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Multiple lines of evidence on the genetic relatedness of the parthenogenetic and bisexual *Haemaphysalis longicornis* (Acari: Ixodidae)

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ABSTRACT

As an obligate hematophagous ectoparasite, the hard tick *Haemaphysalis longicornis* exhibits two reproductive strategies, bisexual reproduction, and obligate parthenogenesis, which have attracted a widespread attention. However, the speciation of parthenogenetic population remained ambiguous due to its similarity in morphology but the remarkable differences in cytogenetics as compared with those of the bisexual ones. In the present study, we explored several new lines of genetic evidence to resolve this controversial issue. The number of the chromosomes in two lineages was checked by classical methods and their total DNA levels were determined utilizing flowcytometry. In addition, the sequences of 12S rDNA, 16S rDNA, cytochrome c oxidase I and II (COI, COII) and internal transcribed spacer-2 (ITS-2) genes were used to assess their phylogenetic relationship. We observed that the chromosome ploidy of bisexual and parthenogenetic *H. longicornis* collected by our laboratory was diploid and triploid, respectively. Flowcytometry analysis indicated a ratio close to 2:3 in the DNA contents of bisexual to parthenogenetic *H. longicornis*. Although the chromosome ploidy is different, their gene sequences are extremely similar. Analogous to the intra-species genetic difference of other invertebrates, sequence differences of all loci examined are below 2%. Phylogenetic trees constructed from 12S rDNA, 16S rDNA, COI, and ITS-2 genes revealed that they were all in the same monophyletic clade instead of splitting independently into evolutional branches. Moreover, according to 4× Rule, the K/θ ratio of two reproductive populations calculated based on COI was much smaller than four, strongly supporting that they belong to the same species. Therefore, we conclude that the evolutionary process just disturbs the chromosome ploidy and the sexual determination of parthenogenetic population and that it would be better to consider parthenogenetic *H. longicornis* as a metapopulation rather than a cryptic species.

1. Introduction

Ticks are obligate hematophagous ectoparasite of a variety of vertebrates, including amphibians, reptiles, birds, and mammals. As vector for numerous pathogens, they have attracted much scientific and public attention due to their medical and veterinary importance (Jongejan and Uilenberg, 2004). Their vectorial competence should be attributed to the long period (e.g. 4–14 days for ixodid ticks) of attachment to their hosts (Kaufman, 2010).

The hard tick *Haemaphysalis longicornis* Neumann, 1901, which is widely distributed in China, New Zealand, Korea, Japan and Australia (Hoogstraal et al., 1968; Teng and Jiang, 1991), can transmit a large variety of pathogens including *Theileria* (Li et al., 2009), *Babesia* (Guan et al., 2010; Ros-García et al., 2013), *Rickettsia* (Zou et al., 2011) and tick-borne encephalitis virus (Kovalev and Mukhacheva, 2013). This tick species exhibits two reproductive strategies, i.e. bisexual and parthenogenetic strategies. The parthenogenetic individuals are known from certain areas in China, Australia, New Zealand, New Caledonia, Fiji, New Hebrides, Tonga, Hokkaido, and Honshu Islands of Japan (Herrin and Oliver, 1974), whereas the distribution of the parthenogenetic population in China has been reported only in Sichuan province (Yang et al., 2007), and was occasionally found in one zoo of Shanghai without a population established (Zhou et al., 2004), and hence, the vectorial role of this

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parthenogenetic population in China is still unknown. Parthenogenetic *H. longicornis* used in this study originated from the field-collected obligatory parthenogenetic population in Sichuan province of China, and has been maintained for several generations in our laboratory since 2007. They exhibited strictly reproductive isolation with the bisexual population, and no males were observed throughout the whole life cycle. Previous studies in our laboratory have investigated the morphological, biological, and molecular characteristics of bisexual and parthenogenetic *H. longicornis* (Chen et al., 2012a), and further illustrated the structure of Haller's organ and its development in parthenogenetic lineages (Wang et al., 2013). Compared to bisexual ones, parthenogenetic females produce about twice as many offspring. Karyological analysis revealed the diploid chromosome set in obligatory bisexual *H. longicornis* and triploid set in obligatory parthenogenetic individuals (Oliver et al., 1973). These attributes make the parthenogenetic *H. longicornis* one of the special cytogenetic cases as a valuable model for us to study the reproductive development and evolution of arthropods. However, the two reproductive populations are extremely similar in their morphology except for some differences in Haller's organ (Wang et al., 2013), genital apron and life cycle (Chen et al., 2012a). Therefore, in this study, we focused on assessing the relationships among parthenogenetic and bisexual *H. longicornis* with a number of molecular markers by comparing the similarities and discrimination of these genetic markers between parthenogenetic and bisexual *H. longicornis*, aiming to gain better understanding on the relatedness of these populations, and to identify the specific genetic markers used for rapid identification of parthenogenetic *H. longicornis*.

