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# Molecular Characteristics of Subterranean Termites of the genus *Reticulitermes* (Isoptera: Rhinotermitidae) From Korea

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**ABSTRACT** Molecular and taxonomic studies of subterranean termites in the genus *Reticulitermes* (Isoptera: Rhinotermitidae) have suggested that *Reticulitermes speratus kyushuensis* Morimoto is the only species that occurs in Korea. However, the specimens studied to date were collected from a limited number of locations, and basic information about the distribution, taxonomy, and molecular characteristics of this genus in Korea is still lacking. In this study, we collected *Reticulitermes* specimens from 50 sampling sites distributed throughout the country. By comparing the cytochrome oxidase subunit II gene of mitochondrial DNA sequences, we conclude that *Reticulitermes speratus* is represented by two haplotypes in Korea. In addition, we report the first occurrence of another *Reticulitermes* species in this country.

**KEY WORDS** *Reticulitermes*, Korea, cytochrome oxidase II gene, internal transcribed spacer, subterranean termite

Subterranean termites (Isoptera: Rhinotermitidae) are the most widely distributed family of termites, occurring throughout tropical and subtropical areas as well as temperate regions, in which they are abundant. They are important in natural ecosystems, affecting soil formation and stabilization and the quality and distribution of organic matter, and playing a key role as decomposers of wood. However, subterranean termites are also one of the most important insect pests due to their attack on wooden structures and products, which has a major economic impact on several countries because of direct damage and termite control costs (UNEP/FAO/Global IPM Facility 2000, Su 2002, Szalanski et al. 2004).

The genus *Reticulitermes* comprises globally distributed subterranean termite species. In Japan, seven *Reticulitermes* species have been recognized (Takematsu 1999, Yasuda et al. 2000, Kitade and Hayashi 2002). Of these species, *Reticulitermes speratus* (Kolbe) was subdivided into five subspecies by Morimoto (1968), and later grouped into three subspecies [*Reticulitermes speratus speratus* (Kolbe), *Reticulitermes speratus leptolabralis,* and *Reticulitermes speratus kyusuensis* Morimoto] based on taxonomic and chemotaxonomic (cuticular hydrocarbons) characters (Takematsu 1999, Takematsu and Yamaoka 1999). Basic information about the distribution, taxonomy and molecular characteristics of subterranean termites in Korea is, however, still lacking.

A review of the literature shows that *R. speratus* is the only subterranean termite species that occurs in Korea (Becker 1969, Park and Bae 1997, Han et al. 1998, Lee et al. 2001). Becker (1969) first reported that the specimens of *R. speratus* from Korea belong to the subspecies *kyushuensis*, which also is distributed in southern Japan. Other studies (Park and Bae 1997, Han et al. 1998, Lee et al. 2001) reached the same conclusion by comparing the morphology of the specimens from southern Korea with those of *R. speratus* subspecies occurring in Japan. In addition, the chromatographic profile of cuticular hydrocarbons of the specimens from Korea was not different from that of *R. s. kyusuensis* from Japan (Takematsu 1999).

Recently developed methods now enable a more accurate classification at the species level in complex taxonomic structure and make more comprehensive genetic surveys possible. Among these methods, mitochondrial DNA (mtDNA) has been used for the study of phylogenetic relationships within the genus *Reticulitermes* (Jenkins et al. 2001, Marini and Mantovani 2002). Austin et al. (2002) conducted an extensive genetic study of *Reticulitermes* species from various countries by sequencing the cytochrome oxidase II (COII) region of the mitochondrial genome, and these results, along with those from Park et al. (2006), showed that *R. speratus* specimens from Korea corresponded to *R. speratus* from southern Japan, indicating that the only *Reticulitermes* species in Korea is *R. s. kyushuensis.*

Previous studies, however, have only used *Reticulitermes* specimens from a limited number of locations in Korea, such as Gyeongnam and Gyeonggi, and this

