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[Thi-Thu Nguyen](#) , Mi-Sun Yoo , [Hyang-Sim Lee](#) , [So Youn Youn](#) , Se-Ji Lee , Su-Kyoung Seo , Jaemyung Kim , [Yun Sang Cho](#) \*

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## Article

# Molecular Identification and Prevalence of the Mite *Carpoglyphus lactis* (Acarina: Carpoglyphidae) in *Apis mellifera* in the Republic of Korea

Thi-Thu Nguyen <sup>1,2,†</sup>, Mi-Sun Yoo <sup>1,†</sup>, Hyang-Sim Lee <sup>1</sup>, So Youn Youn <sup>1</sup>, Se-Ji Lee <sup>1</sup>, Su-Kyoung Seo <sup>1</sup>, Jaemyung Kim <sup>1</sup> and Yun Sang Cho <sup>1,\*</sup>

<sup>1</sup> Laboratory of Parasitic and Honeybee Diseases, Bacterial Disease Division, Department of Animal and Plant Health Research, Animal and Plant Quarantine Agency, Gimcheon, Republic of Korea; nguyenthush.hua@gmail.com (T.-T.N.); msyoo99@korea.kr (M.-S.Y.); leehs76@korea.kr (H.-S.L.); syoun@korea.kr (S.Y.Y.); dltpwl86@korea.kr (S.-J.L.); ssk922@korea.kr (S.-K.S.); kimjm88@korea.kr (J.K.)

<sup>2</sup> Institute of Biotechnology, Vietnam Academy of Science & Technology, Ha Noi, Viet Nam

\* Correspondence: choys@korea.kr; Tel.: +82(10) 7345 5072

† These authors contributed equally to this work.

**Simple Summary:** Honeybees (*Apis mellifera*) are crucial for our ecosystem, but they face various threats. This study investigates the detection of a new mite species emerging in Korean honeybees, *Carpoglyphus lactis*. Using the polymerase chain reaction method to amplify the Cytochrome *c* oxidase subunit 1 gene region, we examined the presence of *C. lactis* in honeybee colonies across nine provinces of Republic of Korea. We found *C. lactis* in eight provinces in ROK, particularly during winter. This is the first identification of *C. lactis* in Korean honeybees. Identifying new threats is essential for beekeepers and researchers. Understanding the prevalence of *C. lactis* mite species and its impact on honeybee health will help develop strategies to protect these harmful insects.

**Abstract:** *Apis mellifera*, especially weak ones, are highly vulnerable to *Carpoglyphus lactis* mites, which can rapidly infest and consume stored pollen, leading to weakened colonies and potentially colony collapse. This study aimed to ascertain and investigate the prevalence of this mite in honeybee colonies across 9 provinces in the Republic of Korea (ROK). A total of 615 honeybee colonies were collected, from 66 apiaries during the spring and 58 apiaries during the summer of 2023. A 1,242 bp segment of the the Cytochrome *c* oxidase subunit 1 (COI) gene was amplified using the polymerase chain reaction method. The detection levels of *C. lactis* in the honeybees were compared between winter and summer. Based on the COI sequence analysis, the nucleotide sequence similarity of *C. lactis* mites isolated in the ROK with those from China (NC048990.1) was found to be 99.5%, and with those from the United Kingdom (KY922482.1) was 99.3%. This study is the first report of *C. lactis* in Korean apiaries. The findings in this study demonstrate a significantly higher detection rate in winter, which is 4.1 times greater than that in summer ( $p < 0.001$ ). Furthermore, the results underscore the usefulness of molecular diagnostic techniques for detecting *C. lactis* mites.

**Keywords:** *Apis mellifera*; *Carpoglyphus lactis*; cytochrome *c* oxidase subunit 1 (COI) gene; molecular identification; Republic of Korea

## 1. Introduction

*Apis mellifera*, western honeybee, play a crucial role in the process of pollination and stability of the ecosystem [1,2]. Basically, they produce economic and health benefit products such as honey, royal jelly, propolis, and beeswax. Unfortunately, the rate of honeybee colony losses has been increasing annually in many countries in the world [3–5]. According to a collaborative survey

conducted by the Rural Development Administration, Animal and Plant Quarantine Agency, local authorities, and Korean Beekeeping Association in the Republic of Korea (ROK), 416,409 beehives disappeared between October 2021 and March 2022. Numerous factors influence the health of honeybee colonies, and one of devastating factors to colony loss is parasitic infection, especially mite species [1,6–12]. In the ROK, significant harm to honeybee colonies is attributed to *Varroa* mite [1,11,13,14]. Other harmful mite species have been identified morphologically and/or genetically, including *Tropilaelaps mercedesae*, *Tyrophagus curvipennis*, and *Acarapis* mites in the ROK [12,14]. However, there has been no research about detecting *Carpoglyphus lactis* within the Korean honeybee population.

