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Genetic Typing, Based on the 56-Kilodalton Type-Specific Antigen Gene, of *Orientia tsutsugamushi* Strains Isolated from Chiggers Collected from Wild-Caught Rodents in Taiwan▽†

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***Orientia tsutsugamushi* is the etiological agent of scrub typhus, a mite-borne, febrile illness that occurs in the Asia-Pacific region. We conducted strain characterization of *O. tsutsugamushi* isolates from chiggers obtained from rodents based the nucleotide sequence of the 56-kDa outer membrane protein gene. With the use of PCR, a total of 68 DNA sequences of 56-kDa antigen genes were amplified. Phylogenetic analysis revealed that there were at least six definable clusters among the 68 isolates: 37% Karp-related strains (25/68), 27% TA763 strains (18/68), 12% JG-related strains (8/68), 19% Kato-related strains (13/68), 4% divergent strains (3/68), and 1% representing a Gilliam prototype strain (1/68). Overall, the *O. tsutsugamushi* genotypes exhibited a high degree of diversity, similar to that seen in strains from the rest of the areas where scrub typhus is endemic. Moreover, the 56-kDa protein sequence similarity between *O. tsutsugamushi* isolates from mites and those from human patients (H. Y. Lu et al., Am. J. Trop. Med. Hyg. 83:658–663, 2010) were striking, thus highlighting potential risk factors for this emerging zoonotic disease.**

Orientia tsutsugamushi is an obligate intracellular, Gram-negative bacterium that causes scrub typhus, an acute febrile illness characterized by a typical primary lesion (eschar), lymphadenopathy, and nonspecific symptoms such as chills, headache, and malaise (10, 24). The disease is endemic in Asian countries, including Japan, South Korea, China, Thailand, and Taiwan (6, 13). An estimated 1 million cases occur annually (22). However, the precise incidence of this disease is unknown, as the available diagnostic facilities in most of the region of endemicity are limited (32). In nature, the bacterial agent is maintained within a life cycle involving wild mammals and a mite vector, primarily of the *Leptotrombidium* genus (14). Humans are accidentally infected by the bites of a larval mite, known as a chigger, during feeding (30).

The 56-kDa type-specific antigen (TSA) gene encodes a dominant membrane protein, which accounts for 10% to 15% of the total protein of *O. tsutsugamushi* (17). The deduced size of the 56-kDa protein varies from 516 to 541 amino acids, according to gene sequences deposited in the GenBank (11). It is highly immunogenic, and the sera of most patients with scrub typhus are reactive. Serological analyses show that the antigenicity of the 56-kDa protein is diverse and includes variants such as the representative Karp-, Gilliam-, and Kato-type strains and other isolates (2, 8, 18). The immune responses to the 56-kDa protein provide protection against Scrub typhus, suggesting that it is a potential candidate for vaccine development (3, 16, 33). No commercially licensed vaccine for scrub typhus is currently available.

In the present study, we cloned and sequenced the 56-kDa TSA gene of *O. tsutsugamushi* isolated from the chigger mite obtained from a rodent in order to understand the genetic types as well as the relationships with other known strains. Our results demonstrated that the genotypes of *O. tsutsugamushi* isolates appeared highly diverse and widely and geographically distributed in Taiwan.

MATERIALS AND METHODS

Study site and arthropod collection. All the arthropods used in this study were collected from rodents captured by randomly setting live traps in the fields of Taiwan's mainland and off shore islands, such as Penghu, Kinmen, and Lan-Yu. The rodent species found in this study were diverse, including *Bandicota indica*, *Rattus norvegicus*, *Rattus losea*, *Rattus flavipectus*, *Rattus mindanensis*, and *Suncus murinus*; the predominance of the rat species depended on the geographic location. *R. losea* was commonly found in all study sites except Lanyu. The dominant rodent species consist of *R. flavipectus* in Kinmen and *R. mindanensis* in Lanyu.

The mites were collected from the captured rodents by brushing the animal's fur thoroughly using a metal comb over the plastic bag. The collected mites were placed in an Eppendorf tube and delivered to the laboratory.

DNA extraction and isolation of bacteria. The mites carried in each individual rodent were grouped and were processed for DNA extraction in accordance with the procedures described previously (28). Briefly, the mites (10 to 50 mites) collected from individual rodents were pooled, their surfaces were disinfected in a solution of 70% methanol containing 0.2% iodine triturated in a 0.2- to 1-ml sucrose-phosphate-glutamate buffer, and were then subjected to DNA extraction and cell culture. The DNA was extracted using a QIAamp DNA minikit (Qiagen, Hilden, Germany) and then stored at −70°C for later use. *O. tsutsugamushi* was isolated from the mite homogenates by inoculation into L929 cells by use of the Shell-vial method (12), and infected monolayer cells were examined for *O. tsutsugamushi* using an indirect fluorescence assay (IFA).

