Multiplex PCR assay for identifying five species of chigger mites in the Republic of Korea

Hyeon Seung Lee
School of Life Science, Kyungpook National University

Kwang Shik Choi (✉ ksc@knu.ac.kr)
School of Life Science, Kyungpook National University

Method Article

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Abstract

Background

Molecular date of chigger mites, disease vectors of scrub typhus, are lacking; therefore, they are typically identified morphologically. However, owing to the small size of chigger mites, they are easily damaged. Moreover, factors such as spontaneous mutation and the morphological similarity of adults make accurate classification and identification difficult. Therefore, this study aimed to develop a rapid and accurate molecular method for identifying five chigger mite species (*Leptotrombidium pallidum*, *L. palpale*, *L. scutellare*, *L. orientale*, and *Euschoengastia koreaensis*) that transmit scrub typhus in the Republic of Korea.

Methods

Species-specific primers for multiplex polymerase chain reaction (PCR) were designed using the internal transcribed spacer regions of 106 chigger mite samples from five species.

Results

Pieces of multiplex PCR products with unique size were created as follows: *L. palpale*, 933 bp; *L. orientale*, 747 bp; *L. pallidum*, 567 bp; *E. koreaensis*, 487 bp; *L. scutellare*, 244 bp.

Conclusions

A multiplex PCR-based molecular method was successfully developed for identifying five chigger mite species (mainly collected from rodents) that transmit scrub typhus in Korea and are distribute nationwide. This multiplex PCR assay will complement morphological classification, providing an accurate species identification method that will be useful for monitoring chigger mites.

Background

Vector-borne diseases cause more than 700,000 deaths annually and are a global health problem; moreover, the spread of such diseases is acceleration due to globalization and climate change [1–4]. Scrub typhus, a vector-borne infectious diseases, is an acute febrile disease caused by *Orientia tsutsugamushi*, which is transmitted to humans by chigger mites [5, 6]. This disease is a serious public health issue in the Asia-Pacific region, including Korea and Japan [7]. In the Republic of Korea (ROK), scrub typhus was first reported in 1951; thereafter, its occurrence was not reported until 1986, and many patients with this disease have been reported since then [8]. The Korea Centers for Disease Control and Prevention has designated and managed scrub typhus as a Class 3 legal infectious disease since 1994, and > 4,000 patients have been reported annually since 2004 [9].
Chigger mites are an abnormal disease vector that parasitizes only in the larval stage; they progress through a life cycle including eggs, larvae, nymphs, and adults, only requiring animal tissue fluid to grow from the larval stage into nymphs [10–12]. Chigger mites mainly inhabit humid and grassy environments, such as forests, grasslands, and shrubbery, and their spawning behavior is affected by humidity and temperature [8, 13, 14]. Chigger mite numbers tend to increase in spring and autumn, reflecting the pattern of scrub typhus incidence in patients [10, 15, 16].

Although chigger mites are classified according to the classification key established by Ree [17], they are easily damaged owing to their small size, exhibit natural variation, and can only be classified and identified at the larval stage owing to the morphological similarity of adults [18]. Indeed, the presence of morphologically similar species can lead to misidentification [19]. Although > 50,000 species of chigger mites are known, molecular data are available for < 100 species; thus, species are typically identified morphologically rather than by molecular identification [20, 21].

In the ROK, 63 species of chigger mites across 15 genera are known, and accurate species identification and regular monitoring are required to successfully control disease transmitted by these mites [20]. Therefore, in this study, we propose a new molecular method using the ribosomal RNA Internal transcribed spacer (ITS) region of five chigger mite species (Leptotrombidium pallidum, L. palpale, L. scutellare, L. orientale, Euschoengastia koreaensis) that transmit scrub typhus in Korea, are mainly collected from rodents, and are distributed nationwide [22].

