SEQUENCE ANALYSIS OF *ORIENTIA TSUTSUGAMUSHI* DNA FROM MITES COLLECTED IN THE XISA ARCHIPELAGO, CHINA

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Abstract. The genotype of *Orientia tsutsugamushi* DNA from mites in the Xisa archipelago of China were identified. A natural focus of tsutsugamushi disease in the archipelago was found. The DNA sequence that codes for the 56 kDa protein of *O. tsutsugamushi* was amplified by nested polymerase chain reaction (N-PCR). The purified positive products were cloned into a pGEM-T vector and sequenced. The DNA sequence was compared with various sequences on the internet for sequence homology. A 507 bp DNA fragment encoding the 56 kDa protein was amplified from the samples. The sequence homology was 85% (Karp strain), 68% (Gilliam strain), 65% (Kato strain), and 67% (Yonchon strain). *Orientia tsutsugamushi* is carried by the mites of the Xisa archipelago; the main genotype is the Karp strain.

INTRODUCTION

Orientia tsutsugamushi is the etiological agent of tsutsugamushi disease, a major rickettsial disease of humans that is of concern throughout Asia (Sugita *et al*, 1992; Shieh *et al*, 1996; Tay, 1996; Horinouchi *et al*, 1997) and that is sometimes fatal. Tsutsugamushi disease is transmitted by infected mites. *O. tsutsugamushi* is a very small coccobacillus and an obligate intracellular parasite of humans. The antigenic types of *O. tsutsugamushi*, referred to as Gilliam, Karp and Kato strains, are often recognized and are therefore called the prototype strains. Additional antigenic types, such as Shimokoshi (Tamura *et al*, 1984), Kawasaki (Yamamoto *et al*, 1986), Kuroki (Yamamoto *et al*, 1989; Ohashi *et al*, 1990), Boyong (Kim *et al*, 1993), and Yonchon (Seong *et al*, 1997) have also been isolated. These variants are distinguishable from one another by serological cross-tests with strain-specific polyclonal or monoclonal antibodies. The serotype variation of *O. tsutsugamushi* depends on the antigenicity of an immunodominant 56 kDa major protein, which was shown to be a type-specific antigen, that is located on the outer membrane of located on *O. tsutsugamushi* (Murata *et al*, 1986; Ohashi *et al*, 1992). In recent years, the polymerase chain reaction (PCR), restriction fragment length polymorphism (RFLP), and sequence analysis have proved to be valuable diagnostic tools (Kelly *et al*, 1994; Murai *et al*, 1995; Horinouchi *et al*, 1996; Ohashi *et al*, 1996; Tselentis *et al*, 1996).

Xisa archipelago is situated on China's southern coast; the islands belong to Hainan Province. In recent years, the incidence of tsutsugamushi disease in the south sea islands of China has increased (Wang *et al*, 2000) and the health of the people of the area has been affected. Because the islands are far from the mainland, the isolation of *O. tsutsugamushi* is difficult. PCR and sequence analysis are valuable approaches; in this study, we describe the amplification and sequencing of *O. tsutsugamushi* DNA from mites that were collected in the Xisa archipelago.

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MATERIALS AND METHODS

Study area

The Xisa archipelago lies at latitude 15º43′ ~17º18′N, longitude 111º6′~112º54′E. Xisa archipelago consists of four islands. The area has a southern tropical sea climate. The average temperature is 26.4ºC. The average annual rainfall is 1509.8 mm, the relative humidity is 70-80%.

Sample collection

The mites were collected from moist marshland soil in the study area in May 2000. The mites were placed in cryotubes and stored in liquid nitrogen and 95% ethanol. Chick embryo yolk sac liquid (Karp, Kato, and Gilliam strains) was purchased from the Beijing Biotransformation Institution.

DNA extraction

The method advanced by Peng *et al* (2000) was used. Each pool, consisting of 50~100 mites, was homogenized in 1.5 ml of protease K lysis buffer (0.5% sodium dodecyl sulfate, 25 mmol/l sodium acetate, 2.5 mmol/l ethyenediaminetetraacetic acid), protease K (1 mg/ml) was then added; the mixture was then incubated at 50ºC for six hours. The DNA was purified by repeated phenol-chloroform extraction followed by ethanol precipitation. The DNA pellets were resuspended in 50 μl of 1 x TE buffer (pH8.0) and used as the templates for PCR.