PCR amplification. Nested PCR was performed to detect *O. tsutsugamushi* DNA in the DNA extract. The primer sequences were designed based on the DNA sequence from the Karp strain (GenBank accession no. AY956315) coding for the 56-kDa TSA gene, as described previously (5, 7). The two pairs of primers used were as follows: pair 1, comprising F6 (5′-GTTGGAGGAATGAATTAC

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TABLE 1. Description of *Orientia tsutsugamushi* isolates and reference strains examined[a]

Isolate	Mo/yr	Source	Location	Country	No. of bases	GenBank accession no.	Strain	Source or reference
HL01	01/2008	Chiggers	Hualien	Taiwan	1,566	GU120139	TA763	This study
HL02-1	04/2008	Chiggers	Hualien	Taiwan	1,605	GU120140	Karp related	This study
HL02-2	04/2008	Chiggers	Hualien	Taiwan	1,566	GU120141	TA763	This study
HL03-1	01/2008	Chiggers	Hualien	Taiwan	1,566	GU120142	TA763	This study
HL03-2	01/2008	Chiggers	Hualien	Taiwan	1,596	GU120143	Kato related	This study
HL04	01/2008	Chiggers	Hualien	Taiwan	1,605	GU120144	Karp related	This study
HL05	01/2008	Chiggers	Hualien	Taiwan	1,605	GU120145	Karp related	This study
KM01	2001	Chiggers	Kinmen	Taiwan	1,611	GU120146	Karp related	This study
KM02	2002	Chiggers	Kinmen	Taiwan	1,575	GU120147	Gilliam	This study
KM03	2003	Chiggers	Kinmen	Taiwan	1,590	GU120148	Kato related	This study
KM04	01/2008	Chiggers	Kinmen	Taiwan	1,587	GU120149	TA763	This study
KM05	03/2008	Chiggers	Kinmen	Taiwan	1,611	GU120150	Karp related	This study
KM06	03/2008	Chiggers	Kinmen	Taiwan	1,575	GU120151	Kato related	This study
KM07	03/2008	Chiggers	Kinmen	Taiwan	1,608	GU120152	Karp related	This study
KM08	03/2008	Chiggers	Kinmen	Taiwan	1,605	GU120153	TA763	This study
KM09	03/2008	Chiggers	Kinmen	Taiwan	1,608	GU120154	Karp related	This study
KM10-1	10/2009	Chiggers	Kinmen	Taiwan	1,605	GU446588	TA763	This study
KM10-2	10/2009	Chiggers	Kinmen	Taiwan	1,605	GU446589	TA763	This study
KM11-1	10/2009	Chiggers	Kinmen	Taiwan	1,611	GU446590	Karp related	This study
KM11-2	10/2009	Chiggers	Kinmen	Taiwan	1,608	GU446591	Karp related	This study
KM12	10/2009	Chiggers	Kinmen	Taiwan	1,605	GU446592	Karp related	This study
KM13	10/2009	Chiggers	Kinmen	Taiwan	1,608	GU446593	Karp related	This study
KM14	10/2009	Chiggers	Kinmen	Taiwan	1,572	GU446594	JG related	This study
KM15-1	10/2009	Chiggers	Kinmen	Taiwan	1,593	GU446595	Divergent	This study
KM15-2	10/2009	Chiggers	Kinmen	Taiwan	1,593	GU446596	Divergent	This study
KM16-1	10/2009	Chiggers	Kinmen	Taiwan	1,632	GU446597	Divergent	This study
KM16-2	10/2009	Chiggers	Kinmen	Taiwan	1,596	GU446598	JG related	This study
KM17-1	10/2009	Chiggers	Kinmen	Taiwan	1,611	GU446599	Karp related	This study
KM17-2	10/2009	Chiggers	Kinmen	Taiwan	1,611	GU446600	Karp related	This study