**Methods**

**Sample collection and species identification**

Wild rodents were trapped to collect chigger mites. In 2020 (Hapcheon-gun, Gyeongsangnam-do) and 2021 (Gimcheon-si, Gyeongsangbuk-do), chigger mites were collection once a month in March, April, October, and November, with 20 Sherman traps (H.B. Sherman traps, USA) installed at each environment (Fig. 1). The installed trap was recovered after 24 h, and the chigger mites were obtained from the captured wild rodent and transferred to a 2-ml vial containing 70% ethanol. Using the method reported by Lee and Choi [18], the bodily fluid of chigger mites was extracted and used for molecular experiments, whereas the mite body from which the fluid was extracted were used for morphological species identification [17, 23].

**ITS amplification**

Universal primers were designed to identify the sequences of the five species of Korean chigger mites, i.e., L. palliudm, L. palpale, L. scutellare, L. orientale, and E. koreaensis (forward primer: 5’-TCGTAACAAGGTTTCCGTAGG-3’; reverse primer: 5’-CTTTCTTTTCCTCCGCTGAA-3’). Each reaction mixture (25 µL) contained genomic DNA (1 µL), 1×polymerase chain reaction (PCR) buffer (Mg²⁺ plus), forward primer and reverse primer (0.4 µM), 0.2 mM each dNTP, and 0.5 units of Hot start Taq DNA
polymerase (TaKaRa, R007AM). PCR amplification was performed using a Thermal Cycler Dice (TaKaRa, TP350) with the following conditions: 95°C for 5 min; 40 cycles at 95°C for 30 s, 50°C for 50 s, 72°C for 50 s; and final extension at 72°C for 10 min. Each PCR product was separated via electrophoresis on a 1.5% (wt/vol) agarose gel, stained with ethidium bromide (VWR Life Science), and visualized. Subsequently, the nucleotide sequence was analyzed in both directions by Macrogen (Macrogen Daejeon, Korea). The ITS sequence was analyzed using Bioedit v7.2.6.1 [24], and the sequence information was registered in GenBank (accession numbers: *L. pallidum*, OQ129374; *L. palpale*, OQ129376; *L. scutellare*, OQ129378; *L. orientale*, OQ129408; and *E. koreaensis*, OQ129409).

**Species-specific primers**

We designed five multi primer sets consisting of species-specific forward primers and universal reverse primers for the ITS region (Fig. 2; Table 1). Each reaction mixture (25 µL) contained genomic DNA (1 µL), 1× PCR buffer (Mg²⁺ plus), each primer (0.4 µM), 0.2 mM of each dNTP, and 0.5 units of Hot start Taq DNA polymerase (TaKaRa, R007AM). PCR amplification was performed using a Thermal Cycler Dice (TaKaRa, TP350) with the following conditions: 95°C for 5 min; 40 cycles at 95°C for 30 s, 55°C for 50 s, 72°C for 50 s; and final extension at 72°C for 10 min. Each PCR product was separated by electrophoresis on a 2% (wt/vol) agarose gel, stained with ethidium bromide, visualized, and sequenced.

<table>
<thead>
<tr>
<th>Species</th>
<th>Forward primer (5′-&gt;3′)</th>
<th>Universal reverse primer (5′-&gt;3′)</th>
<th>Product length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Leptotrombium palpale</em></td>
<td>AGAAGCTGGGGTGAAAGGTGTT</td>
<td>CTTTCTTTTCCTCCGCTGAA</td>
<td>933</td>
</tr>
<tr>
<td>Leptotrombium orientale</td>
<td>GTGGTGAGGGGCAAAATGAG</td>
<td></td>
<td>747</td>
</tr>
<tr>
<td>Leptotrombium pallidum</td>
<td>CGGCTGCAGAGTAGATTAC</td>
<td></td>
<td>567</td>
</tr>
<tr>
<td>Euschoengastia koreaensis</td>
<td>GGTGGCTGGTGGAAGTACTT</td>
<td></td>
<td>487</td>
</tr>
<tr>
<td>Leptotrombium scutellare</td>
<td>TGCTGGCTTTAGAACTCGCAA</td>
<td></td>
<td>244</td>
</tr>
</tbody>
</table>

**Results And Discussion**

**Molecular species identification**
In total, 106 DNA samples extracted from five species of chigger mites in the ROK were analyzed: *L. pallidum*, 25; *L. scutellare*, 26; *L. palpale*, 20; *L. orientale*, 22; and *E. koreaensis*, 13. The multiplex PCR analysis results for five species are shown in Fig. 3 (*L. palpale*, 933 bp; *L. orientale*, 747 bp; *L. pallidum*, 567 bp; *E. koreaensis*, 487 bp; and *L. scutellare*, 244 bp). The molecular analysis results were consistent with the morphological observation results.