2. Materials and methods

2.1. Collection and rearing of ticks

Nymphs of parthenogenetic *H. longicornis* and *Haemaphysalis doenitzi* were collected from vegetation by blanket dragging in Cangxi county (31°37′–32°10′N, 105°43′–106°28′E) in Sichuan province, while the bisexual *H. longicornis* ticks were collected from the naturally infested sheep in Xiaowutai National Natural Reserve Area (39°50′–40°07′N, 114°47′–115°30′E) in Hebei province, China. Location data were converted to keyhole markup language (KML) file, which are available as Supplementary Data to be visualized in Google Earth (http://earth.google.com) (Supplementary KML file). All colonies of these ticks were fed on rabbits in our laboratory as described previously (Liu et al., 2005; Chen et al., 2012b). During the off-host periods, ticks were maintained in cotton-plugged glass tubes filled with folded filter paper in an incubator with 80% relative humidity and 6/18 h of light/dark (L/D) cycle at 26 ± 1 °C. Procedures involving animals were carried out by following Guidelines of Institutional Animal Care and Use Committee of Hebei Normal University.

2.2. Chromosome preparations

Single tick cells were acquired from tick embryos. In details, the eggs with a mass of 500 mg were collected from 10 females at 8–15 days after the onset of oviposition, disinfected with 70% ethanol for 5 min and crushed with a flattened end of the glass rod in PBS solution (pH 7.4). Resultant was filtrated and centrifuged at 1500*g* for 5 min to gain the single tick cells. Metaphase chromosomes were prepared following the protocols developed for the karyology of tick cell line according to Esteves et al. (2008) with minor modification. Single cells were incubated in PBS containing 0.4 μg/mL colchicines at 30 °C for 45 min, then treated with hypo-osmotic solution (75 mM KCl) and fixed with methanol-glacial acetic acid solution in 3:1 proportion. The suspension was dropped onto cold glass slides and stained with Giemsa. Chromosome photos were taken by Digital Microscope (VHX-600ESO, Keynce, Japan) and optimized by Adobe Photoshop CS (Adobe Systems Inc., Seattle, WA, USA). To accurately determine the chromosome number, more than 100 sets of well-separated metaphase chromosomes were examined and counted, respectively.

2.3. Flow cytometry analysis

Flow cytometry was used to quantify the number and relative size of cells or cellular components based on the fluorescent intensity of fluorochrome-labeled material (Cram, 2002). Total DNA content of ticks was assessed using a modified version of the methods according to DeSalle et al. (2005a) and Geraci et al. (2007). Single tick cell suspension was prepared as described in Section 2.2. of chromosome preparations, and labeled by propidium iodide (PI) binding buffer (trisodium citrate, 1 mg/mL; PI, 50 μg/mL; tritonX-100, 1%; and RNase A, 20 μg/mL) for 30 min in the dark at 4 °C, then centrifuged at 1500*g* for 5 min and re-suspend with PBS. Three samples of two reproductive populations were conducted independently. DNA content analysis was performed by flowcytometry using Epics-XL II FACS Caliber flowcytometer (Beckman Coulter, CO, USA) and Multicycle AV Software.

2.4. DNA and RNA extraction and cDNA synthesis

Unfed adult ticks of two reproductive populations of *H. longicornis* and *H. doenitzi* were used for amplification of gene fragments. Ten unfed adult ticks of each strain were disinfected with 70% ethanol for 5 min and rinsed with PBS three times before they were used for DNA or RNA extraction. Genomic DNA was isolated using DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Total RNA was extracted with AxyPrep mRNA Extract Kit (Axygen Biotechnology Limited, Hangzhou, China) and reversed transcription was performed with SuperScript First-Strand cDNA synthesis Kit (Invitrogen, China) according to the manufacturer's instructions.

2.5. PCR and DNA sequencing

A combination of mitochondrial DNA-encoded genes and the nuclear DNA-encoded ribosomal genes is particularly suitable for molecular phylogenetic analysis because of maternal inheritance and different evolutionary rates (Casati et al., 2008). We amplified multiple genes, including the mitochondrial DNA-encoded 12S rDNA, cytochrome c oxidases I (COI) and COII, as well as the nuclear DNA-encoded ribosomal loci 18S rDNA and internal transcribed spacer 2 (ITS-2), from *H. longicornis* and *H. doenitz*. Additionally, nuclear protein coding genes including elongation factor 1-alpha gene (*EF-1α*) and heat shock protein gene 82 (*hsp82*), which are usually used for phylogenetic analysis in insects and mites, and 17-beta-hydroxysteroid dehydrogenase (*17β-HSD*), which is hypothetically responsible for catalyzing the sex hormone and involved in fatty acid elongation (Moeller and Adamski, 2009), were also amplified.

The primer sets include the previously published and newly designed sequences for the corresponding genes and were synthesized by Invitrogen (Beijing, China) (Table 1). PCR reactions of each gene were performed with high fidelity polymerase (Invitrogen, China) and repeated three times. All amplified PCR products were purified using QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany), and cloned into pMD19-T vector (TaKaRa, Dalian, China). At least three positive clones per gene were picked and sequenced by Invitrogen Sequencing Service (Beijing, China). All