KM18	10/2009	Chiggers	Kinmen	Taiwan	1,611	GU446601	Karp related	This study
KM19-1	10/2009	Chiggers	Kinmen	Taiwan	1,611	GU446602	Karp related	This study
KM19-2	10/2009	Chiggers	Kinmen	Taiwan	1,584	GU446603	TA763	This study
KM20	10/2009	Chiggers	Kinmen	Taiwan	1,605	GU446604	TA763	This study
KM21-1	10/2009	Chiggers	Kinmen	Taiwan	1,575	GU446605	Kato related	This study
KM21-2	10/2009	Chiggers	Kinmen	Taiwan	1,560	GU446606	TA763	This study
KM21-3	10/2009	Chiggers	Kinmen	Taiwan	1,605	GU446607	TA763	This study
MZ01-1	07/2007	Chiggers	Matsu	Taiwan	1,602	GU120155	Karp related	This study
MZ01-2	07/2007	Chiggers	Matsu	Taiwan	1,602	GU120156	Karp related	This study
MZ02	07/2007	Chiggers	Matsu	Taiwan	1,572	GU120157	JG related	This study
OI01	12/2009	Chiggers	Lan-Yu	Taiwan	1,572	GU446608	Kato related	This study
OI010	12/2009	Chiggers	Lan-Yu	Taiwan	1,566	GU446620	TA763	This study
OI011	12/2009	Chiggers	Lan-Yu	Taiwan	1,566	GU446621	TA763	This study
OI02	12/2009	Chiggers	Lan-Yu	Taiwan	1,572	GU446609	JG related	This study
OI03-1	12/2009	Chiggers	Lan-Yu	Taiwan	1,566	GU446610	Kato related	This study
OI03-2	12/2009	Chiggers	Lan-Yu	Taiwan	1,566	GU446611	Kato related	This study
OI04	12/2009	Chiggers	Lan-Yu	Taiwan	1,578	GU446612	Kato related	This study
OI05-1	12/2009	Chiggers	Lan-Yu	Taiwan	1,605	GU446613	TA763	This study
OI05-2	12/2009	Chiggers	Lan-Yu	Taiwan	1,611	GU446614	Karp related	This study
OI06-1	12/2009	Chiggers	Lan-Yu	Taiwan	1,581	GU446615	Kato related	This study
OI06-2	12/2009	Chiggers	Lan-Yu	Taiwan	1,602	GU446616	Karp related	This study
OI07	12/2009	Chiggers	Lan-Yu	Taiwan	1,602	GU446617	Karp related	This study
OI08	12/2009	Chiggers	Lan-Yu	Taiwan	1,608	GU446618	Karp related	This study
OI09	12/2009	Chiggers	Lan-Yu	Taiwan	1,575	GU446619	Kato related	This study
PH01	07/2007	Chiggers	Penghu	Taiwan	1,608	GU120158	Karp related	This study
PH02	05/2008	Chiggers	Penghu	Taiwan	1,608	GU120159	Karp related	This study
PH03	05/2008	Chiggers	Penghu	Taiwan	1,605	GU120160	TA763	This study
PH04	05/2008	Chiggers	Penghu	Taiwan	1,608	GU120161	Karp related	This study
PH05	05/2008	Chiggers	Penghu	Taiwan	1,605	GU120162	TA763	This study
TT01-1	07/2008	Chiggers	Taitung	Taiwan	1,575	GU120163	Kato related	This study
TT01-2	07/2008	Chiggers	Taitung	Taiwan	1,572	GU120164	JG related	This study
TT02-1	10/2008	Chiggers	Taitung	Taiwan	1,572	GU120165	JG related	This study
TT02-2	10/2008	Chiggers	Taitung	Taiwan	1,572	GU120166	JG related	This study
TT03-1	12/2008	Chiggers	Taitung	Taiwan	1,593	GU120168	Divergent	This study
TT03-2	12/2008	Chiggers	Taitung	Taiwan	1,572	GU120169	Kato related	This study
TT04	12/2008	Chiggers	Taitung	Taiwan	1,566	GU120170	Kato related	This study
TT05	12/2008	Chiggers	Taitung	Taiwan	1,572	GU120171	JG related	This study
TT06-1	12/2008	Chiggers	Taitung	Taiwan	1,605	GU120172	TA763	This study