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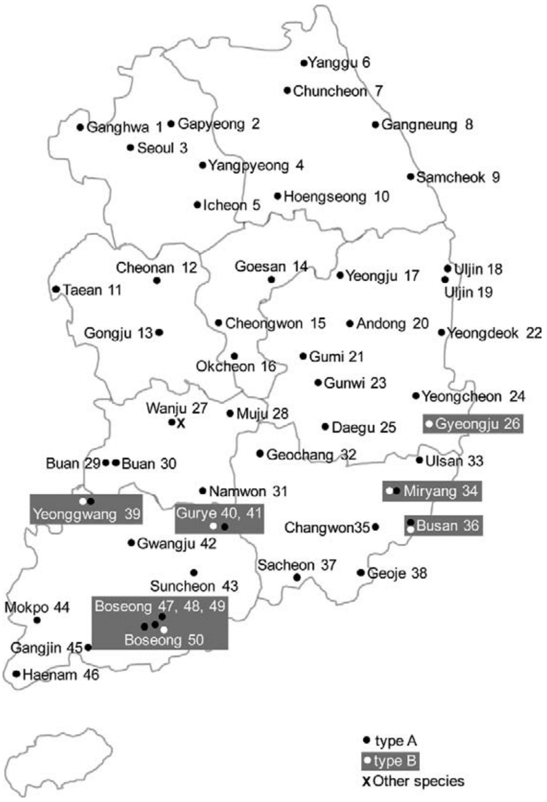

**Fig. 1.** Map showing the collection sites and site numbers of *R. speratus* samples in Korea.

is possibly not enough to represent all *Reticulitermes* species and subspecies that occur in the country. Analysis of mtDNA known to inherit from the mother is also insufficient to show general genomic information that is given from both parents. In this study, we analyzed the molecular characteristics of *Reticulitermes* species from Korea and compared them with those from China and Japan. The objectives of the current study were to 1) collect the number of *Reticulitermes* species inhabiting Korea through an extensive survey, and 2) understand their molecular characteristics based on the analysis of the COII region of the mtDNA and 18S and 28S gene comprising internal transcribed spacer (ITS) of the genomic DNA.

## Materials and Methods

**Sample Collection.** The *Reticulitermes* specimens were collected from 50 sampling sites in Korea (Fig. 1). Ten voucher specimens for DNA extraction and preservation were collected from a randomly selected colony in each of the sites. They were submerged in pure ethanol and stored at −80°C in Korea University Termite Collection ([KUTC], Seoul, Korea).

**DNA Extraction, Amplification, and Purification.** Ethanol-preserved vouchers were mixed by inverting the sample tubes several times. The specimens were then washed by distilled water and allowed to dry on filter paper. Total genomic DNA was extracted from individual heads and legs after grinding using the genomic DNA purification kit (Promega, Madison, WI). Extracted genomic DNA was stored at −20°C and used as a polymerase chain reaction (PCR) template. PCR was conducted with two primer sets. The mtDNA primer set, TL-J-3037 (5′-ATGGCAGA TAGTGCAATGG-3′) (Liu and Beckenbach 1992) and TK-N-3785 (5′-GTTTTAAGAGACCAGTACTTG-3′) (Simon et al. 1994) was used for the entire mtDNA COII gene. Another primer set, CAS18sF1 (5′-TACACACCGCCCGTCGCTACTA-3′) and CAS28sB1d (5′-TTCTTTTCCTCCSCTTAYTRATATGCTTAA-3′) (Ji et al. 2003) amplified the entire region of ITS 1, 5.8S, and ITS 2. PCR was performed on a MyCycler thermal cycler system (Bio-Rad Laboratories, Hercules, CA) by using the same amplification method as described by Austin et al. (2002). PCR products were purified using Accuprep PCR purification kit (Bioneer Inc., Daejeon, Korea). Sequencing was performed at the MACROGEN DNA Synthesis Sequencing Facility (Seoul, Korea). All representative sequences used in this study were deposited in GenBank (Table 1). GenBank accession numbers of COII and ITS regions were HM560009–560013 and JF810209–810213, respectively.

**DNA Sequence Data Analysis.** All mtDNA COII and ITS sequences were subjected to a BLAST search of GenBank database (Altschul et al. 1994) and aligned using MUSCLE 3.8.31 (Edgar 2004). Neighbor-joining (NJ), maximum parsimony (MP), and maximum likelihood (ML) analysis were conducted in the PAUP version 4.0b10 software package (Swofford 2001), and gaps were treated as missing. Modeltest 3.7 (Posada and Crandall 1998) was implemented to apply the best-fit model. NJ topologies were inferred using HKY