*C. lactis*, commonly known as the cheese mite or milk mite, belongs to the genus *Carpoglyphus* within the family Carpoglyphidae of Astigmata [15–17]. This mite is primarily associated with cheese and other milk products [16]. The previous reports mainly focus on the relationship between the mite and milk products, as well as its impact on cheese quality. The *C. lactis* species has been observed in the Holarctic, Oriental, Australian, and Indian regions [15,18–21]. Additionally, *C. lactis* mites are known to be relatively common in beehives among the observed regions. They are primarily found in stored pollen and aged beehives, potentially posing a threat to stored honey [22]. Moreover, they are present in beehive debris, in hive materials, on dead bees, and on beehive frames, serving as a source of pollen, honey, and essential bee substances stored in queen bee cells [23,24]. The damage caused by these mites is particularly severe for beehives stored during the winter season. Germany and the United States highlight widespread cases of beehive destruction due to *C. lactis* mites during winter storage [20,25,26]. In Zander, Germany, in 1947, 250 beehives were destroyed by *C. lactis* mites in a single winter storage area [27]. In Alabama, the US, beehives were found heavily infected with *C. lactis* mite after winter storage by Baker and Delfinado, 1978 [21]. *C. lactis* mites devastate honeybee combs, consuming stored pollen and polluting the hive with a brownish-yellow powder composed of mite carcasses, debris, and pollen. Research on the detection rate and prevalence of this mite on honeybees is still limited. Considering the severity of *C. lactis* mite on honeybees, understanding their presence and prevalence in honeybee colonies is crucial for beekeepers.

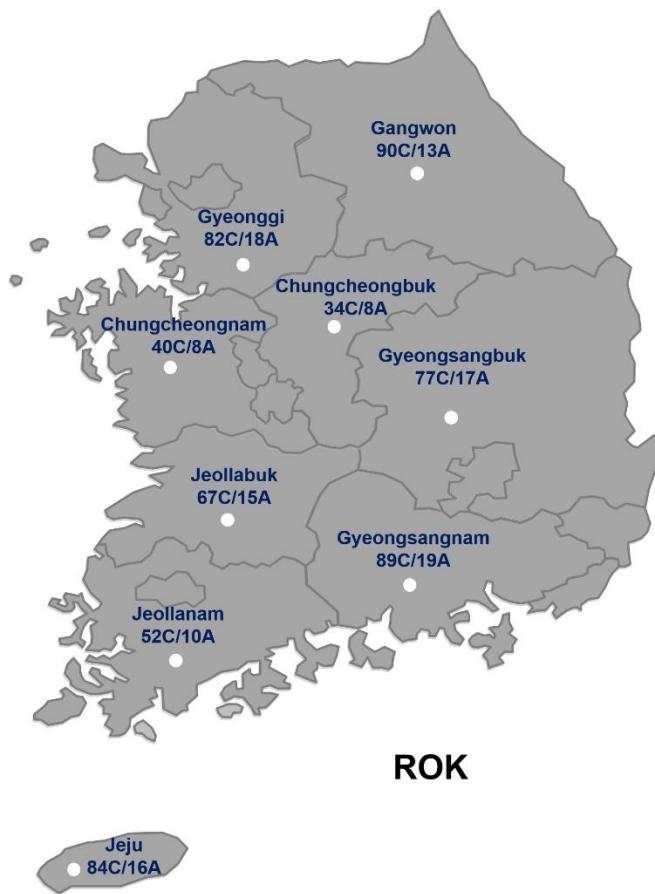
This study aimed to identify the emergence of *C. lactis* in honeybee colonies in the ROK in 2023. The detection rate of this mite was surveyed across 9 provinces in the ROK. This marks the initiation of investigations for future reference and encourages the development of effective management strategies against *C. lactis* in honeybee colonies.