Continued on following page

TABLE 1—*Continued*

Isolate	Mo/yr	Source	Location	Country	No. of bases	GenBank accession no.	Strain	Source or reference
TT06-6	12/2008	Chiggers	Taitung	Taiwan	1,605	GU120173	TA763	This study
Hualien-1			Hualien	Taiwan	1,842	AY243357	JG related	11
Hualien-3			Hualien	Taiwan	1,875	AY636101	Kato related	11
Hualien-4			Hualien	Taiwan	1,742	AY714315	Kato related	11
Hualien-7			Hualien	Taiwan	1,857	AY834393	JG related	11
Hualien-8			Hualien	Taiwan	1,863	DQ323174	JG related	11
Hualien-11			Hualien	Taiwan	1,889	DQ323175	TA763	11
Hualien-12			Hualien	Taiwan	1,890	DQ323176	Karp related	11
Hualien-13			Hualien	Taiwan	1,856	DQ789360	JG related	11
Hualien-14			Hualien	Taiwan	1,851	DQ852664	TA763	11
Taitung-1			Taitung	Taiwan	1,889	AF516948	Karp related	11
Taitung-2			Taitung	Taiwan	1,857	AY335819	JG related	11
Taitung-4			Taitung	Taiwan	1,889	AY787232	TA763	11
Taitung-5			Taitung	Taiwan	1,863	AY834392	JG related	11
CDC Gilliam				Taiwan	1,860	DQ485289	Gilliam	11
CDC Karp				New Guinea	1,884	AY956315	Karp	11
CDC Kato				Japan	1,875	AY836148	Kato	11
TA 763	1963	Rodent		Thailand	1,581	U80636	TA763	11
Kawasaki				Japan	1,569	M63383	JG related	
UT176	07/2004	Human	Udorn Thani	Thailand	1,602	EF213081	Karp related	4
UT302	08/2004	Human	Udorn Thani	Thailand	1,587	EF213078	TA763	4
UT316	10/2004	Human	Udorn Thani	Thailand	1,611	EF213078	Karp related	4
UT329	07/2005	Human	Udorn Thani	Thailand	1,596	EF213099	JG related	4
UT336	07/2005	Human	Udorn Thani	Thailand	1,599	EF213089	Karp related	4
TW-1	05/2006	Human	Kinmen	Taiwan	1,608	GQ332742	Karp related	15
TW-2	10/2006	Human	Taoyuan County	Taiwan	1,605	GQ332743	Karp related	15
TW-3	07/2006	Human	Taipei County	Taiwan	1,605	GQ332744	Karp related	15
TW-6	09/2006	Human	Kaohsiung City	Taiwan	1,608	GQ332747	Karp related	15
TW-7	06/2006	Human	Kaohsiung City	Taiwan	1,608	GQ332748	Karp related	15
TW-8	11/2007	Human	Changhua County	Taiwan	1,692	GQ332749	Karp related	15
TW-11	07/2007	Human	Nantou County	Taiwan	1,584	GQ332752	TA763	15
TW-12	05/2007	Human	Taitung County	Taiwan	1,593	GQ332753	Divergent	15
TW-13	11/2007	Human	Nantou County	Taiwan	1,557	GQ332754	JG related	15
TW-14	11/2007	Human	Taitung County	Taiwan	1,551	GQ332755	JG related	15
TW-15	12/2007	Human	Pingtung County	Taiwan	1,569	GQ332756	JG related	15
TW-16	07/2007	Human	Kaohsiung City	Taiwan	1,572	GQ332757	JG related	15
TW-17	07/2007	Human	Taipei City	Taiwan	1,596	GQ332758	JG related	15
TW-18	06/2007	Human	Kaohsiung City	Taiwan	1,596	GQ332759	JG related	15
TW-20	05/2006	Human	Hsinchu County	Taiwan	1,572	GQ332761	Kato related	15
TW-21	07/2006	Human	Kinmen	Taiwan	1,590	GQ332762	Kato related	15
TW-22	06/2006	Human	Kaohsiung City	Taiwan	1,575	GQ332763	Kato related	15

[a] "Gilliam" refers to the Gilliam prototype; "JG related" refers to the Gilliam variant.

TGG-3′) (nucleotides 406 to 425) and R6 (5′-AGCGCTAGGTTTATTAGCA T-3′) (nucleotides 1059 to 1040), and pair 2, comprising F7 (5′-AGGATTAGA GTGTGGTCCTT-3′) (nucleotides 369 to 388) and R7 (5′-ACAGATGCACTA TTGGCAA-3′) (nucleotides 1175 to 1156). Approximately 4% of the first PCR products were used as a template DNA for the second PCR; purified Karp strain DNA and distilled water were used instead of the specimen DNA as positive and negative controls, respectively. PCR targeting DNA was visualized with a UV transilluminator after agarose gel electrophoresis and staining with ethidium bromide (see Fig. S1 in the supplemental material).

PCR amplification of the complete 56-kDa TSA gene was performed using primers TF1 (5′-AGAATGAAAAAAATTATGTTAATTGC-3′) and TR1 (5′-AAACTAGAAGTTATAGCGYACAC-3′). The PCR program was started with denaturation at 94°C for 3 min, followed by 35 cycles consisting of 40 s at 94°C, 30 s at 55°C, and 1 min 40 s at 72°C, with a final extension step of 10 min at 72°C. The PCR was conducted in a PTC-200 peltier thermal cycler (MJ Research, Reno, NV). The PCR products were purified using a PCR purification kit (Qiagen, Hilden, Germany) and cloned using a TOPO TA cloning kit (Invitrogen, Carlsbad, CA). PCR products were sequenced by using an ABI 3730 XL DNA analyzer at Genomics BioSci & Tech, Inc., Taipei, Taiwan. Strands were sequenced in the forward and reverse directions using primers T7P (5′-TAA TAC GAC TCA CTA TAG G-3′) and T3P (5′-GCAATTAACCCTCACTAA AGGG-3′), corresponding to the priming sites on TA cloning vector.

Phylogenetic analysis. Multiple sequence alignment and analysis of the genetic relationships between isolates and reference strains were performed using Bionumerous version 4.6 and the CLUSTAL_X program (26), and the resulting dendrogram was constructed with the software program MEGA4 (25), using the neighbor-joining method and bootstrap test on 1,000 replicates. The complete nucleotide sequences of the 56-kDa gene for the *O. tsutsugamushi* strains isolated from different regions and the references for each type of strain are listed in Table 1. A percentage nucleotide identity matrix was constructed using the DNAStar MEGALIGN 6.1 program (DNAStar, Inc., Madison, WI).

Nucleotide sequence accession numbers. All the DNA sequences used in this study were deposited in the GenBank database under the accession numbers indicated in Table 1.