**Table 1. Termite collection data**

| Isolate | Species | Collection site | GenBank | |
|---|---|---|---|---|
| | | | ITS | COII |
| KUTC27 | *Reticulitermes* sp. | Yongjin-myeon, Wanju-gun, Jeonbuk | JF810209 | HM560009 |
| KUTC1 | *R. speratus* | Ganghwa-eup, Ganghwa-gun, Incheon | JF810207 | HM560007 |
| KUTC26 | *R. speratus* | Yonggang-dong, Gyeongju-si, Gyeongbuk | JF810208 | HM560008 |
| KUTC30 | *R. speratus* | Jusan-myeon, Buan-gun, Jeonbuk | JF810210 | HM560010 |
| KUTC36 | *R. speratus* | Haeundae-gu, Busan, Gyeongnam | JF810211 | HM560011 |
| KUTC38 | *R. speratus* | Jangmok-myeon, Geoje-si, Gyeongnam | JF810212 | HM560012 |
| KUTC47 | *R. speratus* | Boseong-eup, Boseong-gun, Jeonnam | JF810213 | HM560013 |

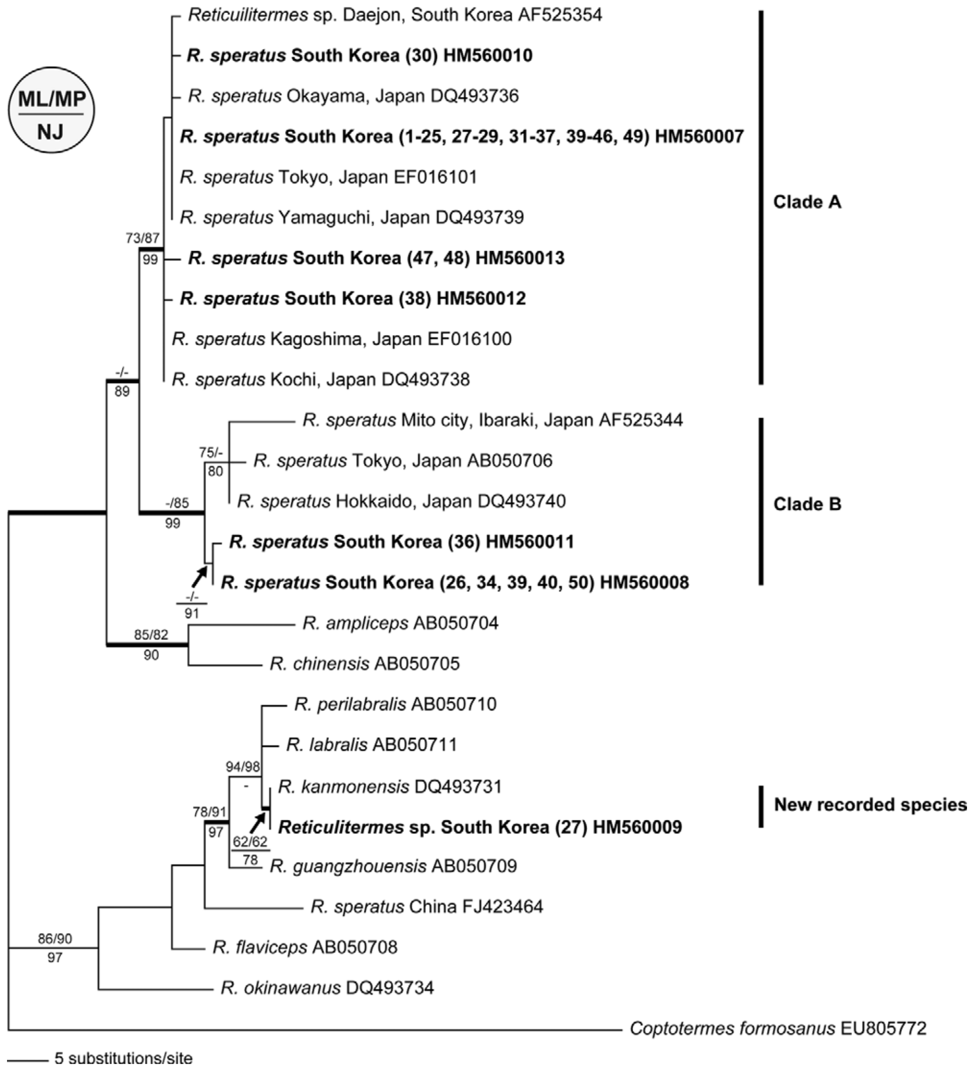

**Fig. 2.** MP tree inferred from COII mtDNA sequences. Bootstrap values for 1,000 replicates are shown above the branches and supported at ≥70%. Sampling site numbers are given in parentheses. Branches maintained in three different analyses (MP, ML, and NJ analyses) are marked by bold lines. Numbers above the branches that are before the slash are ML bootstrap proportions, and those that are after the slash are MP bootstrap proportions. Values below the branches are NJ bootstrap proportions. Termites collected in this study are indicated by bold type.

