and WA3) were closely related to *R. s. speratus* populations (Fig. 2). Further surveys of *R. s. leptolabralis* need to be conducted since sampling error may have resulted from the geographic characteristics of their distribution since most regions occupied by *R. s. leptolabralis* overlapped with the eastern margin of the *R. s. kyushuensis* distribution and the southern margin of the *R. s. speratus* distribution (Morimoto, 1968; Takematsu and Yamaoka, 1999).

According to  $p$ -distance analyses, the average  $p$ -distance value between the two major populations of *R.*

*speratus* (0.0317) was higher than the *p*-distance values at species-level between some other species (0.009 between *R. okinawanus* and *R. yaeyamanus*; 0.014 between *R. guangzhouensis* and *R. kanmonensis*; 0.017 between *R. guangzhouensis* and *R. miyatakei*; 0.020 between *R. kanmonensis* and *R. miyatakei*) (Table 5). It has long been known that the genealogical history of orthologous genomic regions of species is not necessarily identical to the history of the species itself (Neigel and Avise, 1986; Pamilo and Nei, 1988; Rosenberg, 2002; Templeton, 2001). Thus, the deep divergence of the two *R. speratus* populations found here need not mean that the two populations warrant species-level distinction. Since there has been insufficient work on genetic exchange and intrinsic mating barriers between the two mtDNA lineages of *R. speratus*, further studies of ecological and behavioral aspects are needed to understand the species status of the two *R. speratus* populations. Better conserved gene regions like 18S should also contribute to understanding the level of speciation (Yoon and Kim, 2005).

**Phylogenetic relationships** Intra-/interspecific phylogenetic comparisons of *R. speratus* with other *Reticulitermes* species have also aided understanding of the variation between populations. Like the analyses of population genetic structure above, the branch lengths from BI analyses also suggest relatively long divergence time between the two major populations (Clade *a* and *b*), followed by rapid and relatively recent radiation of both lineages (Fig. 2). These results, however, need not imply separation of the two major populations into species. They may simply reflect divergence and radiation of maternal lines within a single species. The occurrence of both haplotypes in Ashizuri and southern Honshu may have resulted from more recent range expansion of the two populations.