RESULTS

Prevalence of *O. tsutsugamushi* in chigger mites from rodents. Figure 1 shows the Taiwan map indicating the geographic areas of *O. tsutsugamushi* strains isolated from chigger mites collected from rodents as well as the strains isolated from a human scrub typhus patient (15) that were used in this study.

FIG. 1. Map of the geographic areas in Taiwan showing *O. tsutsugamushi* strains isolated in this study and from a human scrub typhus patient. For each study site, the number of captured rodents and the prevalence of *O. tsutsugamushi* DNA fragment in mite-hosting rodents are indicated.

The numbers of mites carried by each individual rodent, i.e., the mite indexes, differed drastically, from a few to several hundreds. Mite pools collected from 130 out of 257 (51%) rodents were found to be positive for the *O. tsutsugamushi* 56-kDa gene fragment based on nested PCR. The prevalence of *O. tsutsugamushi* in rodents among the study sites ranged from 35 to 95%. A relatively high prevalence rate in Penghu was observed in the present study. Among the nest PCR-positive specimens, 49/95 (52%) mite homogenates that had been isolated from 19 *R. losea* organisms, 13 *R. mindanensis* organisms, 13 *R. flavipectus* organisms, 3 *Suncus murinus* organisms, and 1 *Bandicota indica* organism were successfully cultured for bacterial pathogens as examined by IFA using *O. tsutsugamushi*-specific antibody. A total 68 complete 56-kDa TSA gene sequences were amplified from these samples by PCR.

***O. tsutsugamushi* characteristics.** The results of analysis of the 56-kDa TSA gene sequences of the *O. tsutsugamushi* isolates used in this study are shown in Tables 1 and 2. These *O. tsutsugamushi* strains are related to either the Karp- or Gilliam-type strains or Kato and the Thai-type strain TA763 (Fig. 2). The majority of isolates (38%; 25/68) were related to the Karp-type reference strain (percent nucleotide identity range [PNIR] for Karp strain, 94.4% to 96.8%). Only one isolate was related to the Gilliam prototype strain (PNIR for Gilliam strain, 99.8%). The amino acid sequence identities ranged from 73.2% to 100% among the 68 isolates.

***O. tsutsugamushi* distribution by geographical region.** The majority of *O. tsutsugamushi* isolates (29/68) studied were from Kinman Island, which represented a high proportion of Karp-related strains (44%; 13/29), eight TA763 strains (28%; 8/29), two Gilliam-related strains (7%; 2/29), one single Gilliam isolate, and two divergent strains. In contrast, 14 isolates (6 [43%; 6/14] Kato-related strains, 4 [29%; 4/14] Karp-related strains, 3 [21%; 3/14] TA763-related strains, and 1 JG-related strain) were found on Lan-Yu, a small island in the Pacific Ocean southeast of Taiwan. Ten isolates (four [40%; 4/10] JG-, three [30%; 3/10] Kato-, and two TA763-related strains and 1 divergent strain) were from Taitung County. Seven isolates were from Hualien (three [43%; 3/7] TA763 strains, two Karp-related strains, and one Kato-related strain). Five isolates (three Karp and two TA763 strains) were from the Penghu islands. Three isolates (two Karp-related strains and one JG-related strain) were from the Matsu islands. The genotypes of *O. tsutsugamushi* isolates from Taiwan varied widely, with the main Karp-type strains widely distributed in different geographic regions.

Taiwanese *O. tsutsugamushi* isolates and international strains. The phylogenetic relationships among Taiwan *O. tsutsugamushi* isolates and strains from other countries were investigated using MEGA4. Figure 2 shows that the phylogenetic tree based on the 56-kDa TSA gene sequences is divided into six definable groups. The analysis showed that the Taiwan isolates within the group of Karp-related strains were more

TABLE 2. Comparison of the amino acid homologies of the entire 56-kDa TSA gene between the *Orientia tsutsugamushi* strains isolated from Taiwan and the reference strains[a]

