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Molecular Epidemiology of an *Orientia tsutsugamushi* Gene Encoding a 56-kDa Type-Specific Antigen in Chiggers, Small Mammals, and Patients from the Southwest Region of Korea

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Abstract. A phylogenetic analysis of *Orientia tsutsugamushi* was performed to elucidate its antigenic diversity in chiggers, small mammals, and patients. Between September 2014 and December 2016, a total of 3,816 chiggers were identified within nine species of four genera in the southwest region of Korea: *Leptotrombidium scutellare* (49.9%; 1,907/3,816), *Leptotrombidium orientale* (21.1%; 804/3,816), *Leptotrombidium pallidum* (12.4%; 474/3,816), *Euchoengastia koreaensis* (7.2%; 273/3,816), *Leptotrombidium palpale* (6.7%; 256/3,816), *Neotrombicular gardellai* (1.3%; 50/3,816), *Leptotrombidium zetum* (0.8%; 32/3,816), *Walchia fragilis* (0.5%; 18/3,816), and *Neotrombicular japonica* (> 0.1%; 2/3,816). Twelve chiggers (11 *L. scutellare* and one *L. palpale*) tested positive for *O. tsutsugamushi* by polymerase chain reaction and, except for 1 chigger (KY266830), were part of the Boryong strain cluster. Of the 413 small mammals that were analyzed for *O. tsutsugamushi*, *Apodemus agrarius* was the most common rodent species (89.5%; 370/413), followed by *Crocidura lasiura* (6.8%; 28/413) and *Myodes regulus* (3.6%; 15/413). The sequence identity of an *O. tsutsugamushi* sample obtained from the *A. agrarius* sample population belonged to the Saitama strain cluster. Furthermore, a phylogenetic analysis in 125 patients revealed four clusters (Boryong cluster: 82.4% [103/125], Karp: 13.6% [17/125], Kawasaki: 3.2% [4/125], and Saitama: 0.8% [1/125]). This study clarified the phylogenetic relationship for *O. tsutsugamushi* in chiggers, small mammals, and patients. The Boryong strain was the most common strain in chiggers and patients. In addition, various strains were identified, except for the Boryong strain, in the southwest region of Korea. Overall, the data presented here will be helpful for the establishment of prevention strategies for scrub typhus.

INTRODUCTION

Orientia tsutsugamushi is a rod-shaped, gram-negative, intracellular bacterium that belongs to the order Rickettsiales and is the causative agent of scrub typhus or Tsutsugamushi disease.[1,2] Unlike other bacteria, the cell walls of *O. tsutsugamushi* lack a peptidoglycan and lipopolysaccharide layer. Scrub typhus has been recognized as a major cause of acute febrile disease that is encountered in rural regions such as bushes and abandoned grain field.[3] This disease is endemic to the Western Pacific region, northern Australia, and Central Asia. Clinical characteristics of scrub typhus include eschar, fever, rash, meningitis, intracellular coagulation, lymphadenopathy, and multiple organ failure. Moving forward, if patients are not treated, the fatality rate will continue to increase.[4]

Furthermore, *O. tsutsugamushi* can be transmitted by the larval trombiculid mite (chigger) while feeding, which then can propagate to its progeny through transovarian transmission. The *Leptotrombidium* species are known to act as the primary vector for *O. tsutsugamushi*,[5,6] where the major species, *Leptotrombidium pallidum* and *Leptotrombidium scutellare*, can be found in the central and southern regions of Korea, respectively.[7,8]

Wild rodents, such as *Apodemus agrarius*, are also natural hosts of scrub typhus as well as for chiggers.[9,10] At the same regions where this study was performed, a serosurveillance for scrub typhus in small mammals was performed in 2014–2015 by Park, and a seropositive rate of 25% (37/145) in *A. agrarius* was observed.[11]

The 56-kDa type-specific antigen (TSA) is an immunodominant protein located on the surface of *O. tsutsugamushi* that is not expressed in other bacteria. Therefore, phylogenetic analysis of the 56-kDa TSA gene sequence is useful to clarify variations of scrub typhus.[12–15]

Worldwide, more than 20 variations of scrub typhus are caused by various prototype strains of *O. tsutsugamushi*. The Boryong strain is predominant in Korea. The Giliam, Karp, Kato, TA678, TA686, TA716, TA763, and TH1817 strains have been reported in Southeast Asia, Australia, and Taiwan. In addition, the Gilliam, Karp, Kato, Kawasaki, Kuroki, and Shimokoshi strains have been found in Japan.[1] Furthermore, differences in virulence according to genotype variations of *O. tsutsugamushi* have been identified.[16]

Recently, climate change may have affected the life cycle of wild rodent and chiggers.[17] Therefore, clarifying the relationship between the vector and environment may help predict future cases of scrub typhus.[18–23] In northern China, a phylogenetic analysis for scrub typhus in chiggers, rodents, and patients was conducted, and consistency of circulating strains in the hosts was demonstrated.[24]

According to the Korea Centers for Disease Control and Prevention, 10,000 cases of scrub typhus were reported annually between 2013 and 2015, as well as 6,000 cases in 2005. The distribution of chiggers has also expanded from the southern region to the central region for the past decade.[25] Therefore, scrub typhus is an increasingly important disease in Korea.[26–29]

For the development of strategies to control this disease, a better understanding of the vector's life cycle is required as well as knowledge of the *O. tsutsugamushi* strain in the host.