Primers and PCR amplification

The method of Peng *et al* (2000) was adopted. The specific primers for DNA amplification used in this study were selected on the basis of the gene encoding of the antigenic 56 kDa protein. Amplification of the DNA was by nested PCR (N-PCR). Briefly, the first amplification was carried out by primers 1 (5′-GATCAAGCTTCCTCAGCCTACTATAATGCC-3′) and 2 (5′-CTAGAAGTTATAGCGTAC ACCTGCACTTGC-3′). The second amplification was carried out using primers 1 and 3 (5′-CTAGGGATCCGACAGATGCACTATTAGGC-3′). All primers were designed to amplify the part sequence that codes the 56 kDa type-specific antigen protein. Both the first and second amplifications continued for 35 cycles. After the first denaturing step of 3 minutes at 95ºC, the polymerase chain reactions were carried out for 35 cycles of denaturing at 94ºC for 45 seconds, annealing at 58ºC for 1 minute, and extension at 72ºC for 2 minutes with a final extension at 72ºC for 10 minutes. The N-PCR products were electrophoresed in 1.5% agarose gels, which were then stained with ethidium bromide and viewed by ultraviolet transillumination. If a 507 bp specific band was detected, then the sample was deemed to be positive for *O. tsutsugamushi* infection.

Restriction fragment length polymorphism (RFLP)

The method of Peng *et al* (2000) was used for RFLP. The restriction endonucleases, HincII and PstI, were selected (referencing prototype *O. tsutsugamushi* strains Gilliam, Karp, and Kato). When the amplified products were digested with PstI, the Karp DNA generated fragments of 129 bp, 162 bp, and 216 bp; the Gilliam DNA generated fragments of 123 bp and 357 bp. When the amplified products were digested with HincII, the Kato DNA generated fragments of 173 bp and 307 bp. In conclusion, when the N-PCR products were digested with HincII and PstI, the Kato, Karp, and Gilliam strains could be differentiated by their restriction endonuclease profiles. PCR products (15 μl) were digested overnight with the restriction endonuclease in a 20 μl volume, following the conditions suggested by the manufacturer. The digested samples were resolved by electrophoresis in 3% agarose gels, stained with ethidium bromide, and viewed by ultraviolet transillumination.

Cloning

Using the method of Peng *et al* (2000), the N-PCR products, which were purified by phenol-chloroform and 70% ethanol precipitation, were cloned into pGEM-T vector using the TA cloning kit (Promega) according to the

manufacturer's instruction. The recombinant plasmid was identified by N-PCR with the above primers and the 507bp products amplified by N-PCR from the positive recombinant plasmids were digested with HincaII and PstI to classify their genotyps.

Extraction of plasmid and sequencing

A positive colony was incubated in 5 ml LB medium for 8 to 12 hours; the DNA of the plasmid was the extracted using the Wizard Plus SV Minipreps DNA Purification System Kit (Promega). The purified plasmid DNA was sent to the Beijing Saibaisheng Company for sequencing.

Sequence analysis

A homology search was made via the internet using BLAST. The nucleotide sequences were aligned and the phylogenetic tree was constructed by CLUSTAL (version 1.74; European Molecular Biology Laboratory, Heidelberg, Germany). Bootstrap re-sampling tests were performed to confirm its reliability.

RESULTS

Mite species

98.50% (1182/1200) of the mites collected were *Leptotrombidium (L.) deliense;* 1.33% (16/1200) were *Walchia chinensis;* 0.17% (2/1200) were *Odontacarus majesticus.*

Analysis of PCR amplification

The amplification products was electrophoresed on 1.5% agarose gels which were then stained with ethidium bromide and observed under ultraviolet transillumination. The 507 bp-specific band was detected.

Analysis of RFLP

When NPCR products from the mites were digested by the restriction endonucleases PstI, the profiles, with bands 129 bp, 162 bp and 216 bp, were compatible with the profiles of Karp strain. The products can not be digested by restriction endonucleases HincII.

