PNIE PROCEEDINGS OF THE NATIONAL INSTITUTE OF ECOLOGY OF THE REPUBLIC OF KOREA

Original Article

PNIE 2024;5(4):128-133
https://doi.org/10.22920/PNIE.2024.5.4.128
pISSN 2765-2203, eISSN 2765-2211

Species Identification Method via Unique Genetic Markers for *Reticulitermes kanmonensis*

Soon Jae Eum[1,2], Kibeom Park[3], Youngho Cho[1], Youngjun Park[1]*

[1]Invasive Alien Species Team, National Institute of Ecology, Seocheon, Republic of Korea
[2]Department of Marine Biotechnology, Kunsan National University, Gunsan, South Korea
[3]Invirustech, Gwang-ju, Republic of Korea

ABSTRACT

Invasive alien species, along with climate change, are major contributing factors to biodiversity loss, and their unintentional introduction through imported goods is increasing. Termites, as wood-feeding insects, pose a significant threat when introduced into Korea via imported wood, necessitating a rapid and accurate identification method. In this study, we developed a diagnostic method based on species-specific genetic sequences for identifying *Reticulitermes kanmonensis*, a recently identified species. Termite samples were collected from Wanju-gun, Jeollabuk-do, and subjected to whole-genome sequencing to pinpoint species-specific genetic sequences. Utilizing these sequences, we designed primer sets and employed TaqMan-based primer sets and qPCR analysis to select the final primer sets capable of rapidly distinguishing *R. kanmonensis*. The genetic detection method developed here offers a rapid means of identifying alien termite species, likely enhancing termite management and quarantine practices in Korea.

Keywords: Specific markers, Termites, *Reticulitermes kanmonensis*

Introduction

Invasive alien species, in conjunction with climate change, are among the five crucial contributors to biodiversity losses, with unintentional introductions through imported goods rising globally (IPBES, 2023). In 2023, South Korea reported exports of USD 632.2 billion and imports of USD 642.6 billion, marking an increase of 10-20% over the past decade (Biswas, 2023; MOTIE, 2024), thereby heightening the risk of unintentional introduction of alien species. The import of large quantities of agricultural products and raw materials such as timber, which likely introduce wood-feeding insects like termites, has particularly increased (Kim & Lee, 2019; Eyer & Vargo, 2021). Termites play a crucial role as decomposers in ecosystems by breaking down plant materials, yet they also cause significant economic damage to wooden structures, leading to annual losses of $40 billion (Rust & Su, 2012).

In South Korea, concerning the introduction of alien termite species, six ant species from five families and five genera were reported to have been quarantined 67 times in various wood materials and other plants imported from 16 countries between 2003 and 2022; *Coptotermes acinaciformis* and *Porotermes quadricollis* were first identified in 2007, followed by *Coptotermes formosanus* in 2010, *Coptotermes curvignathus* in 2017, and *Coptotermes gestroi* in 2020. These species are targeted as controlled

Received September 23, 2024; **Revised** October 18, 2024; **Accepted** October 19, 2024

***Corresponding author:** Youngjun Park
e-mail aquatic@nie.re.kr
https://orcid.org/0000-0003-3916-5796

pests in South Korea. However, there are 19 quarantine stations at 22 ports across the country, and cases of quarantine involving alien termite species continue to occur, indicating potential nationwide introductions (Kim & Kim, 2024).

Rapid identification of alien termite species (introductions) is crucial for effective pest control; however, taxonomic studies have been insufficiently conducted with only 15 termite cases identified at the species level out of a total of 67 (Kim & Kim, 2024). *Cryptotermes domesticus* and *Incisitermes minor* were discovered in May and September in Gangnam-gu, Seoul, and Changwon-si, Gyeongsangnam-do, South Korea, respectively. It took one week to genetically identify the alien species introduction status of *R. speratus*, which was found in Gangnam-gu. (ME, 2023a, 2023b, 2023c).

This study aims to propose a diagnostic method using species-specific genetic sequences for *Reticulitermes kanmonensis*, a native species in South Korea, to accurately and swiftly determine whether termites found in imported wood are invasive species.