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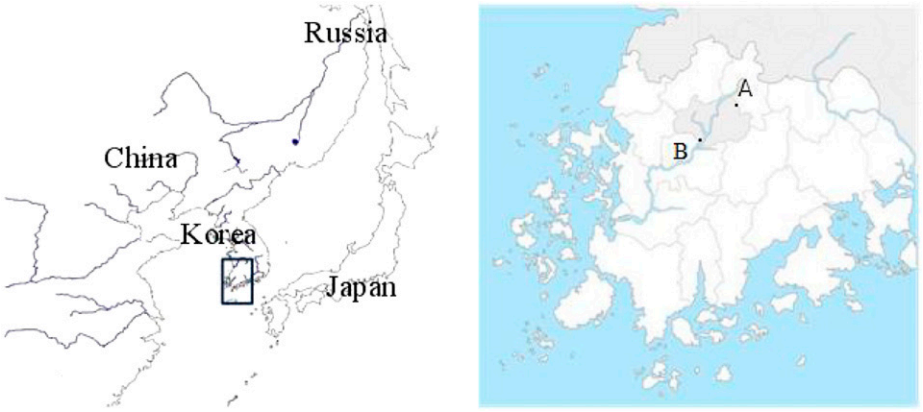

FIGURE 1. Small mammals collection sites at A (35°13′51.7″ N, 126°54′23.8″ E) and B(35°09′19.2″ N, 126°45′05.4″ E) in the southwest area of Korea, from September 2014 to December 2016. This figure appears in color at www.ajtmh.org.

We surveyed a distribution of chiggers and performed a phylogenetic analysis of the 56-kDa TSA gene sequence to identify the genetic characteristics of *O. tsutsugamushi* in chiggers, small mammals, and patients in the southwest region of Korea.

MATERIALS AND METHODS

Collection of small mammals and chiggers. All procedures were conducted under an animal use protocol approved by the Chosun University Animal Ethics Committee. From September 2014 to December 2016, small mammals were trapped monthly using Sherman live traps (3 in × 3.5 in × 9 in, USA) baited with peanut butter–covered biscuits in two southwest regions of Korea (Figure 1). After wild rodents were euthanized, their organs (spleen, kidney) were collected for detection of *O. tsutsugamushi*. Chiggers collected from the rodents were stored in 70% ethyl alcohol until used to acquire sample fluids for DNA extraction.

Patients' blood sample. From 2014 to 2015, blood samples were collected from patients who had visited Chosun University Hospital with suspected scrub typhus in the

TABLE 1

Chigger infestation rates (%) on small mammals (number of chigger infested small mammals/number of captured small mammals) in the southwest area of Korea, from September 2014 to December 2016

	Rodents		Shrew	
Month	*Apodemus agrarus*	*Myodes regulus*	*Crocidura lasiura*	Total (chigger infested/captured small mammals)
September 2014	9/10	–	–	9/10
October	12/12	–	–	12/12
November	18/22	–	0/1	18/23
December	0/5	1/1	0/1	1/7
January 2015	8/14	3/3	0/1	11/18
February	3/7	1/1	–	4/8
March	5/10	1/1	–	6/11
April	3/9	1/1	–	4/10
May	11/22	1/1	0/3	12/26
June	2/16	–	–	2/16
July	0/18	–	–	0/18
August	4/13	–	–	4/13
September	8/10	–	0/1	8/11
October	15/15	–	1/1	16/16
November	17/17	1/1	–	18/18
December	14/15	–	–	14/15
January 2016	4/6	1/1	–	4/7
February	7/8	–	–	7/8
March	12/13	1/1	0/1	13/15
April	9/10		0/2	9/12
May	6/25	1/1	0/1	7/27
June	0/22	–	–	0/22
July	0/13	–	0/1	0/14
August	0/7	–	–	0/7
September	6/12	1/1	0/1	7/14
October	5/5	–	1/5	6/10
November	12/12	–	0/4	12/16
December	8/25	2/2	0/5	10/32
Total	198/370 (53.5)	15/15 (100)	1/28 (3.5)	214/413 (51.8)

TABLE 2
Species of chiggers collected from small mammals captured in the southwest area of Korea, from September 2014 to December 2016

Host species	Number of chiggers collected (%)									Total
	Leptotrombidium scutellare	*Leptotrombidium pallidum*	*Leptotrombidium orientale*	*Leptotrombidium palpale*	*Leptotrombidium zetum*	*Neotrombicular gardellai*	*Neotrombicular japonica*	*Euchoengastia koreaensis*	*Walchia fragilis*	
Rodents										
Apodemus agrarius	1,866	474	585	237	30	50	2	268	–	3,512 (92.0)
Myodes regulus	38	–	219	19	2	–	–	5	18	301 (7.9)
Shrews										
Crocidura lasiura	3	–	–	–	–	–	–	–	–	3 (> 0.1)
Total	1,907 (49.9)	474 (12.4)	804 (21.1)	256 (6.7)	32 (0.8)	50 (1.3)	2 (> 0.1)	273 (7.2)	18 (0.5)	3,816 (100)

southwest region of Korea. Blood sample analysis was blinded. All experiments were conducted under approval of the Chosun University Medical College (IRB number: 2013-10-001).

Acquisition of fluids from chiggers and species identification. The fluids from the engorged chiggers were acquired as previously described.[30] The chigger's internal contents (organ, fluid, and hemolymph) were squeezed out with two fine pins under a stereomicroscope (Carl Zeiss, Oberkochen, Germany). The chigger exoskeleton was identified by Ree's fauna key.[31]

DNA extraction from chigger samples. DNA from the chigger samples were purified using the G-spin Total DNA Extraction Kit (Intron Biotechnology, Seoul, Korea). The DNA from the rodent's organs as well as the patients' blood was purified using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. DNA was stored at –20°C until use.

Nested polymerase chain reaction (PCR) amplification for the 56-kDa TSA gene. PCR was performed via the INNOPLEX TSUTSU detection kit (catalogue number: IPC10040; Intron Biotechnology). The kit was designed using

TABLE 3
Monthly distribution of chigger species (positive chigger for *Orientia tsutsugamushi*) collected from small mammals captured in the southwest area of Korea