## 2. Materials and Methods

### 2.1. Collection of Samples and Detection of Astigmatid Mites in Honeybees

Honeybee samples were collected from 9 provinces (Gangwon, Gyeonggi, Chungcheonnam, Chungcheonbuk, Gyeongsangnam, Gyeongsangbuk, Jeollanam, Jeollabuk, and Jeju) in the ROK (Figure 1). The honeybee samples were regularly collected throughout the year 2023 from non-migrating honeybee colonies. Honeybee samples were collected from the hive of each opened colony; subsequently, the comb was taken out after carefully observing the comb without the queen bee. A total of 615 honeybee colonies from 124 apiaries were sampled to investigate the presence of Astigmatid mites. The worker bees ( $n = 10\text{--}30$ ) were collected in 50 mL Falcon tubes from colonies in each apiary. Larvae sampling was collected from three random locations within the hive using forceps, checking 10 larvae at each site. Samples were collected using sterile tools to prevent contamination and directly placed into 50 mL Falcon tubes. Each tube was labelled with a unique identifier to ensure sample traceability throughout the testing process. To preserve the integrity of the collected samples, they were promptly transferred to a temperature-controlled storage facility at  $-20\text{ }^{\circ}\text{C}$ . The honeybee samples were used to assess temperature-dependent appearance patterns. Concurrently, these samples were used to evaluate the prevalence, diversity, and simultaneous occurrence of pathogens and Astigmatid mites. The samples were divided into two portions; the first was used for Astigmatid mite examination under a dissecting microscope, and the remained one was used for total nucleic extraction in polymerase chain reaction (PCR). The comb was collected in positive honeybee colonies for mite examination under a dissecting microscope. As this study only

involved honeybees, which are invertebrates not covered by most ethical regulations related to animal research, formal ethical approval was not required.



**Figure 1.** Honeybee colonies collected from 124 Korean apiaries in 9 provinces of the ROK in 2023. The number includes adult bees and larvae. C: colony; A: Apiary.

## 2.2. Total Nucleic Acid Extraction

The honeybee samples were homogenized using a Precellys 24 tissue homogenizer (Bertin Instruments, Montigny-le-Bretonneux, France) in three cycles of 15 s at a speed of 5,000 rpm in 1 mL of phosphate-buffered saline solution. Subsequently, a mixture of 200  $\mu$ L of lysis buffer and 20  $\mu$ L of proteinase K solution was added to the homogenized sample, and the resulting mixture was incubated at 56 °C for 10 min. The total nucleic acids were extracted using the Maxwell® RSC automated system according to the manufacturer's instructions. Finally, 60  $\mu$ L of purified total nucleic acids were employed to detect honeybee pathogens. The total nucleic acids from the honeybee and control samples were stored at -20 °C until further analysis.

## 2.3. Amplification of Astigmatid Mite-Specific Genes Using Polymerase Chain Reaction

DNA sequence-based identification has proven to be the most reliable approach for the species determination. Cytochrome *c* oxidase subunit I (*COI*) gene has been successfully used in the species identification and phylogenetic studies of mites [28]. The primer pairs used in this experiment were described in Table 1.

**Table 1.** Primers used in this study.

Name of primers	Sequences (5' -> 3')	Amplicon size (bp)	Note	References
COI-For	GTTTGGAATCTCTCATAC		Used for Astigmatid mite detection	[28]
COI-Rev	GAGCAACAAACATAARAAGTA TC	377		
CL-For	CTTGAATTGTAGAATGGA		Used for <i>C. lactis</i> mite detection	This study
CL-Rev	CTAATCGAGGTGTCCGAGGT	1242		

The total reaction volume (20  $\mu$ L), including 1  $\mu$ L of DNA, 1  $\mu$ L of each primer (10 pmol), and 17  $\mu$ L ddH<sub>2</sub>O, was used by AccuPower® PCR PreMix and Master Mix (Bioneer Corp., Daejeon, ROK). The PCR thermal cycling conditions were as per those by Nguyen et al. [12]: 94°C (5 min); 5 cycles of 94°C (20 s), 52°C (30 s), and 68°C (30 s); followed by 5 cycles of 94°C (20 s), 50°C (30 s), and 68°C (30 s); 30 cycles of 94°C (20 s), 48°C (30 s), and 68°C (30 s); 30 cycles of 94°C (20 s), 46°C (30 s), and 68°C (30 s); and a final extension step at 68°C (5 min). The amplified products corresponding to the *COI* gene segment of *C. lactis* in the honeybee colonies were visualized using 1% agarose gel electrophoresis, with a determined size of 1,242 bp. Subsequently, the PCR products were purified and sequenced by Cosmogenetech Co., Ltd. (Seoul, ROK). To verify the presence of *C. lactis* mite and assess its genetic diversity within Korean honeybees, the positive control was specifically designed by cloning the *COI* gene from a known Korean *C. lactis* mite.