Materials and Method

Sample collection of *Reticulitermes speratus* and *Reticulitermes kanmonensis*

To design a diagnostic method based on species-specific genetic sequences for *R. kanmonensis*, the native species

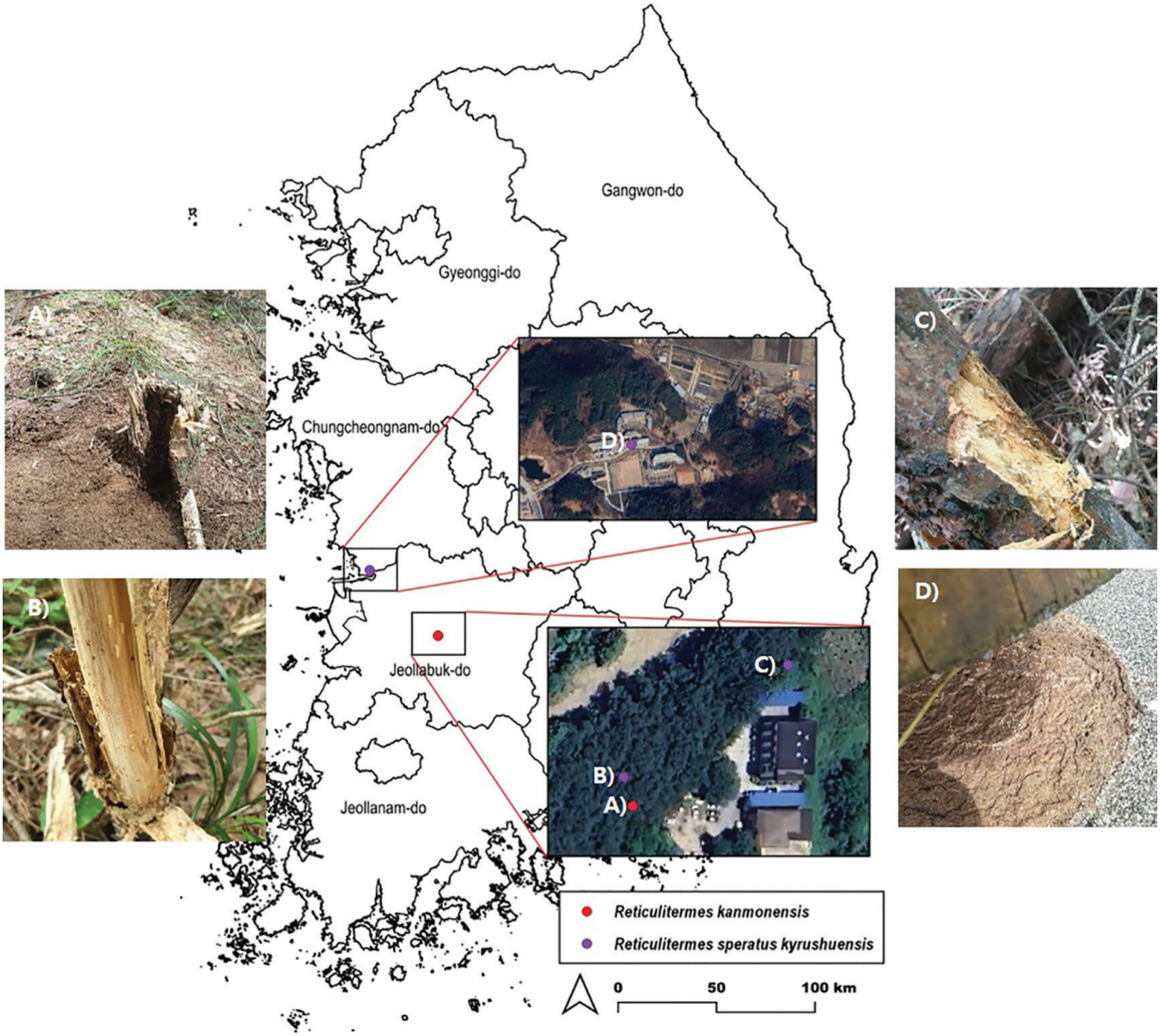

Fig. 1. Collection Sites for *R. kanmonensis* (A) and *R. speratus kyuhuensis* (B, C, D) in Korea.

R. speratus was collected and used as a control for comparative analysis. *Reticulitermes speratus* was collected in June and August 2023 from two locations: Wanju-gun in Jeollabuk-do, where *R. kanmonensis* was first identified in Seocheon-gun in Chungcheongnam-do, South Korea (Lee *et al.*, 2015). *R. speratus* samples were sourced from both standing and fallen dead pine trees or stumps using an axe; *R. speratus* specimens collected from a single stump and tree were treated as a single sample and preserved in 95% ethanol. Four samples, each comprising a minimum of one army ant and two worker ants, were collected and utilized (Fig. 1).