Sequence analysis of positive colonies and their genetic relationship

Two selected colonies were classified as a single strain by PCR-RFLP. The results of sequencing (Fig 1) were compared with the sequences of Karp, Kato, Gilliam, Yonchon, Shimokoshi, TA763,TA686, Boryong, Kuroki, and Kawasaki in the DDBJ. The results revealed 85% homology with the Karp strain, 68% homology with the Gilliam strain, 65% with the Kato strain, and 67% with Yonchon. The phylogenetic tree analysis represents all these colonies' genetic relationships (Fig 2). On bootstrap analysis, the genetic groups were highly reliable.

DISCUSSION

It is warm and humid in the Xisa archipelago, which is the south subtropical island natural foci of tsutsugamushi disease. But the isolation of etiological agent in the area is difficult because these islands are far from the continent and lack of medical condition and instruments. The clinical cases could be easily misdiagnosed due to lack of specificity of presenting symptoms. On the other hand, the symptoms of tsutsugamushi disease appeared after 1 to 2 week-latent period, and the antibody titer against *O. tsutsugamushi* was not high enough to be detected at the initial stage. In recent years, polymerase chain reaction and restriction fragment length polymorphism have provided a valuable diagnostic approach (Tay *et al*, 1996; Seong *et al*, 1997; Peng *et al*, 2000), meanwhile also provided an approach of studying the biological and genetic characteristics of *O. tsutsugamushi* in these areas. We successfully amplified the part-DNA of mites; the sequence that codes the 56 kDa type-specific antigen protein. The mites are small and easy to carry and to preserve, while the mites are the vector of tsutsugamushi disease. The *O. tsutsugamushi* in mites is directly related to illness of human. So these methods are more valuable