#### 2.4. Phylogenetic Analysis and Statistical Analysis

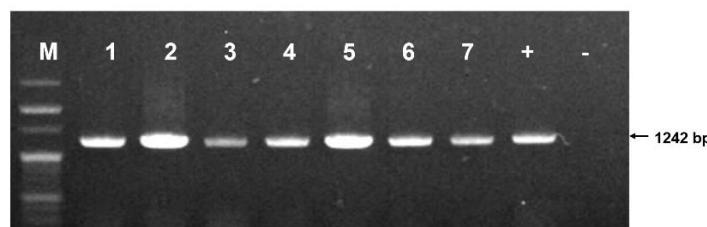
The sequences of the *COI* gene segments of *C. lactis* mites amplified from different regions were sequenced and compared with the *COI* gene of this mite available in the National Center for Biotechnology Information (NCBI). Subsequently, the sequences were aligned using ClustalW multiple alignment in BioEdit version 7.2.5 [29]. A species phylogenetic tree was constructed using MEGA11 based on the nucleotide sequences of the *COI* gene [30]. Simultaneously, a phylogenetic tree was built according to the calculated values obtained from Kimura's two-parameter distance method by neighbour-joining with 1,000 bootstrap replicates and maximum-likelihood method. Three sequences of *C. lactis* mites isolated from China (NCBI accession nos.: NC\_048990.1 and MN073839.1) and the UK (NCBI accession no. KY922482.1) were chosen as representative sequences.

The values were evaluated using the likelihood ratio Chi-square test to measure the strength between the variables of the two groups. Statistical significance was set at  $p < 0.05$ .

### 3. Results

#### 3.1. Molecular Identification of *Carpoglyphus lactis* Mites in Honeybee Colonies

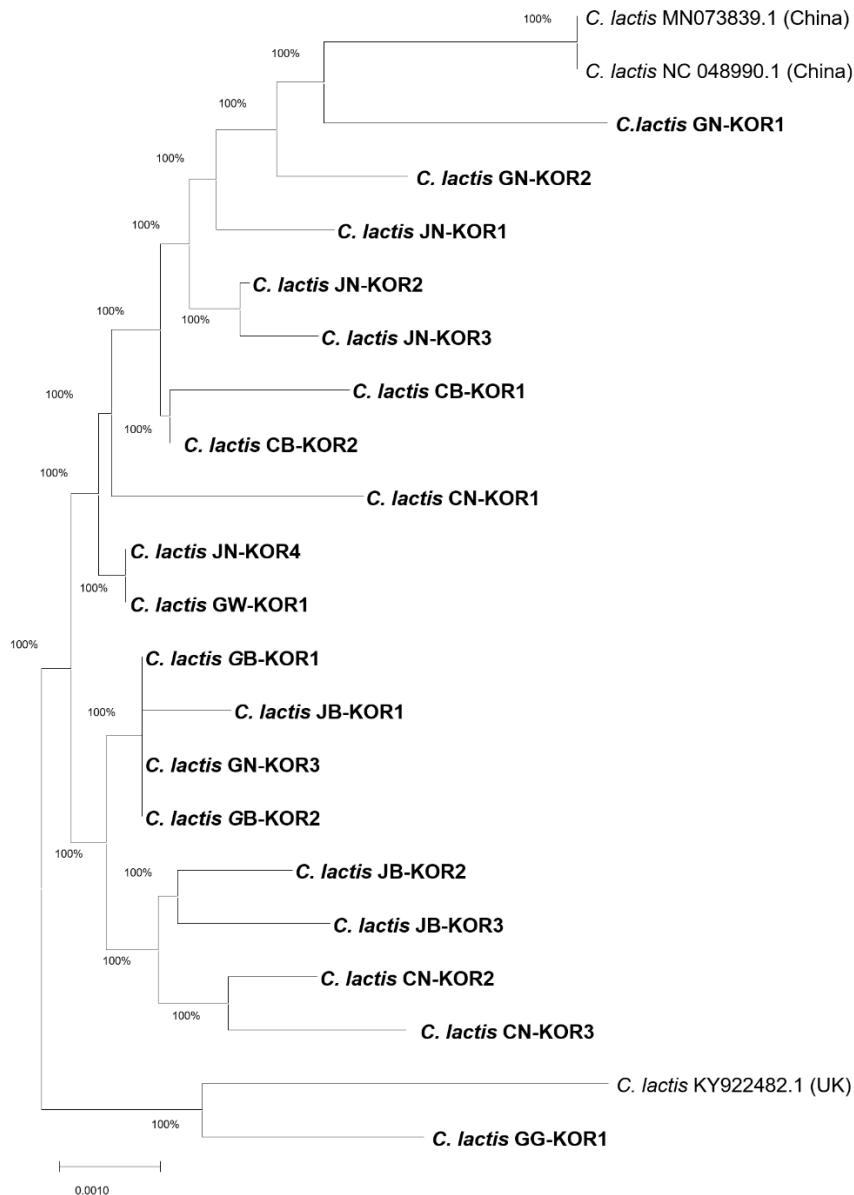
Among 615 honeybee colonies sampled from 124 apiaries, PCR analysis successfully identified the presence of the *C. lactis* mite in a subset of colonies. The characteristic 1,242 bp PCR product confirmed the presence of the mite (Figure 2).