Sequencing

The Clear-S Quick DNA extraction kit (IVT-3002, Invirustech, S. Korea) was used to extract DNA from the collected termite samples according to the manufacturer's protocol.

Production of primers for gene amplification and selection

To design primers for species amplification, this study identified candidate sequences from WGS analysis of genomic DNA. Subsequently, Primer3 software (Untergasser *et al.*, 2012) was employed to design five pairs of primer and probe sets for each species (Table 1), focusing on the precise amplification of species-specific genetic sequences. The properties of these primers were optimized considering Tm values, GC content, and potential secondary structure formation.

qPCR was conducted using extracted and refined genes from two species as templates. The conditions were as follows: 10 µL EzAmp™ HS qPCR 2X Master Mix (SYBR Green, Low Rox), 3 µL (1 ng/µL) of genomic DNA, 0.5 µL of each primer at 10 pmol, and 6 µL of nuclease-free water for a final volume of 20 µL. The cycling condition commenced with an initial denaturation at 95°C for five minutes, followed by 45 cycles of denaturation at 95°C for 10 seconds and annealing/extension at 60°C for 30 seconds. Melting curve analysis was performed, increasing the temperature by 0.5°C/s from 60°C to 95°C. Two primer sets were selected for each species based on their PCR amplification performance and specificity.

Results and Discussion

Whole-genome sequencing (WGS)

WGS was conducted using the Illumina Novaseq platform with 150 bp libraries in a paired-end sequencing format, generating each 150 bp read. This sequencing produced 282,273,060 RAW reads with Q20 (97.09%), Q30 (92.39%), and GC content (46%). WGS data for other termite species were obtained from the SRA database of NCBI GenBank. WGS data estimated the total genome size of *R. kanmonensis* to be approximately 270-277 Mb, of which about 81% was repetitive sequences.

For the effectiveness of the designed primers, it is crucial that they exhibit a low mutation rate. HMMER and BLASTn analyses of the designed primers and amplified sequences revealed that all five sets showed high similarity

Table 1. Development of qPCR primer sets for two termite species

	Primer	Sequence
R. Kan Set 1	>qFw_A1_R.kan_1_134bp	ACTTCTCGGGAATGTTCTCCAG
	>qRev_A1_R.kan_1_134bp	ACTGGCTCTTTGGGACACAG
	>qIO_A1_R.kan_1_134bp	ACGACAACGGACGCAACCTGTCA
R. Kan Set 2	>qFw_A1_R.kan_2_78bp	TAAGTAGGCCCGTCGTAAACAG
	>qRev_A1_R.kan_2_78bp	CGCTATCCTTGCCATCTTTGC
	>qIO_A1_R.kan_2_78bp	TGGTAGGGTGTTTGCCGAAACGTAAGC
R. Kan Set 3	>qFw_A1_R.kan_3_144bp	CGACACATCTACCGCATGAG
	>qRev_A1_R.kan_3_144bp	CTTGTAAGCGTCTGTTGTAATGC
	>qIO_A1_R.kan_3_144bp	CCGGGGTTTCGCTTCTGGGTGGC
R. Kan Set 4	>qFw_A1_R.kan_4_138bp	TTGGAATGCAGAGTGTCAGC
	>qRev_A1_R.kan_4_138bp	GAAGTTAGCGGTGTCAGAGAAC
	>qIO_A1_R.kan_4_138bp	TCAATTAGGCCGACGTCAACTCCGA
R. Kan Set 5	>qFw_A1_R.kan_5_79bp	TGCGATTACTTTAGACCTGCATC
	>qRev_A1_R.kan_5_79bp	CAAAGACACGCCTGGTATCTG
	>qIO_A1_R.kan_5_79bp	ACAGTAGCGTACCGGGTGACTGCA

to protein-coding regions of other termites, suggesting these as potential protein-coding regions of *R. kanmonensis* (Tables 2 and 3). Nevertheless, some primer sets did not align with specific protein-coding regions, indicating the need for further research, including a full genome analysis of *R. kanmonensis*.

Development and selection of species-specific primers

A TaqMan-based probe method, known for its high specificity and sensitivity, was employed to enhance accuracy. DNA was extracted from each termite, followed by WGS to identify species-specific sequences. Subsequently, PCR results were confirmed, and target sequences were found to be well-amplified (Table 4). By selecting and verifying primer sets that efficiently amplify only the target species, R. Kan set 1 and R. Kan set 4 were chosen as species-specific primers for *R. kanmonensis* due to their high amplification efficiency and lack of non-specific bands (Fig. 2).