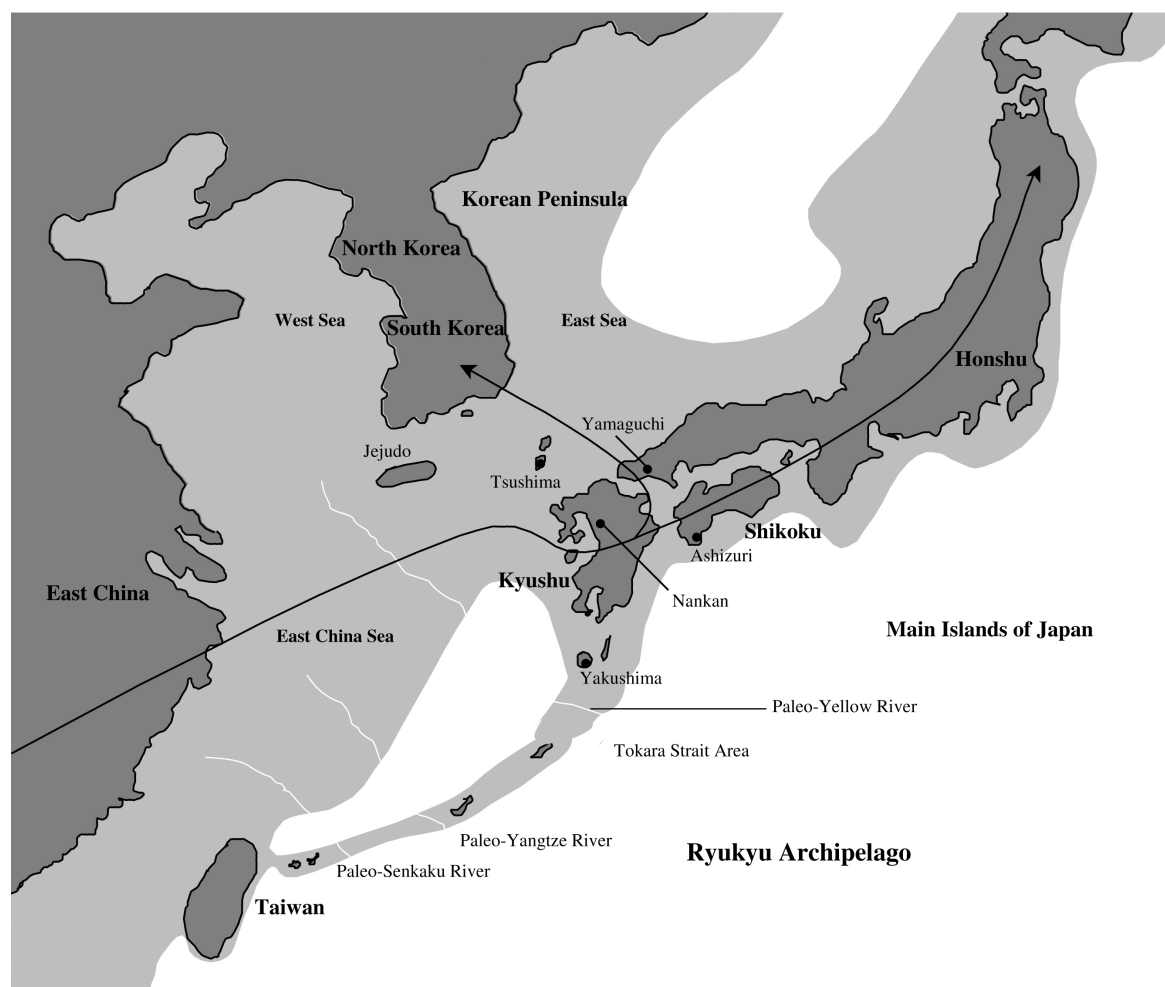
The analysis of interspecific phylogeny revealed an interesting aspect of the intra-specific *R. speratus* relationships: the populations of *R. speratus* formed a sister group to Chinese species (*R. chinensis* and *R. ampliceps*) rather than to the Japanese *Reticulitermes* (Fig. 3). The clade [*R. okinawanus*, *R. yaeyamanus*] was a sister to the clade [*R. speratus* (*R. chinensis*, *R. ampliceps*)]. Since *R. speratus* populations were more closely related to the Chinese species than to the Japanese species, our results support the studies in which *R. okinawanus* and *R. yaeyamanus* were posited as distinct species from *R. speratus* (Takematsu, 1999; Takematsu and Yamaoka, 1999). Furthermore, the distributional regions of *R. speratus* subspecies and *R. okinawanus* and *R. yaeyamanus* are separated by the Tokara Strait, i.e., the former species occurs in northern parts of the Tokara Strait, whereas the latter two species occur only in the southern regions of the strait. The *p*-distance value between *R. okinawanus* and *R. yaeyamanus* was the lowest among the *p*-distance values compared at the species level (Table 5). Their *p*-distance value was

even lower than the average *p*-distance value between populations of the two major clades of *R. speratus*, probably indicating recent divergence of the two species.

**Phylogeography** As for most endemic Japanese terrestrial fauna (Kimura, 2000; Lin *et al.*, 2002; Millien-Parra and Jaeger, 1999), it has been suggested that endemic Japanese termites also had paleogeographic distributions during the Pleistocene, with their origin source pool from the Asiatic continent (Maekawa *et al.*, 1998). Despite uncertainties associated with calculations of this type (Ayala, 1997; Gillespie, 1991), we applied a simple molecular clock to infer an historical origin for the current distributions of *R. speratus* by assuming a divergence rate of 2.3% per million years for insect mitochondrial DNA (Brower, 1994). If our calculation using the COII sequences is correct, the average *p*-distance value ( $0.048 \pm 0.006$ ) between Clade *a+b* and its sister group (*R. chinensis* and *R. ampliceps*) indicates that the two clades diverged about 2.3–1.9 million years ago. The divergence of Clades *a* and *b* may have occurred about 1.5–1.2 million years ago.

Using the estimated divergence times, phylogenetic analysis and paleogeographic hypotheses, we examined three possible land-bridge routes for ancestral *R. speratus* migration (Korean route, Taiwan-Ryukyu Arc route and East China Sea route). The Mamiya route in northern part of Hokkaido Island is in the Subarctic region, and is excluded because termites are limited to temperate through tropical regions (Pearce and Waite 1994). As first proposed by Morimoto (1968), an ancestral *R. speratus* could have migrated into the Japanese main islands from China via the Korean Peninsula (China-Korea-Japanese main islands). According to our phylogenetic analysis, however, some populations from Ashizuri and Tsushima (e.g. AS4/5 and TSU) are more basal than the Korean populations. The estimated divergence time is also incongruent with the above hypothesis, since Clade *a* and *b* diverged far earlier than the divergence time between Korean and southern Japanese populations. Thus, our results suggest the reverse event, rather than migration into the Japanese main islands from Korea.