**Figure 2.** Amplification of *COI* regions from *Carpoglyphus lactis* mites in honeybee samples. PCR products were confirmed on 1% agarose gel. Lane M is a 100 bp DNA ladder (Enzymomic, ROK);

lanes 1 and 8 showcase PCR products of the honeybee samples. Lane “-” indicates a negative control without a DNA template.

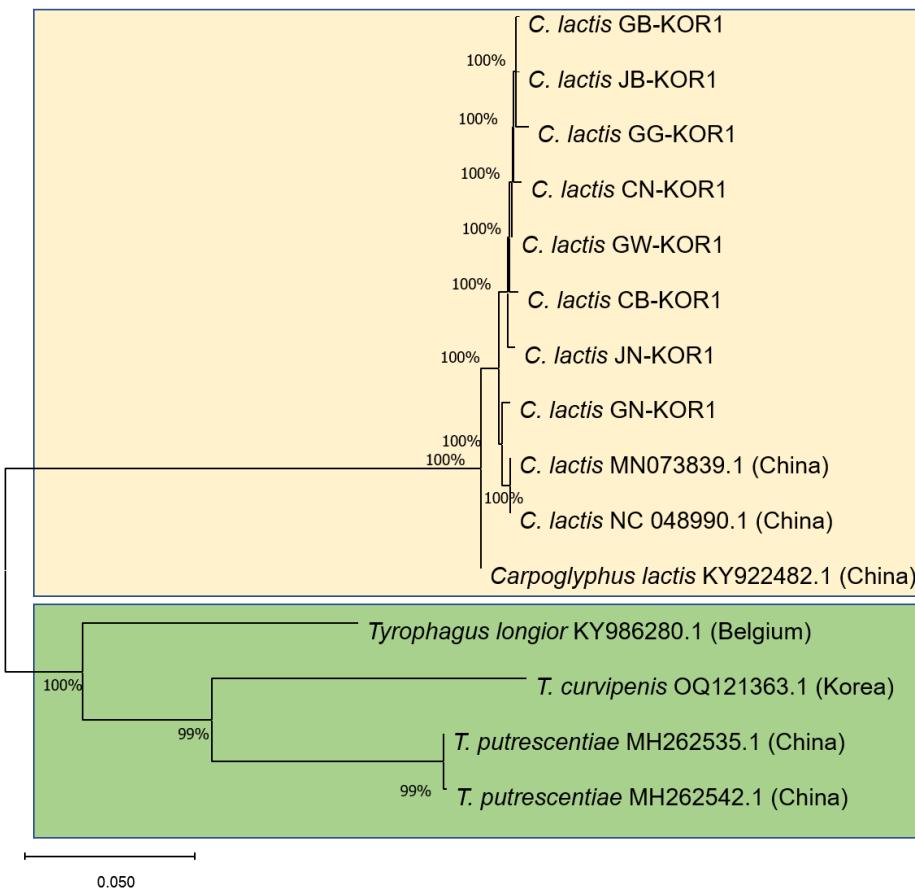
Comparative analysis of the gene segment sequences of *C. lactis* mites compared with the GenBank database (blast.ncbi.nlm.nih.gov/Blast.cgi) unveiled a significant level of nucleotide similarity (99.1~99.5%) between those from this study and those from China (NCBI accession No. NC\_048990.1) and the UK (NCBI accession no. KY922482.1) (Figure S1). Variations in the *COI* gene sequence of *C. lactis*, involving A to G and C to T at identical positions from the UK, were identified. These mutation positions exhibited remarkable consistency among strains isolated nationwide, resembling those from the UK (Figure S1). A phylogenetic tree, constructed by the *COI* gene sequence of *C. lactis* (Figure 3), demonstrated high similarity (99.1~99.5%) between *COI* gene sequences isolated in Korean honeybee colonies and those from the UK and China. Our analysis of the *COI* gene sequence in honeybee samples revealed the widespread presence of *C. lactis* in honeybee colonies across 9 provinces in the ROK.