Specificity and sensitivity of species-specific primers

In qPCR, the Ct value, representing the Cycle Threshold, is used to estimate the initial amount of target DNA. Given the higher amplification sensitivity, even a low initial concentration of target DNA can be detected, resulting in a lower Ct value. To confirm the sensitivity of species-specific primers, the Ct value of each primer set was determined. The primer set with the lowest Ct value was selected as the species-specific marker.

Table 2. Coding protein information of genetic locations for species-specific primer sets of *Reticulitermes kanmonensis*

Primer set	Species	Coding protein	Accession no.
R. Kan Set 1	*Nylanderia fulva* *Camponotus floridanus* *Linepithema humile*	Ras-like GTP-binding protein Rho1	XM_029300460 XM_011255833 XM_012373828
R. Kan Set 2	*Nylanderia fulva*	Defensin-2-like mRNA	XM_029311992
R. Kan Set 3	*Nylanderia fulva*	Uncharacterized mRNA	XM_029304645
R. Kan Set 4	*Nylanderia fulva*	Uncharacterized mRNA	XM_029316608
R. Kan Set 5	*Nylanderia fulva*	Probable chitinase 10 mRNA	XM_029312799

Table 3. Domain information of the genetic regions detected for the primer set of *Reticulitermes kanmonensis*

Primer set	Species	Domain name	Amino acid sequence
R. Kan Set 3	*Tribolium castaneum* *Harpegnathos saltator*	Chitin-binding Peritrophin-A domain	THLPHETDCTKFYKCN WGRPVLMDCPLSGVSL LGGSKNRLHYNRRLQ

Table 4. Sequence of PCR amplicons from species-specific primer sets for native termite species in Korea: *Reticulitermes kanmonensis*

Primer name	Sequence
R. Kan Set 1	ACTTCTCGGGAATGTTCTCCAGGGAATCGGGACTGTCGATGGAGAAGCACATTAGGATGACGTCCGTGTC TGGGTACGACAACGGACGCAACCTGTCATAGTCTTCTTGTCCAGCTGTGTCCCAAAGAGCCAGTT
R. Kan Set 2	TAAGTAGGCCCGTCGTAAACAGCTGGTAGGGTGTTTGCCGAAACGTAAGCTAGTACGGCAAAGATGGCAA GGATAGCG
R. Kan Set 3	CGACACATCTACCGCATGAGACCGACTGCACAAAATTCTACAAATGTAATTGGGGTCGACCAGTATTGATG GATTGTCCTCTTTCCGGGGTTTCGCTTCTGGGTGGCTCGAAGAACAGATTGCATTACAACAGACGCTTAC AAG
R. Kan Set 4	TTGGAATGCAGAGTGTCAGCTATCTTCAATTAGGCCGACGTCAACTCCGAAATTAGTAAATCCTACGACAT CATCGATTTCGCCATCAAGTCTTTTAACGACTACGATTCCACCAAGTTCTCTGACACCGCTAACTTC
R. Kan Set 5	TGCGATTACTTTAGACCTGCATCTCCGGCAGCTACAGTAGCGTACCGGGTGACTGCAACAGATACCAGGC GTGTCTTTG