The Korean (YON and CHE) and some southern Japanese populations (NA1, NA2, YA1) did not differ in COII haplotypes, and differed by only one substitution in the even more variable COIII haplotypes (Table 2). This may indicate that their divergence is recent rather than a paleogeographic episode. Since the Korean populations are located, along with the populations from Yamaguchi (YA1) and Nankan (NA1/2), in more terminal clades than the other southern Japanese populations, they may have diverged from one of the populations in the Yamaguchi and Nankan regions. To explain the presence of *R. speratus* in the Korean Peninsula, a likely scenario is an alate dispersal by a stepping-stone model of gene flow through



**Fig. 5.** Paleogeographic map of East Asia and migration route of ancestral *R. speratus*. Heavy shaded areas indicate current geographic topography, and light shaded areas indicate paleogeographic topography of East Asia during sea level alterations due to glaciations (Kimura, 2000; Lin, 2002; Toda *et al.*, 1999). An ancestor of Japanese *R. speratus* may have migrated to the southern Japanese main islands via the East China Sea basin exposed during early Pleistocene, as indicated by the arrow line, and then migrated to more northern areas of Japanese main islands. Korean *R. speratus* may have been introduced recently from southern Japanese islands.

the smaller islands stretching between the southern regions of the Korean Peninsula and the western Kyushu/southwestern Honshu. Tsushima Island could be important as a middle-bridge for an alate dispersal in that it is the closest of the Japanese islands to the southern Korean Peninsula (Fig. 1). Shimonoseki port in Yamaguchi has been a traditional international port since the 15th century and has been proposed as an artificial route of introduction of *R. kanmonensis* into Japan (Kitade and Hayashi, 2002). Since the populations from Yamaguchi have similar haplotypes to Korean populations (Table 2), a human-mediated movement from the region is another plausible explanation for the presence of *R. speratus* in Korea.

The Taiwan-Ryukyu Arc route (China-Taiwan-Ryukyu Arc-Japanese main islands) is often regarded as the Pleistocene migration route for Japanese terrestrial fauna, i.e., *Hodotermopsis* (Maekawa, 1998), lacertid lizards (Ota *et*

*al.*, 2002), Japanese wildcats (Masuda & Yoshida 1995; Masuda 1996) and grass lizards (Lin *et al.*, 2002). If the Taiwan-Ryukyu Arc route is considered the migration route of ancestral *R. speratus*, however, there is a drawback since *R. speratus* has not been found in Taiwan and Ryukyu islands, being restricted to the northern regions of the Tokara Strait.

The East China Sea (China/East China Sea/Japanese main islands) is thought to be less likely than the Taiwan-Ryukyu Arc route in the paleogeographic history of Japanese terrestrial fauna, but recent studies have suggested that this might have been an important migration route, as shown in migrations of some terrestrial fauna via the area during the glaciations (Lin *et al.*, 2002; Takahashi *et al.*, 2001). According to paleogeographic hypotheses suggested recently, the East China Sea basin and most of the Okinawa Trough were exposed between 1.6–1.3 million

years ago (Kimura, 2000; Lin *et al.*, 2002; Toda *et al.*, 1999) which agrees well with the divergence time between Clades *a* and *b*. At that time, the Paleo-Yellow River that was formed in current Tokara Strait might work as a barrier that prevented the migration of *R. speratus* from the Japanese main islands into the Ryukyu Islands. Thus, the East China Sea route would also explain the fact that *R. speratus* populations are absent from Taiwan and the Ryukyu islands in the southern part of the Tokara Strait.