**Application of multiplex PCR molecular methods**

Chigger mites can be classified only in the larval stage through optical microscopic observation owing to the morphological similarity of adults. They are classified according to the Ree classification key [17] based on characteristics such as the shape of galeal seta, shape of scutum, number of strands in humeral seta-H, number of strands in 1st row of posthumeral setae (1st PHS), and location of setae in coxa. However, without substantial experience, the classification is challenging. Additionally, chigger mites are small; hence, they are easily damaged and subject to natural mutations, and extracting their DNA for morphological species identification is difficult. However, the multiple PCR analysis method developed in this study, which incorporates the chigger mite ITS region, is simple and reduces the misrecognition rate; therefore, it represents a new chigger mite classification method that does not rely on morphological species identification. Moreover, the simultaneous application of molecular and morphological species identification methods is possible.

Scrub typhus is transmitted by chigger mites mainly in eastern regions, including South Korea, Southeast Asia, and northern Australia; however, because overseas exchanges have increased, outbreaks have also been reported in South America, including Chile [6, 25–27]. The multiple PCR method developed for molecular-level identification in the present study is expected to be useful for national vector monitoring because it will enable accurate chigger mite species identification, which will in turn help prevent the spread of vector-borne diseases.

**Conclusions**

In this study, five species of chigger mites (*L. pallidum*, *L. palpale*, *L. scutellare*, *L. orientale*, and *E. koreaensis*) that transmit scrub typhus in ROK and are distributed nationwide were collected from rodents and used to develop a new molecular method for the identification of these species. This new multiplex PCR analysis method enables accurate chigger mite classification, which previously relied only on morphological classification. This study is the first in which molecular species identification of chigger mites was achieved through multiplex PCR in ROK, and this method could be used as a surveillance tool to monitor the distribution of chigger mites transmitting scrub typhus.

**Abbreviations**

PCR: polymerase chain reaction; ROK: Republic of Korea; ITS: internal transcribed spacer; PHS: row of posthumeral setae
Declarations

Acknowledgments

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Funding

Not applicable.

Availability of data and materials

The sequences used in this study are deposited in the NCBI GenBank database (https://www.ncbi.nlm.nih.gov/genbank/) under accession numbers OQ129374, OQ129376, OQ129378, OQ129408, and OQ129409.

Author's contributions

HSL design the research, developed the new multiplex PCR method, and drafted the manuscript. KSC assisted with data analysis and improved the manuscript. Both authors have approved the final manuscript.

Ethics declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Author information

Affiliations

1School of Life Science, Kyungpook National University, Daegu, Korea.

Hyeon Seung Lee and Kwang Shik Choi.

2Research Institute for Dokdo and Ulleungdo Island, Kyungpook National University, Daegu, Korea.

Hyeon Seung Lee and Kwang Shik Choi.
Research Institute for Phylogenomics and Evolution, Kyungpook National University, Daegu, Korea.

Kwang Shik Choi.

Corresponding author

Correspondence to Kwang Shik Choi.

References


**Figures**
Figure 1

Wild rodent collection sites for chigger mites (A: Mountain path, B: Reservoir, C: Rice paddy, D: Field, E: Ditch)
**Figure 2**

Figure 3

Results of multiplex PCR analysis for five species of chigger mites. Lanes: M 100 bp molecular marker, 1 *Leptotrombidium palpale* (933 bp), 2 *Leptotrombidium orientale* (747 bp), 3 *Leptotrombidium pallidum* (567 bp), 4 *Euschoengastia koreaensis* (487 bp), 5 *Leptotrombidium scutellare* (244 bp)