Isolate	% Homology to indicated *O. tsutsugamushi* reference strain							
	KARP	KATO	Gilliam	UT316	TW-22	TA763	UT329	TW-12
HL01	80.9	75.9	81.3	89.7	78.5	**90.6**	76.2	76.9
HL02-1	**93.0**	76.9	84.4	**96.3**	76.0	84.0	78.2	76.3
HL02-2	82.3	77.5	82.6	90.1	78.7	**91.9**	77.6	78.3
HL03-1	81.3	76.3	81.7	89.9	78.9	**91.0**	76.6	77.3
HL03-2	77.2	**89.9**	78.2	85.1	81.0	76.6	78.0	76.9
HL04	**93.0**	76.9	84.4	**96.5**	76.0	84.2	78.2	76.3
HL05	**93.0**	76.9	84.4	**96.5**	76.0	84.2	78.2	76.3
KM01	**93.2**	77.8	85.7	**96.3**	76.3	83.4	79.2	76.3
KM02	84.1	77.0	**99.6**	89.6	75.1	82.8	87.8	77.5
KM03	75.5	**99.6**	76.6	84.4	79.3	75.0	75.8	77.4
KM04	79.6	75.2	80.3	87.8	77.2	**81.7**	74.5	76.1
KM05	**93.2**	77.8	85.7	**96.2**	76.3	83.4	79.2	76.3
KM06	76.1	79.5	74.9	85.7	**99.4**	77.2	73.9	73.5
KM07	**92.2**	75.4	82.9	**98.4**	75.2	85.2	77.5	75.0
KM08	80.0	74.9	81.5	88.6	75.9	**82.2**	73.9	76.1
KM09	**92.2**	75.4	82.9	**98.4**	75.2	85.2	77.5	75.0
KM10-1	80.0	74.9	81.5	88.5	75.9	**82.2**	73.9	76.1
KM10-2	80.0	74.9	81.5	88.5	75.9	**82.2**	73.9	76.1
KM11-1	**93.2**	77.8	85.7	**96.3**	76.3	83.4	79.2	76.3
KM11-2	**92.4**	76.6	83.7	**98.4**	75.9	84.5	78.5	76.1
KM12	**90.6**	74.2	83.8	**94.8**	73.4	81.3	75.7	73.3
KM13	**92.2**	75.4	82.9	**98.4**	75.2	85.2	77.5	75.0
KM14	76.8	77.4	85.3	85.8	73.5	75.8	**96.7**	76.3
KM15-1	73.4	77.6	77.5	86.0	73.5	75.2	75.3	**99.6**
KM15-2	73.0	77.2	76.9	85.9	72.9	74.9	74.7	**99.2**
KM16-1	**91.0**	74.6	84.0	**95.0**	72.7	83.1	77.1	74.0
KM16-2	77.4	75.8	86.2	85.7	72.7	76.4	**95.7**	76.3
KM17-1	**93.2**	77.8	85.7	**96.2**	76.3	83.4	79.2	76.3
KM17-2	**92.8**	77.4	85.3	**96.2**	75.9	83.0	78.8	76.0
KM18	**93.2**	77.8	85.7	**96.2**	76.3	83.4	79.2	76.3
KM19-1	**93.2**	77.8	85.7	**96.2**	76.3	83.4	79.2	76.3
KM19-2	78.0	74.0	79.5	87.2	75.6	**80.9**	73.4	75.6
KM20	80.0	74.9	81.5	88.5	75.9	**82.2**	73.9	76.1
KM21-1	76.1	79.5	74.9	85.6	**99.4**	77.2	73.9	73.5
KM21-2	80.7	75.5	82.2	89.6	78.7	**89.8**	76.9	75.9
KM21-3	80.0	74.9	81.5	88.5	75.9	**82.2**	73.9	76.1
MZ01-1	**94.5**	75.5	82.6	**95.5**	74.6	83.2	76.1	76.3
MZ01-2	**94.5**	75.7	84.5	**95.3**	74.4	83.2	77.2	75.2
MZ02	76.0	76.0	86.6	85.1	72.5	75.2	**97.3**	74.5
OI01	75.9	**89.3**	78.5	84.5	79.7	74.5	76.1	77.6
OI02	76.0	76.0	86.2	85.2	72.5	75.2	**96.9**	74.1
OI03-1	74.2	**82.0**	73.9	84.6	78.4	71.7	72.3	72.8
OI03-2	73.6	**82.0**	73.5	84.8	78.0	71.7	72.3	72.8
OI04	78.1	**83.8**	76.5	83.5	80.4	75.3	75.1	73.5
OI05-1	79.8	74.7	81.3	88.5	75.7	**81.8**	73.8	75.9
OI05-2	89.5	74.9	82.1	**98.5**	74.9	83.0	76.2	75.6
OI06-1	76.8	**83.3**	75.4	84.8	83.6	77.9	75.5	74.5
OI06-2	**94.2**	75.3	82.3	**95.4**	74.4	83.2	75.9	75.8
OI07	**94.4**	75.5	82.6	**95.3**	74.6	83.0	76.1	76.1
OI08	**91.4**	74.5	82.1	**97.8**	74.5	84.7	76.2	75.0
OI09	75.5	79.0	74.3	85.3	**99.0**	76.6	73.3	72.9
OI10	80.7	75.5	80.9	89.7	78.1	**90.4**	75.8	76.6
OI11	80.9	75.9	81.3	89.7	79.1	**90.6**	76.4	76.9
PH01	**91.6**	74.9	82.3	**98.2**	74.7	84.7	76.9	74.4
PH02	**91.8**	75.0	82.5	**98.3**	74.9	84.9	77.1	74.6
PH03	80.2	74.7	81.6	88.7	76.1	**82.0**	74.1	75.9
PH04	**91.8**	75.0	82.5	**98.3**	74.9	84.9	77.1	74.6
PH05	80.0	74.9	81.5	88.5	75.9	**82.2**	73.9	76.1
TT01-1	75.9	79.3	74.7	85.5	**99.4**	77.0	73.5	73.3
TT01-2	76.8	77.4	85.3	85.8	73.5	75.8	**96.6**	76.3
TT02-1	77.9	77.2	86.6	85.4	74.3	76.8	**98.7**	75.9
TT02-2	77.2	76.6	87.2	85.2	74.1	76.0	**99.4**	75.1
TT03-1	73.6	77.6	77.5	86.0	73.5	75.4	75.3	**99.8**
TT03-2	78.1	**79.3**	78.7	86.7	77.6	76.8	76.1	82.7
TT04	74.2	**82.4**	73.9	84.8	78.4	72.1	72.7	73.2
TT05	77.0	77.6	85.5	86.0	73.7	76.0	**96.9**	76.4
TT06-1	80.0	74.9	81.5	88.5	75.9	**82.2**	73.9	76.1
TT06-6	80.0	74.9	81.5	88.5	75.9	**82.2**	73.9	75.9