Month	*Leptotrombidium pallidum*	*Leptotrombidium scutellare*	*Leptotrombidium palpale*	*Leptotrombidium orientale*	*Leptotrombidium zetum*	*Neotrombicular gardellai*	*Neotrombicular japonica*	*Euchoengastia koreaensis*	*Walchia fragilis*	Total
September 2014	–	56	–	2	1	27	–	68	–	154
October	12	257 (2)	–	2	–	–	–	7	–	278 (2)
November	17	265	15	70	–	1	–	12	–	380
December	–	6	1	12	1	–	–	–	–	20
January 2015	1	1	35	105	3	–	–	1	–	146
February	–	–	7	52	2	–	–	–	–	61
March	5	–	–	60	1	–	–	–	–	66
April	–	–	–	62 (1)	–	–	–	–	–	62
May	1	–	1	68	–	–	–	9	–	79
June	–	–	–	2	–	–	–		–	2
July	–	–	–	–	–	–	–		–	
August	–	–	–	8	–	–	–	2	–	10
September	1	56	–	3	–	5	–	30	–	95
October	41	272 (2)	–	4	1	2	–	87	–	407
November	45	378	–	19	–	–	1	4	–	447
December	65	40	84	25	–	–	–	2	–	216
January 2016	9	–	57	57	–	–	–	3	–	126
February	31	–	15	34	11	–	–	2	–	93
March	102	–	10	98	10	–	–	8	–	228
April	44	–	24	74	–	–	–	5	–	147
May	10	–	–	7	–	–	–	–	18	35
June	–	–	–	–	–	–	–	–	–	–
July	–	–	–	–	–	–	–	–	–	–
August	–	–	–	–	–	–	–	–	–	–
September	19	42	–	2	–	15	–	6	–	84
October	1	132 (6)	–	–	–	–	–	18	–	151
November	51	258 (1)	3	4	–	–	1	9	–	326
December	19	144	4	34	2	–	–	–	–	203
Total	474	1,907 (11)	256	804 (1)	32	50	2	273	18	3,816 (12)

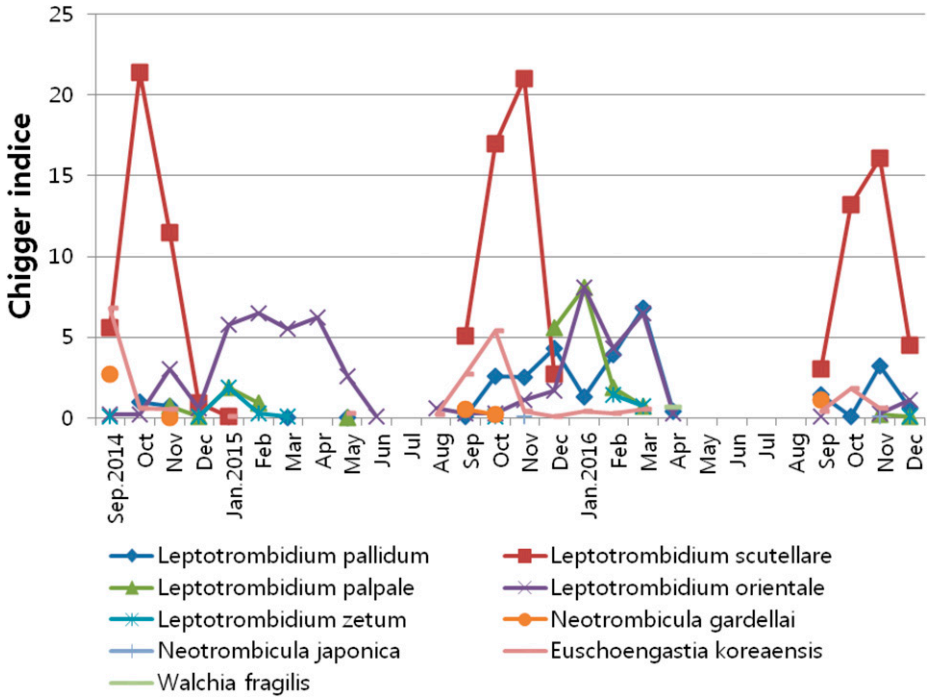

FIGURE 2. Monthly chigger indices by species collected from small mammals captured in the southwest area of Korea, from September 2014 to December 2016. This figure appears in color at www.ajtmh.org.

primer sets (First F: 5′-GCA ATA TTG CTA GTG CAA TGT CTG C-3′, First R: 5′-ATG CAT GCA TGR CGC TKC AAT TTA-3′; Second F: 5′-ATA GGC CTA TAA GTA TWG CKG ATC G-3′, Second R: 5′-CAT CTA GAY GCA CTA TTA GGC AAA-3′) to detect the 56-kDa TSA gene of *O. tsutsugamushi*. The target was a 426–862-bp region that includes variable domain 2, 3 in the total TSA 56 gene (1,566 bp). The first PCR was amplified at 94°C for 5 minutes, followed by 40 cycles at 94°C for 30 seconds, 58°C for 30 seconds, and 72°C for 40 seconds, and a final elongation at 72°C for 5 minutes. The second PCR was amplified at 94°C for 5 minutes, followed by 30 cycles at 94°C for 30 seconds, 58°C for 30 seconds, 72°C for 40 seconds, and a final elongation at 72°C for 5 minutes using the GeneAmp 9700 Biosystem (ABI, California). The final 475-bp PCR products were evaluated by 1.5% agarose gel electrophoresis and visualized with ethidium bromide and an ultraviolet transilluminator.

Sequence and phylogenetic analysis. The amplified PCR products were sent to Cosmogenetech (Daejeon, Korea) for sequencing using the ABI 3730XL DNA Analyzer (Applied Biosystems, Foster City, CA). The nucleotide sequences were aligned using ClustalW to a reference sequence downloaded from the National Center for Biotechnology Information database, and phylogenetic analyses were conducted via the MEGA6 program. A neighbor-joining tree with 1,000 bootstrap replicates was constructed using the Kimura's two-parameter model. Sequences obtained from chiggers, rodents, and human samples were submitted to GenBank (accession number:

TABLE 4
Distribution of *Orientia tsutsugamushi* strains in chigger, small mammals, and humans in the southwest area of Korea

			Cluster				
			Boryong	Kawasaki	Saitama	Karp	Etc.
Host	Test	Positive (%)	114	4	2	17	1
Chiggers	3,816	12 (0.3)	11	–	–	–	1
Leptotrombidium scutellare	1,907	11 (0.6)	10	–	–	–	1
Leptotrombidium pallidum	474	–	–	–	–	–	–
Leptotrombidium orientale	256	1 (0.4)	1	–	–	–	–
Leptotrombidium palpale	804	–	–	–	–	–	–
Leptotrombidium zetum	32	–	–	–	–	–	–
Neotrombicular gardellai	50	–	–	–	–	–	–
Neotrombicular japonica	2	–	–	–	–	–	–
Euchoengastia koreaensis	273	–	–	–	–	–	–
Walchia fragilis	18	–	–	–	–	–	–
Small mammals	413	1 (0.2)	–	–	1	–	–
Apodemus agrarius	370	1 (0.3)	–	–	1	–	–
Myodes regulus	15	–	–	–	–	–	–
Crocidura lasiura	28	–	–	–	–	–	–
Human	402	125 (50.5)	103	4	1	17	–

KY266824-KY266830, KX363954, and KY946003-KY946128, respectively).