According to our hypothesis for the current distribution of *R. speratus* populations in the Japanese islands (Fig. 5), an ancestral group of *R. speratus* and their Chinese sister group (*R. amplexipes* and *R. chinensis*) may have already diverged from an ancestral group about 2.3–1.9 million years ago in their source region, probably Southeast China. Right after the divergence, the ancestor of *R. amplexipes* and *R. chinensis* may have diverged into current *R. amplexipes* and *R. chinensis*, whereas the ancestral *R. speratus* may have migrated to the Japanese islands via the East China Sea basin, while it was exposed during the early Pleistocene (1.6–1.2 MY ago). The southern Japanese main islands are geographically closer to East China than the northern main islands. Thus, ancestral *R. speratus* may have first reached the southern main islands, and subsequent dispersed to northern regions. On contrast to the northern regions, warm-temperate evergreen broadleaf forest was dominant in southern Kyushu and Shikoku adjacent to the coastline of the Pacific Ocean during the glaciations, and this may have helped the success of an early colonization. The *p*-distance value revealed that the southern populations rather than the northern populations are more closely related to the sister clade (*R. amplexipes* and *R. chinensis*). In haplotype analyses (Table 3), the transversion/transition ratio is higher within Korean/southern Japanese than within northern Japanese populations, probably indicating more recent radiation of the latter populations. As such, the results above may reflect that the ancestral group reached the southern regions first. The Paleo-Yellow river and the Tokara Strait formed thereafter separated between the southern and the northern region of the strait during most of the glaciations as well as the estimated divergence time. Thus, migration of *R. speratus* into southern islands of Ryukyu Arc might have been blocked, resulting in distributions of *R. speratus* restricted to the only northern regions of the Tokara Strait.

Northern and southern Japanese populations might have had opportunities to exchange gene pools several times, as inferred from land-bridge connections among Japanese main islands during the Pleistocene (Dobson, 1994; Millien-Parra and Jaeger, 1999; Millien-Parra and Loreau, 2000). Their co-distribution might be expected as well. However, the two major populations have been geologically well separated, with the exception of the overlap

of their distribution in some regions (southern Honshu and Ashizuri) where the two populations interface (Fig. 4B). Thus their distinct distributions might be influenced by other causes as well as a geological vicariance event.

The Japanese archipelago extends over a distance of 2000 km from north to south, and ranges from 22°N to 45°N in latitude. Such a variation in latitude is accompanied by a wide range in climatic environment, ranging from warm tropical in the south to cool-temperate and subarctic in the north (Millien-Parra and Jaeger, 1999; Millien-Parra and Loreau, 2000). Thus the climate environment, like the yearly minimum temperature regime, might be considered as one possible ecological factor to influence the geographic distributions of *R. speratus* (refer to Morimoto, 1975). In Korea, the distribution of *R. speratus* ranges from the southern to the mid-regions of the Peninsula, about 35°N to 37°N in latitude. Southern populations of *R. speratus* in Japan are distributed in southern regions of about 35°N in latitude. Their distributions range from the subtropical (Yakushima island and southern regions of Kyushu) to the warm-temperature regions (southern Honshu, Shikoku and mid-northern Kyushu) where a warm-temperate evergreen broadleaf forest is dominant. The northern populations of Japanese *R. speratus* are distributed in the cool-temperate zone (southern Honshu to southern Hokkaido) characterized by temperate deciduous broadleaf forest (about 35°N to 44°N in latitude). Difference of average temperature between these two zones is over about 6 degree during winter season (5.3°C and –1°C, respectively, in Millien-Parra & Jaeger 1999). Since cooler climates would have predominated during the glaciations, the main islands of Japan would have been dominated by temperate deciduous forest (Kyushu-southern Honshu) and boreal coniferous forest (middle Honshu-middle Hokkaido), with the exception of a small evergreen broadleaved forest in only southeastern of Kyushu and eastern Shikoku (Dobson, 1994; Millien-Parra and Jaeger, 1999). After migration into Japan, a portion of the ancestral group which moved to more northern regions might have been exposed repeatedly to harsher cold conditions during glacial episodes of the Quaternary. In northern populations, some life historical plasticity accompanying with morphological and genetic differentiation might have been selected to be better adapted to cool climates.

Although our estimation of the divergence time corresponds relatively well to paleogeographic events, the hypothesis for the migration time and the route of ancestral *R. speratus* could be narrative and problematic since more samples from East China were not included in the current studies. To assess our hypothesis more thoroughly, more paleoenvironmental studies and geographical surveys need to be conducted in the East China. The intrinsic/extrinsic factors that have facilitated the differentiation of the two Japanese gene pools still remain as a sub-

ject to be considered in the further studies as well.

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