[a] The homologies between the 56-kDa TSA protein sequences of the reference strains and the strains within the same genotype group are indicated in bold.

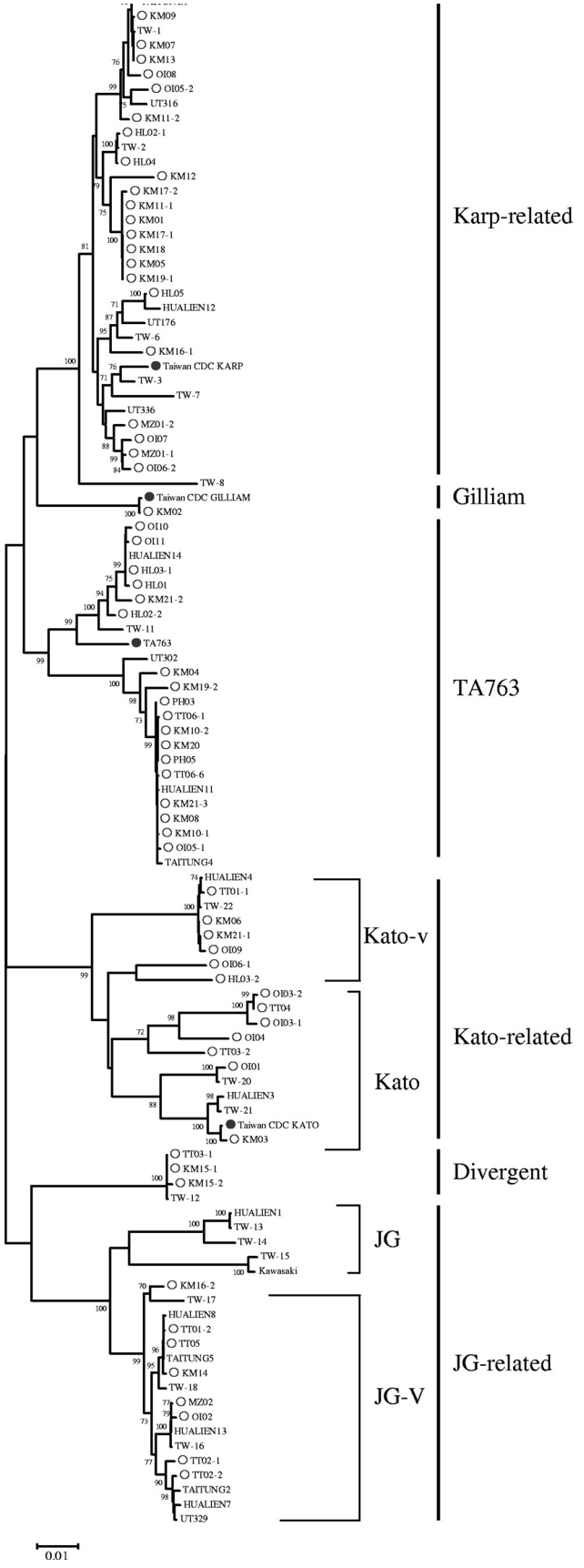

FIG. 2. Phylogenetic tree of *O. tsutsugamushi* based on the nucleotide sequences of the 56-kDa cell surface antigen gene. The subset of the phylogenetic tree is made up of isolates (strains from this study are indicated by open circles) associated with clades with sequence divergence from the reference strains (filled circles), such as Karp, Gilliam-related strains, and Kato, and strains isolated from humans (no mark). Isolates are identified by their GenBank accession numbers. Phylogenetic analyses were conducted using MEGA4. The evolutionary history was inferred using the neighbor-joining method and a bootstrap test on 1,000 replicates.

similar to Thailand strain UT316 than to Karp-CDC (New Guinea). Consistently, a large number of isolates belonged to the TA763 clade, a strain type commonly present in Thailand but not in Japan and Korea.