RESULT

Small mammal collection and chigger infestation rate. During the study period, 413 small mammals, including two species of rodents and one species of shrew, were captured. *Apodemus agrarius* were the most common small mammals (370; 89.5%), followed by *Crocidura lasiura* (28; 6.8%) and *Myodes regulus* (15; 3.6%). Furthermore, *A. agrarius* were captured year-round. The infestation rate of chiggers on the small mammals was 51.8% (214/413), *A. agrarius* 53.5% (198/370), *M. regulus* 100% (15/15), and *C. lasiura* 3.5% (1/28). *Crocidura lasiura* were infested with fewer chiggers than other small mammals. The infestation rate for *M. regulus* was 100% during the study period (Table 1).

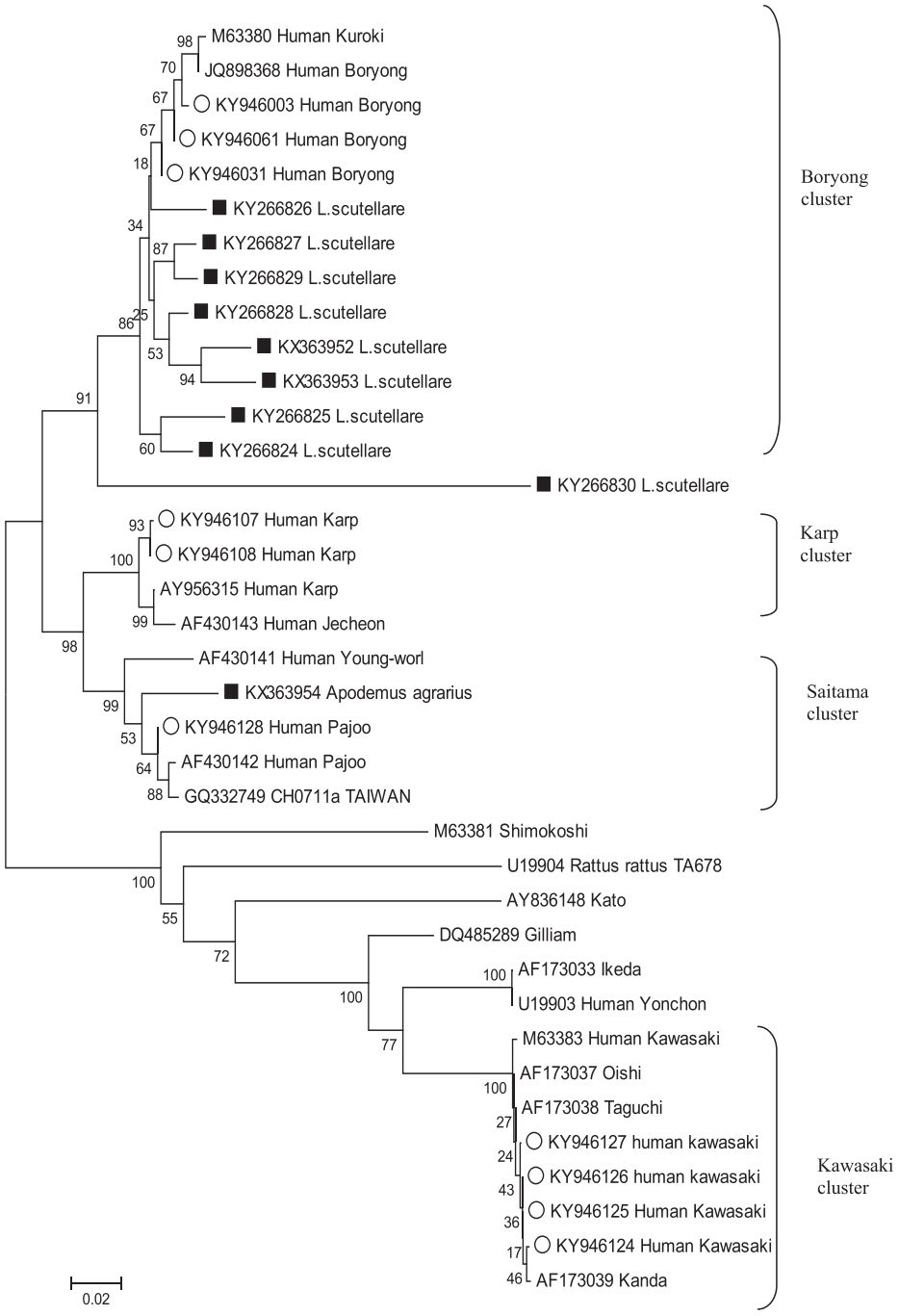

FIGURE 3. Phylogenetic tree of *Orientia tsutsugamushi* based on the 56-kDa type specific gene sequence detected in chiggers; closed rectangle ■ (KX363952, KX363953, KY266824-KY266830), rodent (KX363954), human; open circle ○ (KY946003, KY946031, KY946061, KY946107, KY946108, KY946128, KY946124-KY946127). Phylogenetic tree was constructed by neighbor-joining method with the Kimura's two-parameter model (bootstrap 1,000) using MEGA 6.0. Genbank accession numbers of *O. tsutsugamushi* are indicated for each sequence.