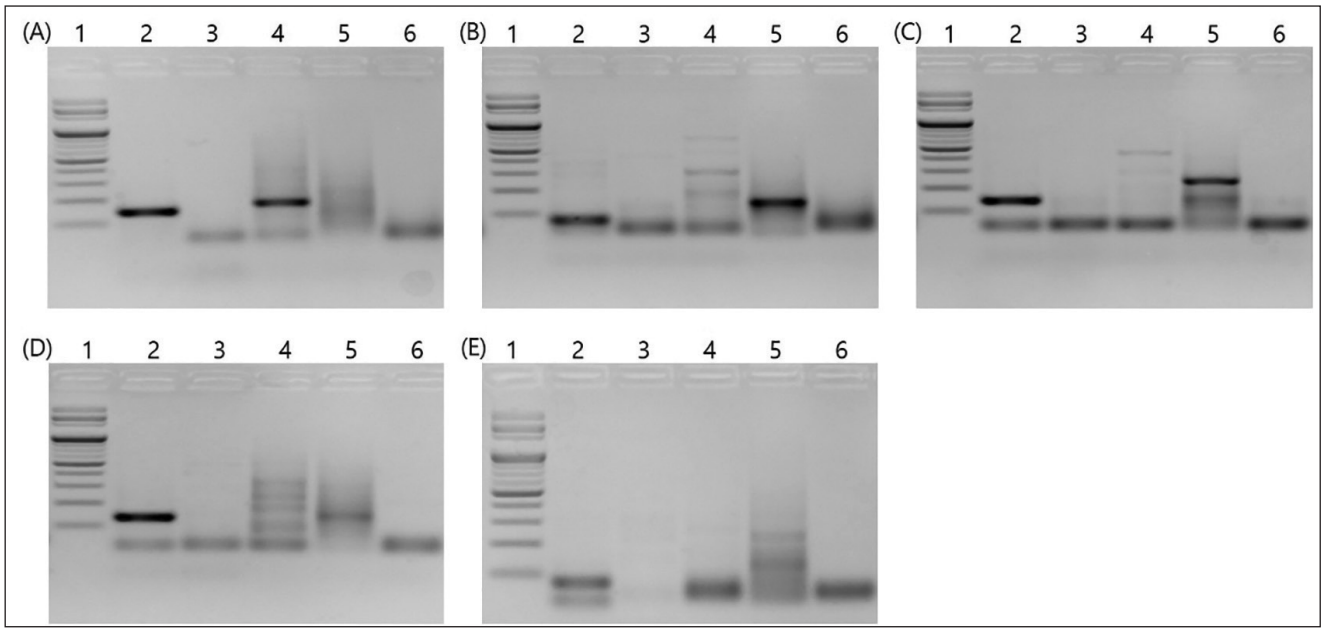

Fig. 2. Amplification Results Using Primer Sets for the Specific Detection of *R. kanmonensis*. Lane 1: 100 bp Marker; lane 2: *R. kanmonensis* DNA; lane 3: *R. speratus* DNA; lane 4: *C. domesticus* DNA; lane 5: *C. gestroi* DNA; lane 6: Distilled Water (D.W). Set designations: (A) R. Kan Set 1, (B) R. Kan Set 2, (C) R. Kan Set 3, (D) R. Kan Set 4, (E) R. Kan Set 5.

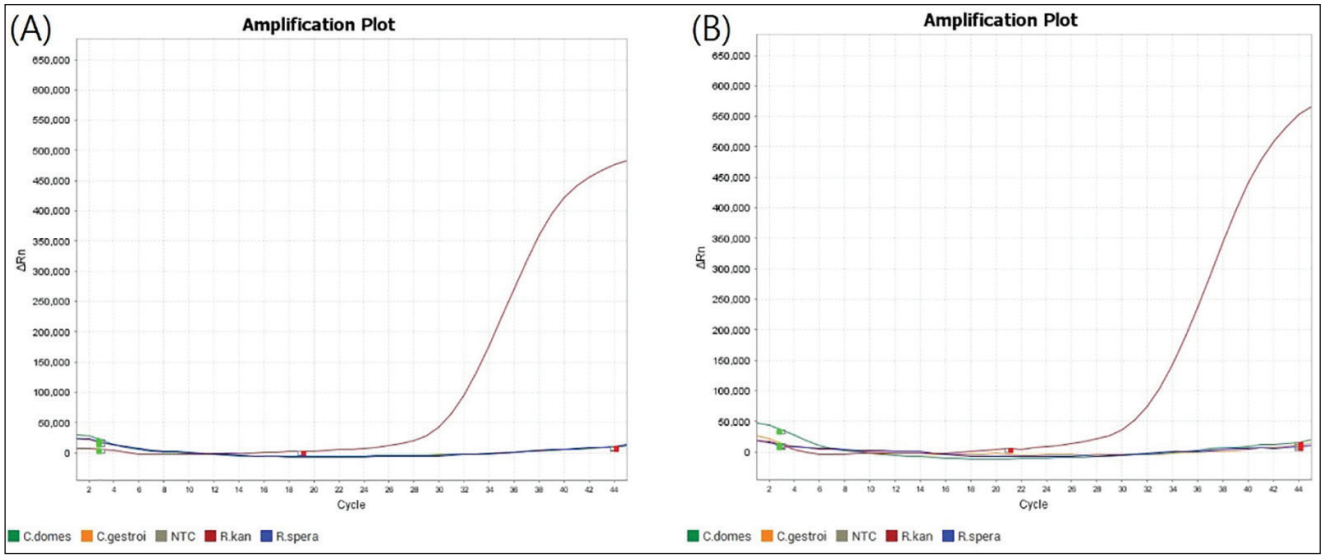

Fig. 3. Ct values from species-specific PCR amplifications. (A) R. Kan Set 1, (B) R. Kan Set 4.