It is noteworthy that the 56-kDa TSA gene sequences detected in this study were closely related to those found in a previous study of blood samples from Taiwan scrub typhus patients (15), suggesting the pathogenic potential of *O. tsutsugamushi* strains. The results are shown in Table 2, indicating that among Kato-related strains, KM-06, KM21-1 OI-09, and TT01-1 were 99% or more identical to the TW-22 strain. Three isolates, KM15-1, KM15-2, and TT03-1, clustered with the divergent group and had 99.2 to 99.8% identity to the TW-12 strain.

DISCUSSION

We demonstrated a high level of genetic diversity for *O. tsutsugamushi* strains isolated in Taiwan. According to DNA sequencing of the 56-kDa type-specific antigen gene among the 68 *O. tsutsugamushi* isolates, all can be grouped into six known genotypes, those representing Karp (37%), TA763 (27%), Kato (19%), JG-related strains (12%), and two minority groups, comprising divergent strains (4%) and one Gilliam-type strain (1%).

Molecular diagnosis of scrub typhus is performed by PCR amplification of target genes from *O. tsutsugamushi*, including a 60-kDa heat shock protein (GroEL) (19, 20, 23) and 47-kDa (9) and 56-kDa outer membrane protein genes (1, 4). Based on the restriction fragment length polymorphism (RFLP) pattern of the 56-kDa gene, Qiang et al. (21) performed a preliminary study of genetic typing of *O. tsutsugamushi* isolates from mites found on wild rodents. These researchers suggested that various types of *O. tsutsugamushi* were indigenous in Taiwan. Our results support this early observation and show that Karp- and TA763-related strains represent two predominant strain types among Taiwan isolates. The TA763 strain, which originated from rodents, is prevalent in Thailand but not Korea and Japan. In addition, the protein sequences of strains KM15-2, KM15-1, and TT03-1 were less than 90% identical to the known sequences, indicating that these variants could be new and region-specific genotypes. Various genotypes are found in different geographic regions in Taiwan. Nonetheless, we found no significant association between strain type and locality, most likely due to limitation of the sample size.

Interestingly, the 56-kDa protein sequences of three Karp-related strains (KM09, KM07, and KM13) were identical, as was the case for four TA763-related strains (KM01, KM17-1,

KM17-3, KM8, and KM19-1). This suggests that these types of strains might be predominant in Kinmen. These similarities were very unlikely to have resulted from cross contamination during PCR amplification, because mite specimens were separately prepared under tightly controlled conditions in a biosafety level 3 cabinet.

The clinical significance of the strains among the isolates is largely unknown. The 56-kDa-protein sequence comparison showed that some strains, such as TT02-1 and TT02-2, were 98.7% or more identical to the human isolate UT329 from Thailand, indicating that these strains might play an epidemiological role for this infectious disease. Similarly, the 56-kDa-TSA-gene sequences of other strains in this study were shown to be highly similar to that isolated from the scrub typhus patient in Taiwan. This finding thus is not only suggesting the pathogenic potential of these *O. tsutsugamushi* isolates but also emphasizing the risk of acquiring scrub typhus due to mite bites.

The rodent species were found to be predominant but geographically specific, widely distributed in offshore islands, especially *R. mindanensis*, which was unique to Lanyu. As no characterization was carried out, the specific mite species were not known. Many mite species, including *Leptotrombidium scutellare*, *Walchia chinensis*, *Leptotrombidium yui*, and *Odontacarus majesticus*, have been identified to be the vector of scrub typhus in Taiwan, but *Leptotrombidium deliense* is the most significant in terms of prevalence (29). However, significant association between bacterial strain and mite species was not found. The average number of mites/rodent (the mite index) was found to be positively related to *O. tsutsugamushi* antibody titers in the rodent sera (data not shown). The mite-hosting rodents coinfested with ticks were observed frequently, ranging from 22.2% in Hualien to 42.1% in Kinmen. Therefore, coinfection of *O. tsutsugamushi* and other important tick-borne pathogens in rodents may have occurred. Recent study demonstrates that the detection of spotted fever group rickettsiae and *Ehrlichia chaffeensis* infection in rodent ticks likely supports this assumption (28, 31). To avoid underestimating the mix-infected bacterial strains within the pooled mite specimens, more than one single amplicon was elected and sequenced during PCR cloning.

In this case, rodents were caught by random sampling via live traps in distinct regions. Thus, ecological factors such as terrain features and seasonal variations (27) could have had a great influence on the rodent capture rate and the numbers of subsequent mite specimens collected, and that could potentially skew the results observed.

This large-scale surveillance of *O. tsutsugamushi* DNA sequences isolated from chigger mites provides an appreciable database for diagnosis for scrub typhus. The result also provides insight into the distribution of this zoonotic pathogen in Taiwan.

In conclusion, this study adds the knowledge of a high degree of diversity of *O. tsutsugamushi* genotypes and strains in wild rodents. The most common type is related to the Karp-type reference strain that is widely distributed in different geographic regions of Taiwan. The isolation from chiggers of *O. tsutsugamushi* strains closely related in genotype to the strains isolated from scrub typhus patients should warrant further investigation of the pathogenicity of these bacteria.

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