TABLE 5

Identity matrix of *Orientia tsutsugamushi* strains in chigger, small mammals, and humans in the southwest area of Korea

		Percent identity																			
Divergence		**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**
	1	–	92.1	92.2	76.7	67.1	67.0	67.0	67.0	85.6	87.4	88.3	93.7	73.8	70.9	72.4	70.0	82.7	66.1	71.8	81.2
	2	0.0	–	99.8	76.7	73.8	74.5	73.8	74.9	83.4	85.2	85.6	86.6	66.4	64.1	65.2	62.8	80.5	58.8	75.8	85.4
	3	0.0	0.0	–	76.5	74.0	74.7	74.0	74.7	83.2	85.0	85.7	86.8	66.6	64.3	65.3	63.0	80.3	59.0	76.0	85.6
	4	8.4	8.9	8.9	–	59.6	59.4	58.7	59.7	82.3	80.9	81.6	71.1	57.2	57.0	57.9	55.1	84.1	51.8	60.5	76.4
	5	34.7	34.7	34.6	37.5	–	99.3	98.4	98.9	64.6	66.2	67.7	61.7	81.6	82.9	91.2	68.4	62.6	77.6	70.6	67.9
	6	34.2	34.3	34.2	37.0	0.2	–	99.1	99.6	64.4	66.1	67.5	61.6	81.4	82.3	90.6	67.7	62.5	77.1	71.3	68.6
	7	33.9	34.3	34.1	36.9	0.2	0.0	–	98.9	63.7	65.3	67.5	61.6	81.4	82.3	90.4	67.5	61.7	77.1	71.3	69.1
	8	33.9	33.9	33.9	36.5	0.5	0.2	0.0	–	64.8	66.4	67.5	61.6	81.4	82.3	90.3	67.3	62.8	77.1	71.3	68.4
	9	8.6	9.2	9.2	5.5	36.5	36.1	36.0	35.7	–	98.2	84.8	82.9	68.8	64.1	67.3	64.4	96.0	62.1	64.4	79.4
	10	8.4	9.0	9.0	5.5	35.9	35.4	35.4	35.1	0.0	–	86.5	84.5	70.6	65.7	69.0	66.1	94.4	63.5	66.1	81.0
	11	9.5	9.5	9.4	0.3	34.8	34.3	34.1	34.1	6.1	6.2	–	84.8	71.3	69.7	68.6	67.3	82.5	64.4	72.0	89.9
	12	1.0	0.0	0.0	8.7	34.7	34.2	33.9	33.9	10.1	9.7	9.2	–	75.3	71.1	69.5	73.6	80.9	70.9	66.6	75.5
	13	25.7	28.0	27.9	31.5	11.1	10.6	10.4	10.4	27.5	26.8	26.7	24.5	–	91.2	89.7	78.2	66.4	89.2	65.0	62.6
	14	31.3	33.9	33.8	33.2	10.0	10.1	9.8	9.8	32.1	31.6	30.6	30.9	7.7	–	88.1	77.4	61.7	94.8	65.5	61.2
	15	31.9	34.7	34.6	34.6	0.2	0.2	0.2	0.5	34.2	33.7	32.5	31.9	10.6	9.3	–	76.5	65.9	85.9	65.7	62.5
	16	30.2	32.6	32.5	33.5	25.9	26.5	26.6	27.0	34.1	33.6	31.6	29.4	23.5	23.9	23.7	–	63.0	76.2	66.6	59.4
	17	8.9	9.5	9.5	5.5	36.0	35.5	35.4	35.1	1.3	1.1	5.5	9.1	27.4	32.2	32.5	32.4	–	61.7	62.3	77.1
	18	31.2	33.9	33.8	33.2	10.0	10.1	9.8	9.8	33.1	32.3	30.6	29.8	7.5	0.0	9.4	23.3	31.1	–	60.3	56.0
	19	31.2	31.5	31.4	33.8	35.6	35.1	34.9	34.9	35.8	35.2	32.0	30.8	32.0	32.1	34.8	28.2	35.6	32.1	–	71.8
	20	14.7	12.2	12.1	2.8	37.4	37.0	37.5	37.1	9.1	9.2	5.1	15.2	35.6	40.0	40.5	39.8	8.6	40.0	37.0	–
	21	**1.0**	**0.0**	**0.0**	**8.7**	**34.7**	**34.2**	**33.9**	**33.9**	**10.1**	**9.7**	**9.0**	**0.2**	**24.6**	**30.6**	**31.9**	**29.4**	**9.1**	**29.3**	**30.8**	**15.2**
	22	10.2	10.5	10.5	3.4	34.8	34.3	34.1	34.1	8.6	8.4	3.4	10.1	25.5	29.7	31.0	29.2	7.3	28.8	32.0	8.0
	23	27.0	28.3	28.3	29.7	30.3	29.8	29.7	29.3	28.2	28.2	28.6	27.0	28.6	31.5	28.2	25.1	27.8	30.9	31.4	33.3
	24	33.3	34.7	34.6	36.2	0.2	0.2	0.2	0.5	35.5	34.9	32.6	32.6	10.7	9.4	0.0	24.1	34.9	9.4	34.8	40.5
	25	32.5	35.0	35.0	34.1	0.5	0.0	0.0	0.2	34.0	33.9	33.5	32.3	10.4	9.8	0.2	25.3	31.9	9.8	35.9	37.8
	26	32.5	35.0	35.0	34.1	0.5	0.0	0.0	0.2	34.2	33.9	33.5	32.2	10.4	9.8	0.2	25.3	31.7	9.8	35.9	37.8
	27	9.3	9.5	9.4	5.8	35.9	35.5	35.2	35.2	1.9	1.7	5.9	9.2	24.2	30.2	32.0	31.4	0.9	29.5	34.5	12.1
	28	9.5	10.0	10.0	0.8	35.2	34.8	34.5	34.5	7.6	7.5	0.6	9.5	26.3	30.8	31.5	31.2	5.9	30.1	32.7	5.6
	29	5.6	4.1	4.1	14.5	35.9	35.5	35.2	35.6	14.0	13.7	15.9	6.0	31.9	38.0	36.6	37.0	14.5	38.0	36.2	17.1
	30	**4.1**	**2.1**	**2.1**	**12.0**	**27.2**	**36.8**	**36.9**	**36.9**	**11.8**	**11.5**	**14.4**	**4.8**	**32.8**	**38.9**	**39.5**	**37.5**	**12.2**	**38.9**	**36.0**	**13.3**
	31	39	1.9	1.9	11.6	36.9	36.5	36.7	36.6	11.5	11.3	14.5	4.6	33.2	39.4	39.3	38.0	11.9	39.4	35.9	14.7
	32	3.0	1.6	1.6	11.4	35.5	35.1	34.9	35.2	11.3	11.1	12.6	3.2	30.1	36.0	36.0	35.5	11.7	36.0	34.3	14.0
	33	4.8	1.9	1.9	11.7	36.3	35.9	36.4	36.0	11.6	11.3	15.0	4.8	32.9	39.1	40.0	38.4	12.0	39.1	36.1	14.9
	34	25.9	22.8	23.1	33.8	57.0	56.4	56.6	56.1	30.4	29.7	35.4	26.9	53.4	60.0	59.6	55.1	31.6	60.0	54.7	32.3
	35	3.9	2.1	2.1	11.8	36.8	36.4	36.6	36.5	11.9	11.7	14.2	6.5	34.5	41.9	38.1	40.8	12.3	43.9	35.9	14.3
	36	6.0	4.3	4.2	14.6	38.4	37.9	38.5	38.1	14.3	14.0	16.6	8.9	36.0	44.1	39.6	44.0	14.8	45.7	38.4	15.3
	37	6.3	4.5	4.5	15.0	38.9	38.4	39.0	38.6	14.7	14.3	16.9	9.1	35.3	43.6	40.0	43.5	15.2	45.7	38.9	15.9
	–	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**