Table 5. Ct values comparison for the sensitivity of two species-specific detection primers

Target species	Primer name	Ct value
R. kanmonensis	R. Kan Set 1	30.945
	R. Kan Set 4	32.212

Although the initially selected primer set was adequate to obtain results, the Ct values were compared to ensure more reliable and accurate outcomes (Fig. 3). In the case of *R. kanmonensis*, the Ct value for R. Kan set 1 was 30.945, indicating higher sensitivity compared to R. Kan set 4 (Table 5). Further analysis of PCR gel loading

results (Fig. 2) showed that in *R. kanmonensis*, R. Kan set 1 amplified more specifically than R. Kan set 4, suggesting that R. Kan set 1 is likely more effective.

Author Contributions

Conceptualization by Soon Jae Eum and Kibeom Park, Research and data collection by Youngjun Park and Youngho Cho.

Conflict of Interest

The author declares that there are no competing interests.

Acknowledgments

This work was supported by the "Nationwide Survey of Non-native Species in Republic of Korea (NIE-A-2024-12)", which was funded by the National Institute of Ecology, Republic of Korea.

References

Biswas, R. (2023). South Korea's exports rebound in October 2023. *Economics Commentary*. Nov 03, 2023.

Eyer, P.A., and Vargo, E.L. (2021). Breeding structure and invasiveness in social insects. *Current Opinion in Insect Science*, 46, 24-30. https://doi.org/10.1016/j.cois.2021.01.004 [PMID: 33549724]

IPBES (2023). The thematic assessment report on Invasive Alien Species and their control. *Intergovernmental Science-Policy Platform on Biodiversity Services*, 1-55.

Kim, S.H., and Lee, S.B. (2019). Introduction of invasive termite and threat of wooden architectural heritage. *Journal of the Society of Cultural Heritage Disaster Prevention*, 4(2), 139-147.

Kim, T., and Kim, S. (2024). Analysis of the invasive termites in plant quarantine and characteristics of Drywood Termites found in South Korea. *Journal of National Heritage*, 9(1), 11-27. https://doi.org/10.69756/jnh.2024.9.1.011

ME (2023a). Post about termites. Ministry of Environment of Korea. https://www.me.go.kr/home/web/board/read.do?pagerOffset=0&maxPageItems=10&maxIndexPages=10&searchKey=title&searchValue=%ED%9D%B0%EA%B0%9C%EB%AF%B8&menuId=10525&orgCd=&boardId=1601930&boardMasterId=1&boardCategoryId=&decorator. *Ministry of Environment*. (last modified 2023.5.19, accessed 2024.08.30).

ME (2023b). Post about termite. Ministry of Environment. https://www.me.go.kr/home/web/board/read.do?pagerOffset=0&maxPageItems=10&maxIndexPages=10&searchKey=title&searchValue=%ED%9D%B0%EA%B0%9C%EB%AF%B8&menuId=10525&orgCd=&boardId=1602970&boardMasterId=1&boardCategoryId=&decorator. *Ministry of Environment*. (last modified 2023.5.24, accessed 2024.08.30).

ME (2023c). Post about termite. Ministry of Environment. https://www.me.go.kr/home/web/board/read.do?menuId=10525&boardMasterId=1&boardCategoryId=39&boardId=1626430. *Ministry of Environment*. (last modified 2023.9.26, accessed 2024.08.30).

MOTIE (2024). https://www.data.go.kr/data/3039964/fileData.do. *Ministry of Trade, Industry and Energy*. (last modified 2023.12.31, accessed 2024.08.30).

Lee, W., Choi, D.-S., Ji, J.-Y., Kim, N., Han, J.M., Park, S.-H., *et al.* (2015). A new record of *Reticulitermes kanmonensis* Takematsu, 1999 (Isopera: Rhinotermitidae) from Korea. *Journal of Asia-Pacific Entomology*, 18, 351-359. http://dx.doi.org/10.1016/j.aspen.2015.04.006

Rust, M.K., and Su, N.Y. (2012). Managing social insects of urban importance. *Annual Review of Entomology*, 57, 355-375. https://doi.org/10.1146/annurev-ento-120710-100634

Untergasser, A., Cutcutache, I., Koressaar, T., Ye, J., Faircloth, B.C., Remm, M., *et al.* (2012). Primer3—new capabilities and interfaces. *Nucleic Acids Res*, 40(15), e115. https://doi.org/10.1093/nar/gks596 [PMID: 22730293 PMCID: PMC3424584]