**Figure 3.** Neighbour-joining phylogenetic tree of *Carpoglyphus lactis* mite based on sequences of *COI* gene (1,137 bp). *C. lactis* mite was isolated from each province of the ROK.

### 3.2. Similarities of COI Gene Sequence of Astigmatid Mites in Honeybee Colonies

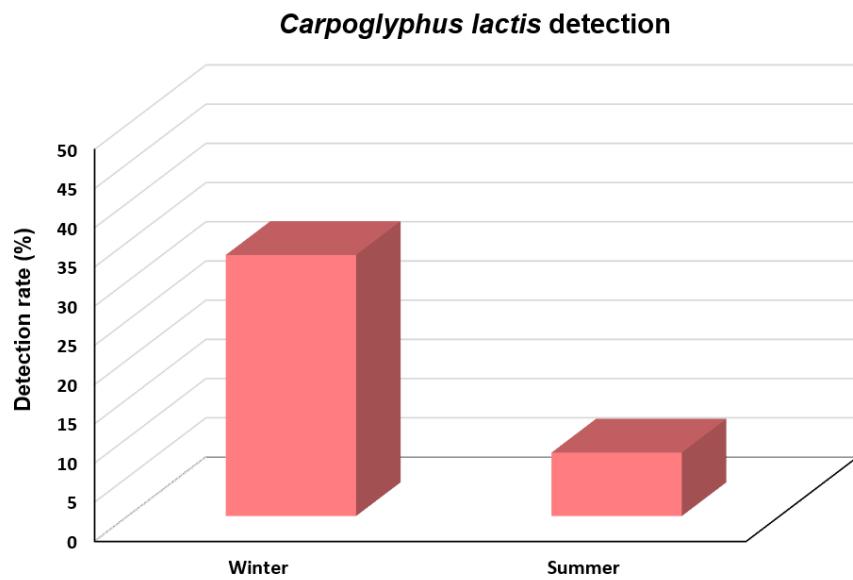
Astigmatid mites are characterized by their tiny size and detrimental impact on honeybee colonies. Species identification was conducted by analysing the phylogeny, utilizing the *COI* gene sequences of *C. lactis* compared to confirm mite species found in honeybee colonies (Figure 4). Our phylogenetic analysis, based on the *COI* gene sequences belonging to two families, aligns closely with current morphological classifications. The mites found in honeybee apiaries belong to the hyporder Astigmatid; however, within this order, the *C. lactis* mite forms a distinct and separate branch with *Tyrophagus* mites. The first branch contains *C. lactis* of the family Carpoglyphidae, while the remaining three species, *T. longior* in Belgium (NCBI accession no. KY986280.1), *T. putrescentiae* in China (NCBI accession nos.: MH262535.1 and MH262542.1), and *T. curvipenis* in ROK (NCBI accession no. OQ121363.1). The nucleotide of *C. lactis* mite isolated from Korean honeybee colonies exhibited a high nucleotide similarity of 80.2% with *Tyrophagus* mites.



**Figure 4.** Phylogenetic maximum-likelihood tree based on sequences of *COI* gene of mites. The species name, NCBI accession numbers, and origin of the accession are given for each sequence. *Carpoglyphus lactis* (*C. lactis*), *Tyrophagus curvipenis* (*T. curvipenis*), and *Tyrophagus putrescentiae* (*T. putrescentiae*).