(continued)

Monthly species distribution of chiggers. A total of 3,816 chiggers were collected from 214 small mammals. *Leptotrombidium scutellare* was the most commonly collected species (1,907; 49.9%), followed by *Leptotrombidium orientale* (804; 21.1%), *L. pallidum* (474; 12.4%), *Euchoengastia koreaensis* (273; 7.2%), *Leptotrombidium palpale* (256; 6.7%), *Neotrombicula gardellai* (50; 1.3%), *Leptotrombidium zetum* (32; 0.8%), *Walchia fragilis* (18; 0.5%), and *Neotrombicular japonica* (2, < 0.1%). Regarding distribution, 3,512 (92.0%) chiggers were found on *A. agrarius*, followed by 301 on *M. regulus* (7.9%), and 3 (< 0.1%) on *C. lasiura*. Eight species of chiggers were observed on *A. agrarius*. Furthermore, *W. fragilis* was only collected from *M. regulus* (Table 2).

Monthly fluctuation of chiggers by host is presented in Table 3 and Figure 2. We observed seasonal differences in chigger species. During the autumn season from September to December, the predominant species was *L. scutellare* whereas, during the spring season between March and May, the predominant species was *L. orientale*.

Prevalence of *O. tsutsugamushi* in samples. Individual nested PCRs were used to screen 3,816 chiggers for *O. tsutsugamushi*. A total of 12 chiggers (0.3%; 12/3,816) were positive for *O. tsutsugamushi*, and 11 samples of *L. scutellare* and one *L. orientale* were confirmed. Furthermore, one *A. agrarius* specimen from the 413 small mammals tested was confirmed to have *O. tsutsugamushi* (0.2%; 1/413). Finally, 125/402) of the patients with suspected scrub typhus tested positive (Table 4).

Phylogenetic analysis for the 56-kDa TSA gene of *O. tsutsugamushi*. The 56 kDa TSA gene sequence identified in 11 chiggers, one rodent, and 125 patients corresponded to four different strains of *O. tsutsugamushi*. The Boryong strain was the most common in chiggers (11/12; 95.8%) and patients (103/125; 82.4%). Interestingly, the sequence (KX363954) identified from the rodents was 84.3% homologous to the CH0711a strain (GQ342749), which is related to the Saitama strain cluster (Table 5). Peculiarly, there was a difference between the sequence (KY266830) identified from the chigger and Boryong cluster (Similarity percent: 74.0–79.8%) (Table 5). In patient samples, the 17 sequences (KY946107-KY946123) identified (17/125; 13.6%) are related to the Karp cluster. In addition, the four sequences (KY946124-KY946127) that were identified (4/125; 3.2%) are related to the Kawasaki cluster, one sequence (KY946128) that were identified (1/125; 0.8%) are related to the Saitama cluster (Figure 3; Table 6). For composition of phylogenetic tree, sequences obtained from chiggers (KX363952, KX363953,