(COII) and F81 (ITS) models with 1,000 bootstrap replicates, and MP analysis was conducted with heuristic method and relative support for different nodes was assessed using 1,000 bootstrap replicates (Felsenstein 1985), with 10 random addition sequences for each bootstrap replicate. ML analysis was implemented using heuristic method with HKY + I (COII) and F81+I (ITS) models. Branch support was assessed using 1,000 bootstrapping with 10 random addition sequences. Bootstrap node support >70% was marked as strong (Hillis and Bull 1993), whereas those with support <70% were not shown. *Coptotermes formosanus* Shiraki (EU805772) was used as an outgroup species for the analysis based on COII region, and *Reticulitermes flaviceps* (Oshima) (EU627785) was used as an outgroup for the analysis of ITS region. In addition, previously published COII sequences of *Reticulitermes* from China and Japan (Austin et al. 2002, Park et al. 2006) were compared with our data.

## Results and Discussion

DNA sequencing of the mtDNA amplicon averaged 677 bp and was used to facilitate comparison with previous GenBank sequences. The average base frequencies were A = 39.6, C = 23.6, G = 13.3, and T = 23.5. The Japanese populations of *R. speratus* studied by Park et al. (2006) comprised a monophyletic group, with the northern Japanese specimens forming four

**Table 2. Tajima–Nei pairwise distances of COII genes between *Reticulitermes* species from East Asia**

| No. | Species | L[a] | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | *R. speratus* EF016101 | J | — | | | | | | | | | | | | | | | | | | | | | | | | |
| 2 | *R. speratus* HM560010 | K | 0.2 | — | | | | | | | | | | | | | | | | | | | | | | | |
| 3 | *R. speratus* DQ493739 | J | 0.0 | 0.2 | — | | | | | | | | | | | | | | | | | | | | | | |
| 4 | *Reticuilitermes* sp. AF525354 | K | 0.2 | 0.3 | 0.2 | — | | | | | | | | | | | | | | | | | | | | | |
| 5 | *R. speratus* HM560007 | K | 0.0 | 0.2 | 0.0 | 0.2 | — | | | | | | | | | | | | | | | | | | | | |
| 6 | *R. speratus* DQ493736 | J | 0.2 | 0.3 | 0.2 | 0.0 | 0.2 | — | | | | | | | | | | | | | | | | | | | |
| 7 | *R. speratus* HM560013 | K | 0.5 | 0.6 | 0.5 | 0.0 | 0.5 | 0.6 | — | | | | | | | | | | | | | | | | | | |
| 8 | *R. speratus* DQ493738 | J | 0.2 | 0.3 | 0.2 | 0.0 | 0.2 | 0.3 | 0.3 | — | | | | | | | | | | | | | | | | | |
| 9 | *R. speratus* EF016100 | J | 0.2 | 0.3 | 0.2 | 0.0 | 0.2 | 0.3 | 0.3 | 0.0 | — | | | | | | | | | | | | | | | | |