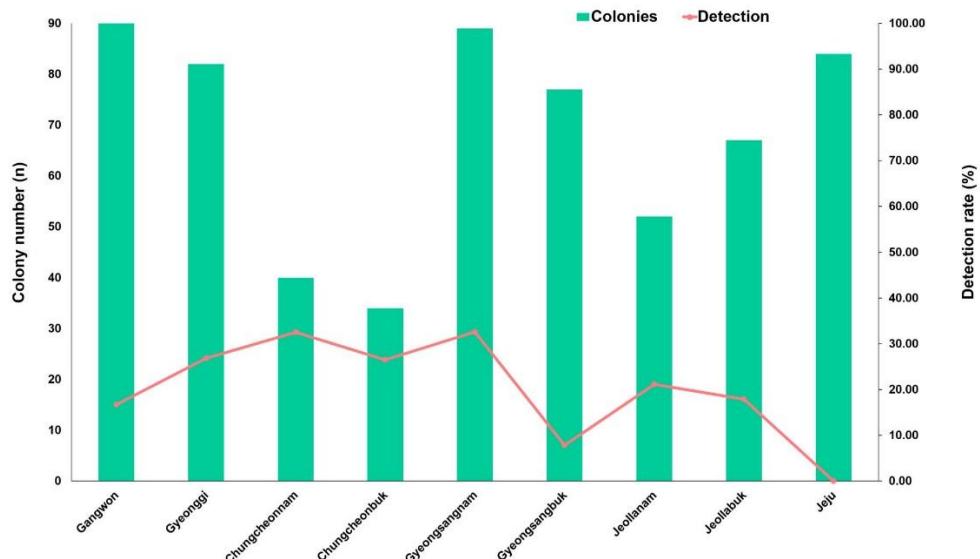
### 3.3. Prevalence of *Carpoglyphus lactis* Mite in Korean Honeybee Colonies

We investigated *C. lactis* mite in honeybee colonies collected during winter and summer in ROK in 2023 (Figure 5). The results indicated a significantly higher detection rate of this mite during winter compared to summer ( $\chi^2 = 62.713$ ,  $df = 1$ ,  $p < 0.001$ ). The detection rate of *C. lactis* in 267 colonies from 66 apiaries collected during winter was 33.3%, whereas this rate during summer was only 8.1% in 348 colonies from 58 apiaries. The highest detection rate was observed in March, with the prevalence rate of 74.7% among the 75 honeybee colonies collected from 23 apiaries.



**Figure 5.** *Carpoglyphus lactis* detection rate during the winter and summer of 2023 in Korean honeybee colonies.

As shown in Figure 6, *C. lactis* mites were found in 8 out of 9 provinces. This mite was not detected in any of the surveyed apiaries in the Jeju province. The detection rates of *C. lactis* in different provinces were as follows: Gangwon at 16.7% (15/90), Gyeonggi at 26.8% (22/82), Chungcheongbuk at 26.5% (9/34), Chungcheongnam at 32.5% (13/40), Gyeongsangbuk at 7.8% (6/77), Gyeongsangnam at 32.6% (29/89), Jeollabuk at 17.9% (12/67), and Jeollanam at 21.2% (11/52). The highest infection rate was observed in the provinces of Chungcheonnam and Gyeongsangnam.



**Figure 6.** Detection rate of *Carpoglyphus lactis* mites in honeybee colonies from 9 provinces in the ROK.

#### 4. Discussion

This study investigated the molecular detection and prevalence of *C. lactis* mites in honeybee colonies in the ROK. This finding provides valuable insight into the occurrence, prevalence, and phylogenetic position of *C. lactis* mites in the ROK, highlighting their significance as a potential pest in honeybee colonies.

We employed molecular techniques to identify *C. lactis* mites in honeybee colonies. To achieve this, we designed specific primers targeting the *COI* gene of *C. lactis* mites, with an amplicon size of 1,242 bp, to minimize non-specific binding of PCR (Table 1). Successful amplification of the targeted *COI* gene segment confirmed the presence of *C. lactis* mites in the tested colonies. The subsequent sequencing and comparative analysis of these gene segments with reference sequences in GenBank database revealed a substantial nucleotide similarity with previously isolated *C. lactis* strains from China and the UK, further supporting the accurate identification of the mite species.

Honeybee mites constitute one of the major factors causing harm to honey bee colonies globally. They parasitize bees and act as disease vectors within the population. Consequently, they adversely impact the health and alter the healthy behaviour of honeybees. As the life cycle of the mite occurs within the beehive, the dense population of these parasites can lead to contaminated honeybee products, reduced food resources, and compromised honeybee health. Recently, *T. curvipenis* mite was reported in Korean honeybee apiaries [12]. *C. lactis* and *Tyrophagus* mites have been found in beehives that primarily feed on stored pollen and other organic materials [22,27,31]. In this study, we detected the new *C. lactis* mite in the Korean honeybee in 8 of 9 provinces in the ROK. The nucleotide of *COI* gene of *C. lactis* mite is similar to that of *Tyrophagus* mite as 80.2%. Building on prior research by Dabert et al. [32], our analysis using the *COI* gene further supports the close relationship between *C. lactis* and *Tyrophagus* mites within the suborder Astigmata. Notably, Dabert et al. [32] also found these two species to cluster together in their phylogenetic tree of Arachnid mites, constructed using the 18S rDNA gene. In 2023, Bowman reported that *C. lactis* and *Tyrophagus* mites can co-occur in UK beehives [15]. The spread of diseases within bee populations infested with these parasites is increasing; hence, effective control measures are challenging. Therefore, investigating the types of parasitic mites and assessing their prevalence is crucial to sustain honeybee health and prevent severe propagation of harmful parasites.