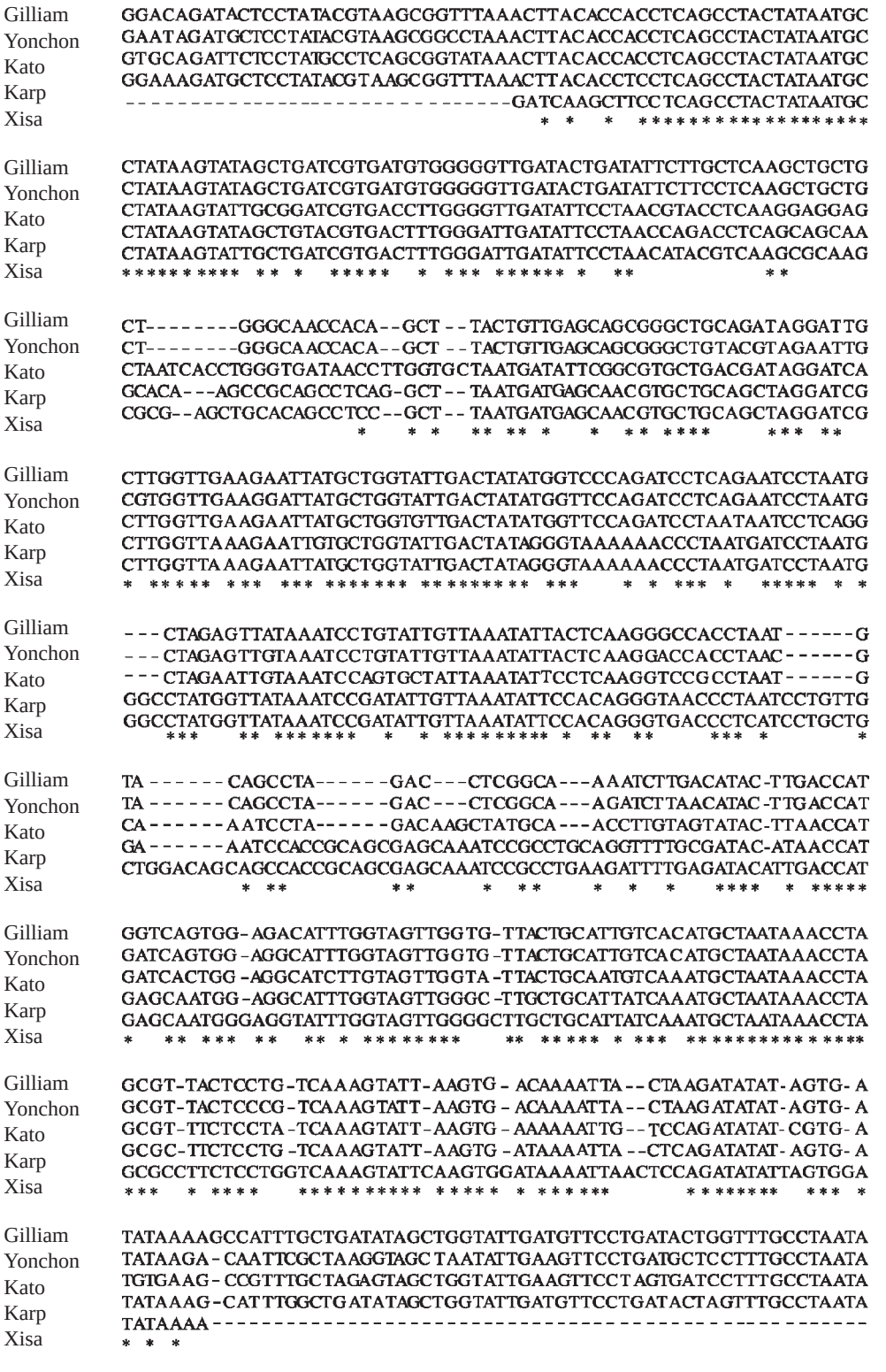

Fig 1–Comparison of sequences of *Orientia tsutsugamushi* strains from Xisa archipelago with reference strains.

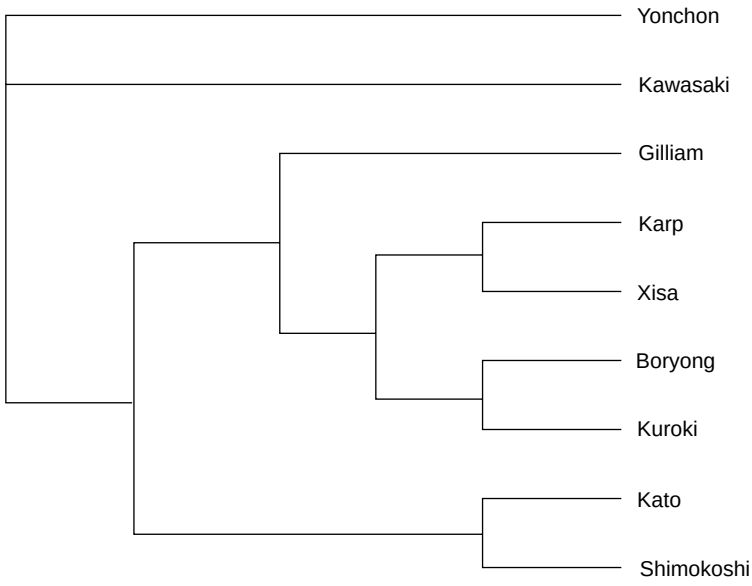

Fig 2–The phylogenetic tree of the *Orientia tsutsugamushi* strains form Xisa archipelago.

in these islands for study of tsutsugamushi disease. Our results showed that the preponderant species of mite in the area is *Leptotrombidium (L.) deliense*. The main *O. tsutsugamushi* in the area is Karp strain, but the gene homology with Karp, only 85%, suggests the possibility of a mutation of *O. tsutsugamushi*.

The islands are far from the mainland. How has the natural focus of tsutsugamushi disease formed? Some researchers have argued that the focus has been caused by migratory birds (Cheng 1987). We suggest that merchant ships from other regions or nations (such as Korea) may have given rise to the focus, these ships may have carried rodents to the islands. In many past years, the mutation of etiological agents took place to adapt themselves to geographical and climatic surrounding on these islands. Finally, the preponderant etiological strain has formed on these islands, although these islands are far from the continent.

The geographical distribution of the preponderant *O. tsutsugamushi* strain in the south sea islands is currently being investigated. This study may provide a useful basis for the control of the disease.

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