| 10 | *R. speratus* HM560012 | K | 0.3 | 0.5 | 0.3 | 0.0 | 0.3 | 0.5 | 0.5 | 0.2 | 0.2 | — | | | | | | | | | | | | | | | |
| 11 | *R. speratus* AF525344 | J | 3.5 | 3.6 | 3.5 | 0.0 | 3.5 | 3.6 | 3.6 | 3.3 | 3.3 | 3.5 | — | | | | | | | | | | | | | | |
| 12 | *R. speratus* AB050706 | J | 2.7 | 2.8 | 2.7 | 0.0 | 2.7 | 2.8 | 2.8 | 2.5 | 2.5 | 2.7 | 1.5 | — | | | | | | | | | | | | | |
| 13 | *R. speratus* DQ493740 | J | 2.0 | 2.2 | 2.0 | 0.0 | 2.0 | 2.2 | 2.2 | 1.9 | 1.9 | 2.0 | 1.5 | 0.6 | — | | | | | | | | | | | | |
| 14 | *R. speratus* HM560011 | K | 2.2 | 2.3 | 2.2 | 0.0 | 2.2 | 2.3 | 2.3 | 2.0 | 2.0 | 2.2 | 2.0 | 0.8 | 0.5 | — | | | | | | | | | | | |
| 15 | *R. speratus* HM560008 | K | 2.0 | 2.2 | 2.0 | 0.0 | 2.0 | 2.2 | 2.2 | 1.9 | 1.9 | 2.0 | 1.9 | 0.6 | 0.3 | 0.2 | — | | | | | | | | | | |
| 16 | *R. chinensis* AB050705 | C | 4.3 | 4.5 | 4.3 | 4.5 | 4.3 | 4.5 | 4.5 | 4.1 | 4.1 | 4.0 | 6.1 | 5.3 | 4.6 | 4.8 | 4.6 | — | | | | | | | | | |
| 17 | *R. ampliceps* AB050704 | C | 5.0 | 5.1 | 5.0 | 5.1 | 5.0 | 5.0 | 5.1 | 4.8 | 4.8 | 4.6 | 6.8 | 6.2 | 5.5 | 5.7 | 5.5 | 3.5 | — | | | | | | | | |
| 18 | *R. kanmonensis* DQ493731 | J | 6.2 | 6.4 | 6.2 | 6.4 | 6.2 | 6.4 | 6.4 | 6.0 | 6.0 | 6.2 | 8.9 | 8.1 | 7.4 | 7.6 | 7.4 | 6.9 | 7.6 | — | | | | | | | |
| 19 | *R.* sp. HM560009 | K | 6.2 | 6.4 | 6.2 | 6.4 | 6.2 | 6.4 | 6.4 | 6.0 | 6.0 | 6.2 | 8.9 | 8.1 | 7.4 | 7.6 | 7.4 | 6.9 | 7.6 | 0.0 | — | | | | | | |
| 20 | *R. perilabralis* AB050710 | C | 6.5 | 6.7 | 6.5 | 6.7 | 6.5 | 6.7 | 6.7 | 6.4 | 6.4 | 6.5 | 9.0 | 8.3 | 7.6 | 7.7 | 7.6 | 6.9 | 7.9 | 0.6 | 0.6 | — | | | | | |
| 21 | *R. labralis* AB050711 | C | 6.0 | 6.2 | 6.0 | 6.2 | 6.0 | 6.2 | 6.2 | 5.9 | 5.9 | 6.0 | 8.7 | 8.0 | 7.2 | 7.4 | 7.3 | 6.7 | 7.5 | 0.5 | 0.4 | 0.8 | — | | | | |
| 22 | *R. guangzhouensis* AB050709 | C | 6.0 | 6.2 | 6.0 | 6.2 | 6.0 | 6.2 | 6.2 | 5.9 | 5.9 | 5.7 | 8.3 | 7.6 | 6.9 | 7.0 | 6.9 | 6.0 | 7.0 | 1.4 | 1.4 | 1.7 | 1.6 | — | | | |
| 23 | *R. flaviceps* AB050708 | J | 5.7 | 5.8 | 5.7 | 5.8 | 5.7 | 5.8 | 5.8 | 5.5 | 5.5 | 5.7 | 8.2 | 7.6 | 6.9 | 7.0 | 6.9 | 6.7 | 7.4 | 2.5 | 2.5 | 2.8 | 2.7 | 2.3 | — | | |
| 24 | *R. speratus* FJ423464 | C | 6.4 | 6.5 | 6.4 | 6.5 | 6.4 | 6.5 | 6.5 | 6.2 | 6.2 | 6.4 | 8.6 | 8.0 | 7.2 | 7.4 | 7.2 | 6.9 | 7.6 | 3.2 | 3.2 | 3.5 | 3.3 | 3.0 | 3.2 | — | |
| 25 | *R. okinawanus* DQ493734 | J | 5.9 | 6.0 | 5.9 | 6.0 | 5.9 | 5.8 | 6.0 | 5.7 | 5.7 | 5.9 | 8.5 | 7.8 | 7.0 | 7.3 | 7.1 | 6.4 | 5.8 | 5.2 | 5.2 | 5.6 | 5.0 | 5.0 | 4.3 | 5.2 | — |

[a] L, locality; C, China; J, Japan; and K, Korea.