The prevalence analysis provided valuable insights into the occurrence and distribution of *C. lactis* mites in honeybee colonies in the ROK. Our survey was conducted in 2023, with samples collected during winter and summer across 9 provinces nationwide. Survey results indicated the presence of this mite species in 8 of 9 provinces, with temporal variations in the infection rate potentially influenced by factors such as environmental conditions and fluctuations in the honeybee population. Our study also revealed a higher infection rate during winter. The initial detection of *C. lactis* in overwintering honeybee colonies was first reported in Germany [27], suggesting a robust development of *C. lactis* mite at lower temperature, particularly within the beehive, making it an ideal habitat for this mite species. This is a significant factor contributing to honeybee colony damage during winter.

Furthermore, we found its significant presence in a considerable portion of the sampled honeybee population in the ROK. The *C. lactis* mites were not detected in honeybee apiaries only in Jeju province among the surveillance areas. In addition, identifying *C. lactis* in both honeybee and beeswax samples indicates that *C. lactis* mites may have a substantial impact on the honeybee health environmentally as well as individually. A more profound investigation into the life cycle of *C. lactis* mites in honeybee colonies is necessary to better understand the effects of mite infestation on honeybee populations and overall hive health. This knowledge will inform timely strategies and protective measures for the sustainability of beekeeping in the ROK.

Although our study successfully identified *C. lactis* mites in Korean honeybee colonies, some limitations warrant consideration. Identifying *C. lactis* mites were not our initial objective, leading to delayed sampling for morphological confirmation during late winter season. Winter sampling focused solely on bees, and excluding mite examination in comb or beehive, thus precluding direct morphological confirmation of *C. lactis*. Summer sampling across different bee stages and materials

also yielded no detections, possibly due to low prevalence in summer or dead mite presence. Further investigation using alternative methods are ongoing. The detected *C. lactis* mites using PCR solely confirms its presence and doesn't necessarily reflect active infection. Further research is needed to assess the impact of different infection levels and potential harm to bee health and honey production. Despite these limitations, our study provides valuable initial insights into the presence of *C. lactis* in Korean apiaries. Further research addressing these limitations will be crucial for understanding its potential impact on honeybee health and developing effective management strategies.

## 5. Conclusions

This study provides the first report of *C. lactis* mites in honeybee colonies within the ROK. Employing molecular detection techniques, we revealed a widespread distribution of *C. lactis* across eight out of nine provinces surveyed. Furthermore, our results demonstrated a seasonal variation in prevalence, with higher detection rates observed during winter. Phylogenetic analysis confirmed the distinct identity of *C. lactis* within the suborder Astigmata and its near relationship with *Tyrophagus* mites. These findings highlight the potential threat posed by *C. lactis* to Korean honeybee health and emphasize the need for further research. Future investigations should focus on elucidating the impact of *C. lactis* infestation on bee colony health and productivity, particularly during winter.

**Supplementary Materials:** The following supporting information can be downloaded at the website of this paper posted on Preprints.org, Figure S1: Multiple alignments of the differing nucleotide positions in the *COI* gene of *Carpoglyphus lactis*. A comparison was conducted between the *COI* sequence (1,137 nucleotides) of *C. lactis* mite isolated from the ROK in this study and the published sequence in GenBank. The highlighted blue frame signified shared nucleotide differences between the isolated strains and those from China (NCBI accession nos.: NC\_048990.1, MN073839.1) and the UK (NCBI accession no. KY922482.1).

**Author Contributions:** Conceptualization, T-T.N., M-S.Y. and Y.S.C.; methodology, T-T.N. and Y.S.C.; software, T-T.N.; Collected honeybee samples and isolated nucleic acids, H-S.L., S.Y.Y., S-J.L. and S-K.S.; writing—original draft preparation, T-T.N.; writing—review and editing, M-S.Y., J.K. and Y.S.C. All authors have read and agreed to the published version of the manuscript.

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**Data Availability Statement:** All data are provided in the manuscript.

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**Conflicts of Interest:** The authors declare no conflicts of interest.

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