TABLE 5
Continued

Percent identity																		
21	**22**	**23**	**24**	**25**	**26**	**27**	**28**	**29**	**30**	**31**	**32**	**33**	**34**	**35**	**36**	**37**		
95.5	87.4	65.7	67.9	68.8	68.4	87.7	87.2	87.2	90.3	89.9	90.1	89.4	72.4	84.1	83.2	82.7	**1**	KY946003_Human_Boryong
88.4	80.0	64.1	67.3	66.8	66.4	80.3	80.0	91.0	94.4	94.4	94.0	93.9	76.9	88.4	87.5	87.0	**2**	KY946031_Human_Boryong
88.6	80.1	63.9	67.5	66.6	66.2	80.5	80.1	91.2	94.6	94.6	94.2	94.0	76.9	88.6	87.7	87.2	**3**	KY946061_Human_Boryong
72.9	77.4	71.5	55.4	61.7	61.4	75.5	78.9	67.9	71.1	71.1	70.8	70.6	57.9	65.3	64.4	63.9	**4**	KY946128_Human_Pajoo
63.5	62.8	64.3	93.3	92.1	91.7	61.9	62.3	68.2	69.3	69.3	69.5	69.1	58.5	63.7	63.5	63.0	**5**	KY946124_Human_Kawasaki
63.4	62.6	64.1	92.8	91.9	91.5	61.7	62.1	69.0	70.0	70.0	70.2	69.9	59.2	64.4	64.3	63.7	**6**	KY946125_Human_Kawasaki
63.4	62.6	63.4	92.6	91.0	90.6	61.7	62.1	69.9	70.8	70.4	71.1	70.4	59.9	65.2	64.8	64.3	**7**	KY946126_human_kawasaki
63.4	62.6	64.4	92.4	91.9	91.5	61.7	62.1	68.8	69.9	69.9	70.0	69.7	59.2	64.3	64.1	63.5	**8**	KY946127_human_kawasaki
84.7	86.8	68.8	60.5	71.7	71.5	91.7	87.2	74.5	77.8	77.8	77.4	77.3	64.1	71.8	70.9	70.4	**9**	KY946107_Human_Karp
86.3	88.3	67.3	62.1	71.8	71.5	93.3	88.6	76.4	79.6	79.6	79.2	79.1	65.5	73.6	72.7	72.2	**10**	KY946108_Human_Karp
86.8	91.9	61.4	70.4	64.8	64.4	88.4	91.7	82.3	83.8	83.6	84.7	83.0	69.9	79.1	77.8	77.6	**11**	AF430142_Human_Pajoo
96.6	87.5	61.9	67.5	66.6	66.6	86.8	85.6	83.2	84.7	84.7	85.6	84.3	68.1	83.4	81.9	81.8	**12**	M63380_Human_Kuroki
75.1	73.8	63.9	86.3	85.9	85.6	73.3	71.1	65.3	65.3	65.0	66.4	65.0	54.9	61.6	61.0	61.4	**13**	DQ485289_Gilliam
69.3	69.1	61.2	89.2	83.8	83.4	67.5	66.2	63.0	63.0	62.6	64.1	62.6	53.1	60.5	59.7	59.9	**14**	AF173033_Ikeda
71.3	71.1	66.1	92.4	95.5	95.1	70.2	70.2	63.5	63.9	63.5	64.6	63.4	53.2	58.5	58.3	57.8	**15**	M63383_Human_Kawasaki
71.3	70.8	62.5	74.5	72.4	72.0	68.2	67.3	61.6	61.7	61.4	62.5	61.0	52.7	59.4	58.1	58.3	**16**	AY836148_Kato
83.8	86.1	70.8	58.5	70.4	70.8	90.8	86.8	71.7	74.9	74.9	74.5	74.4	61.2	69.0	68.1	67.5	**17**	AY956315_Human_Karp
69.9	69.1	58.1	83.9	82.3	82.7	68.1	66.8	57.8	57.8	57.4	58.8	57.4	47.8	57.9	57.4	57.4	**18**	U19903_Human_Yonchon
68.4	67.1	59.6	67.9	63.2	62.8	65.5	66.4	71.7	73.3	72.9	73.3	72.9	62.5	67.3	66.6	66.1	**19**	U19904_Rattus._rattus_TA6.7.8
77.3	83.2	56.9	64.6	60.5	60.1	80.0	84.3	84.7	87.9	86.8	86.8	87.2	74.0	81.4	81.0	80.7	**20**	KX363954_Apod.e.mus_agrarius
–	**90.6**	**63.7**	**66.1**	**68.4**	**68.8**	**90.3**	**88.8**	**85.0**	**86.5**	**86.3**	**87.5**	**86.1**	**69.9**	**81.8**	**80.5**	**80.3**	**21**	**JQ898368_Human_Boryong**
9.8	–	62.8	65.5	68.1	68.4	92.8	94.6	76.9	78.7	78.3	79.6	78.0	64.8	73.5	72.4	72.6	**22**	AF430141_Human_Young-worl
27.0	27.4	–	59.9	69.9	69.5	62.1	62.1	57.8	59.4	59.0	59.0	58.8	51.3	53.1	52.3	52.2	**23**	M63381_Shimokoshi
32.9	32.6	29.7	–	87.9	87.5	63.5	62.8	67.0	66.2	66.1	67.7	65.9	56.5	64.1	63.5	63.4	**24**	AF173039_Kanda
32.3	31.7	27.8	0.2	–	99.6	67.1	67.5	60.8	61.9	61.9	62.1	61.7	51.4	56.3	56.1	55.6	**25**	AF173038_Taguchi
32.1	31.5	27.8	0.2	0.0	–	67.5	67.9	60.5	61.6	61.6	61.7	61.4	51.1	56.0	55.8	55.2	**26**	AF.173037_Oishi
8.9	6.7	28.2	34.0	32.7	32.6	–	93.5	76.4	78.5	78.3	79.2	77.8	64.4	73.1	72.0	71.7	**27**	AF430143_Human_Jecheon
9.4	3.3	28.2	33.6	32.1	31.9	6.2	–	75.3	77.8	77.4	78.2	76.9	63.4	72.2	71.5	70.9	**28**	GQ332749_CH0711a_TAIWAN
6.0	16.8	32.7	37.2	36.3	36.3	15.5	15.6	–	92.4	93.0	93.9	92.6	79.8	90.4	88.8	89.0	**29**	KY266825_L.scutellare
4.8	**14.7**	**30.6**	**39.7**	**37.6**	**37.6**	**14.4**	**14.5**	**7.3**	–	**96.0**	**95.8**	**96.4**	**79.2**	**90.1**	**88.6**	**88.6**	**30**	**KY266826_L.scutellare**
4.8	15.0	31.1	39.8	37.3	37.3	14.5	14.3	6.8	4.4	–	96.2	97.7	79.4	91.0	90.3	89.4	**31**	KY266827_L.scutellare
3.0	12.9	30.0	36.1	35.9	35.9	12.4	12.5	5.4	3.9	3.7	–	96.0	79.1	91.5	91.0	90.8	**32**	KY266828_L.scutellare
4.8	15.3	30.5	39.9	36.7	36.7	15.0	15.4	7.1	3.7	1.8	3.2	–	79.4	90.1	89.4	89.4	**33**	KY266829_L.scutellare
26.9	35.8	49.6	60.6	56.8	56.8	33.9	34.2	24.6	24.7	24.6	25.0	24.4	–	75.5	73.6	74.0	**34**	KY266830_L.scutellare
4.6	15.3	31.9	40.6	37.2	37.2	13.8	13.6	3.9	4.6	3.7	3.2	4.2	24.0	–	92.1	92.4	**35**	KY266824_L.scutellare
6.7	17.4	35.0	42.3	38.8	38.8	16.4	15.7	6.1	7.5	5.1	3.9	5.6	26.4	8.5	–	96.0	**36**	KX363952_L.scutellare
6.9	17.1	34.7	42.7	39.3	39.3	16.4	16.0	6.3	7.0	6.3	4.6	5.6	26.4	8.4	4.3	–	**37**	KX363953_L.scutellare
21	**22**	**23**	**24**	**25**	**26**	**27**	**28**	**29**	**30**	**31**	**32**	**33**	**34**	**35**	**36**	**37**	–	–