different clades. Here, Clade B also encompassed four internal clades, but it was not possible to directly compare our data to these previously published sequences due to differences in their length (the sequences analyzed by Park et al. (2006) were relatively shorter). Still, *R. speratus* sequences from Japan also were partitioned into two clades in the phylogenetic tree, and each clade matched our sequences. *R. speratus* specimens from Korea also were strongly clustered as a monophyletic group in clade B. Further studies, however, are needed to determine whether these specimens represent another subspecies of *R. speratus.*

The results also confirmed that intraspecific molecular differences depended on geographic distribution. Although mtDNA COII sequences from our samples were matched with those of *R. speratus* specimens from Japan, they were different from those of *R. speratus* from China. Austin et al. (2002) found a high degree of intraspecific variation for *R. speratus* depending on the geographic distribution of the specimens, with *R. speratus* from different continents being phylogenetically separated. In this sense, it is interesting to note that our sequence data were subdivided into two clades even though all samples were from the same country. One group of sequences (Clade A, Fig. 2), from specimens distributed throughout Korea, perfectly matched COII sequences of *R. speratus* (EF016101 from Tokyo and DQ493739 from Yamaguchi). Another group of sequences (Clade B, Fig. 2) were closely matched with *R. speratus* (DQ493740) from Hokkaido, Japan. In addition to the observed genetic and geographic differences between the two groups, polymorphic variations also were investigated by examining pairwise distances with the Tajima–Nei method (Table 2). Within *R. speratus*, pairwise distances ranged from 0.0 to 8.6%. The greatest distance was between the specimen from China (AF423464) and the one from Ibaraki, Japan (AF525344). The distance between the specimen from China (AF423464) and the COII sequences belonging to clade B was ≈7%. By comparing *Reticulitermes* specimens from Europe and North America, Austin et al. (2002) also found notable differences within a same species depending on its geographical origin. Here, pairwise distances between Clades A and B of *R. speratus* ranged from 2.0 to 2.3%. The distances between the various *Reticulitermes* species were occasionally lower; for example, the difference between *R. labralis* (AB050711) and *R. kanmonensis* (DQ493731) represented 0.4%. The distances between Clades A and B were considered as substantial intraspecific variations, although the value was not conspicuously high. Therefore, the samples belong to Clade A might correspond to *R. s. kyushuensis*, known until now as the only *Reticulitermes* species in Korea.

Unexpectedly, the samples from Yongjin-myeon, Wanju-gun, and Jeonbuk (Fig. 2) revealed the first occurrence of species regarding as *R. kanmonensis* in Korea. The Sequence of the species showed a complete match with the Japanese *R. kanmonensis* (DQ493731), which was first introduced in Kanmon, Japan (Nawa 1911, 1912a,b, 1917). Although Kitade et al. (2004) have collected this species both on the coast and inland, it was restricted to coastal areas on the Honshu and Kyushu sides of the Kanmon strait. In Korea, this species was collected near a port that frequently offloads wooden products from abroad, suggesting that wooden materials imported from Japan and other countries have contributed to anthropogenic introductions of these species into the country.

In the genomic DNA analysis, we obtained the amplicons averaging 832 bp between 18S and 28S gene, comprising the ITS1, 5.8S, and ITS2 regions. The average base frequencies were A = 21.0, C = 29.0, G = 31.7, and T = 18.3. However, all sequences were not different except one or two base pair according to two of molecular characteristics in COII analysis. The ITS rDNA region having intra- and interspecifically variable sites is considered to be an appropriate tool for studying molecular diversity at subgeneric level. But, it was reported that analysis of ITS2 region in genus *Reticulitermes* was not to clarify variations, because the same consensus sequences were found between the species (Uva et al. 2004). It is difficult to analyze ITS sequences because there is little information about genomic DNA sequences of *R. speratus* in GenBank, and they have similar molecular characteristics between different *Reticulitermes* species. Nevertheless, sequence of the new occurrence sample was the closest matched with *R. flaviceps* (EU622785) that was comparable to ours in size, 677 bp, representing ≈99.0% similarity. This was regarded as *R. kanmonensis* according to COII features, but genomic DNA information of *R. kanmonensis* is not studied yet. Evaluation of sequence data from these taxa demonstrated 98.5% similarity between *Reticulitermes* sequences of new species and *R. speratus.* This result would imply that there is at least two distinct *Reticulitermes* species in Korea.

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