TABLE 6
Strain of *Orientia tsutsugamushi* obtained in rodent, chigger, and humans from September 2014 to December 2016 in the southwest area of Korea

Source	Genbank accession number	Collection date	Cluster	Reference
Chigger (*Leptotrombidium scutellare*)	KX363949	October 2014	Boryong	11
Chigger (*L. scutellare*)	KX363950	October 2014	Boryong	11
Chigger (*Leptotrombidium orientale*)	KX363951	May 2015	Boryong	11
Chigger (*L. scutellare*)	KX363952	October 2015	Boryong	–
Chigger (*L. scutellare*)	KX363953	October 2015	Boryong	–
Rodent (*Apodemus agrarius*)	KX363954	December 2015	Saitama	–
Chigger (*L. scutellare*)	KY266824	October 2016	Boryong	–
Chigger (*L. scutellare*)	KY266825	October 2016	Boryong	–
Chigger (*L. scutellare*)	KY266826	October 2016	Boryong	–
Chigger (*L. scutellare*)	KY266827	October 2016	Boryong	–
Chigger (*L. scutellare*)	KY266828	October 2016	Boryong	–
Chigger (*L. scutellare*)	KY266829	October 2016	Boryong	–
Chigger (*L. scutellare*)*	KY266830	November 2016	–	–
Human	KY946003	2014	Boryong	–
Human	KY946031	2015	Boryong	–
Human	KY946061	2015	Boryong	–
Human	KY946107	2014	Karp	–
Human	KY946108	2014	Karp	–
Human	KY946128	2014	Saitama	–
Human	KY946124	2015	Kawasaki	–
Human	KY946125	2015	Kawasaki	–
Human	KY946126	2015	Kawasaki	–
Human	KY946127	2015	Kawasaki	–

* Different strain from other chiggers.

KY266824-KY266830), rodent (KX363954), human (KY946003, KY946031, KY946061, KY946107, KY946108, and KY946128, KY946124-KY946127) were used (Table 6).

DISCUSSION

Scrub typhus is a common endemic in the southwest region of Korea. The purpose of this study was to confirm the strains of *O. tsutsugamushi* for the 56-kDa TSA gene in chiggers, small mammals, and humans with chigger surveillance.

Seasonal chigger infestation rate for small mammals can be a criterion for the prevalence of scrub typhus. Furthermore, the abundance of chiggers influences the number of cases of scrub typhus. The degree of chigger infestation in small mammals varies from species to species. We found a high infestation rate in *A. agrarius*, which is the main host for scrub typhus in the southwest region of Korea,[9] and which is the most abundant rodent species captured in this study.

Leptotrombidium scutellare, the predominant chigger species in the southern area of Korea, begins to appear in September and reaches a peak in autumn from October to November.[31] In this study, *L. scutellare* was also the predominant chigger species identified during this period and was the primary contributor to the increased chigger infestation rate. However, during spring (March to May), *L. orientale* was the predominant species. Furthermore, the *W. fragilis* strain was identified from *M. regulus*. However, *W. fragilis* has never been reported in chigger species in the southwest region of Korea, but has been previously reported in *M. regulus* in the central region of Korea.[32]

Here, we analyzed for the 56-kDa TSA gene sequence of *O. tsutsugamushi*, and identified the Boryong strain to predominate in chiggers and patients. The Boryong strain is primarily distributed in the southern region of Korea where *L. scutellare* is found.[15] In the present study, the results suggest that the Boryong strain can be transmitted through *L. scutellare* in the southwest region, and that *L. orientale* can act as a vector for scrub typhus in the spring. However, strains of *O. tsutsugamushi* carried by *L. scutellare* can differ between East Asian nations. For instance, in China, *L. scutellare* is considered a vector of the Karp strain,[24] whereas in Japan, *L. scutellare* is considered the main vector of the Kawasaki strain.[14]

Unlike the Boryong strain, the Karp- and Kawasaki-related strains had different genotypes in patients than what was predicted. In the central and southwest regions of Korea, the Karp and Kawasaki strain had already been identified in patients.[33,34] The Karp strain was classified into a higher virulence group, and the Boryong and Kawasaki strains were classified into a lower virulence group.[16]

Furthermore, a Saitama-related strain was found in a single *A. agrarius*, but was not found in chiggers. The Saitama strain is closely related to the Karp strain based on the 56-kDa TSA gene sequence. This strain was first reported in Japan in 1993 by Tamura et al.[35] by identifying it in wild rodents, which tend to carry various strains in East Asia. In addition, Wu et al. (2015) confirmed the Kato, Gilliam, Karp, and TA763 strain in the Guangdong region of China.[36,37] These strains were also reported in Taiwan.[38] Finally, the CH0711a strain was reported in a patient in 2010 in Taiwan.[39]

Our study had several strengths. First, this study was conducted on a monthly basis. Secondly, this is the first study to compare the *O. tsutsugamushi* strains between chiggers, rodents, and patients in the southwest region. Despite a low infection rate, the data presented here provide a foundation for evidence of other strains in chiggers and small mammals. Unlike the strains identified in chiggers and patients, a Saitama-related strain was demonstrated in rodents.

In conclusion, the 56-kDa TSA gene showed variations in *O. tsutsugamushi* strains from different hosts. We confirmed that the Boryong strain is the most common strain in chiggers and patients, whereas wild rodents harbored a Saitama-related strain. In patients, Kawasaki-related strains prevalent in Japan were observed. To identify strains that are circulating in a host at any given point in time, a survey should be conducted continuously. The results provided here will be helpful for minimizing future incidences of scrub typhus by understanding disease transmission vectors.

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