Establishing a new animal model for hepadnaviral infection: susceptibility of Chinese Marmota
species to woodchuck hepatitis virus infection

Bao-Ju Wang,1 Yong-Jun Tian,1 Zhong-Ji Meng,1 Min Jiang,2 Bo-Qing Wei,3 Yuan-Qing Tao,3 Wei Fan,3 An-Yi Li,1 Jun-Jie Bao,1 Xin-Yu Li,1 Zheng-Mao Zhang,1 Zhong-Dong Wang,3 Hu Wang,3 Michael Roggendorf,4 Meng-Ji Lu4 and Dong-Liang Yang1

Correspondence
Dong-Liang Yang
dlyang@tjh.tjmu.edu.cn
Meng-Ji Lu
mengji.lu@uni-due.de

1Division of Clinical Immunology, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, PR China
2Department of Microbiology, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, PR China
3Qinghai Institute for Endemic Disease Prevention and Control, Xining, PR China
4Institut für Virologie, Universitätsklinikum Essen, Essen, Germany

Hepatitis B virus infection (HBV) is a major medical problem in China. The lack of a suitable infection model in China is recognized as an obstacle for research on HBV in China. Chinese Marmota-species is phylogenetically closely related to Marmota monax, thus, it might be suitable to serve as an animal model for HBV infection. Therefore, we attempted to prove the claim about the existence of woodchuck hepatitis virus (WHV)-like viruses in Chinese Marmota-species and to determine the susceptibility of these species to experimental WHV infection. In the present study, 653 sera from three Chinese Marmota-species, Marmota himalayana, Marmota baibacina and Marmota bobak, were screened for WHV-like viruses by serological and molecular assays. The susceptibility to WHV of three species was investigated by experimental infection and monitored by testing of anti-WHc and WHsAg by ELISA, detection of WHV DNA by PCR, and detection of WHV replication intermediates and antigens in liver samples. No evidence for the existence of a genetically closely related virus to WHV in three Chinese Marmota-species was found by serological assays and PCR. M. himalayana was susceptible to WHV infection as inoculated animals became positive for anti-WHc, WHsAg and WHV DNA. Further, WHV replication intermediates and proteins were detected in liver samples. In contrast, M. baibacina remained negative for tested virological parameters. M. bobak species showed a limited susceptibility to WHV. Our data do not support early reports about WHV-like viruses in China. M. himalayana is suitable for the establishment of a model for hepadnaviral infection.

INTRODUCTION

Hepatitis B virus (HBV) causes acute and chronic infections and is one of the major factors for high incidence of hepatocellular carcinoma worldwide (Beasley et al., 1981; Jung & Pape, 2002; Kremsdorf et al., 2006; Shepard et al., 2006; Williams, 2006; Yim & Lok, 2006). The animal models play a crucial role in research on HBV. From the understanding of the HBV replication cycle to the evaluation of antiviral agents against HBV, progression was strongly dependent on the availability of suitable animal models. The chimpanzee is the best model due to its close relationship to humans and its susceptibility to HBV (Guidotti et al., 1999; Muchmore, 2001; Murray et al., 2005; Ogata et al., 1999; Prince & Brotman, 2001; Thimme et al., 2003). However, the chimpanzee model is expensive and could not be used widely. The Pekin duck served as an excellent model for studies on the molecular biology of hepadnaviruses and for drug testing (Freiman et al., 1988; Mason et al., 1980, 1982; Molnar-Kimber et al., 1983; Rollier et al., 1999; Schultz et al., 2004; Summers & Mason, 1982; Triyatni et al., 1998). Recently, the woodchuck model became attractive since the infection of woodchuck hepatitis virus (WHV) in woodchucks strongly resembles the HBV infection in humans in the major virological, pathological and immunological features (Galibert et al.,...
The experimental infection of adult woodchucks leads to an acute self-limiting course with transient viraemia and antigenemia. Woodchucks with chronic WHV infection exist in nature, probably due to the high rate of vertical transmission. Neonates could be persistently infected with WHV if they were exposed to WHV early after birth (Cote et al., 2000). Infected woodchucks develop hepatitis and also regularly develop hepatocellular carcinoma at a high rate (Hsu et al., 1988; Tennant, 2001). The immunological response to WHV in woodchucks is comparable to that of HBV in humans. It should be mentioned that the serological profiles in HBV and WHV are exactly the same, either in the acute self-limiting or in the chronic course of infection. Further, the cellular immune responses to WHV in acute self-limiting infection are usually strong and multispecific, while absent in chronic WHV infection (Cote & Gerin, 1995; Menne et al., 1997, 1998). These features are similar to those in humans. Thus, the woodchuck model is an informative animal model and the results generated from this model are particularly useful since they reflect the clinical situation. Recently, this model has been used for the testing of antiviral drugs (Colonnio et al., 2001; Korba et al., 2000; Mason et al., 1998; Peek et al., 2001) and vaccine prototypes (Fiedler et al., 2001; Lu et al., 2007; Lu & Roggendorf, 2001), studying pathogenesis of hepadnaviral infections (Guo et al., 2000; Kajino et al., 1994; Lu et al., 2001, 2002; Mason et al., 2005; Michalak et al., 2004; Nakamura et al., 2001; Summers & Mason, 2004), and as a model for liver transplantation and reinfection (Dahmen et al., 2002).

The analysis of cytochrome (cyt) b sequences of the Marmota-species revealed that the European-Asian species were probably evolved from a common ancestor with Marmota monax (Steppan et al., 1999). It is proposed that the ancestor of the Marmota-species crossed the Bering Bridge during the late Pliocene and then radiated into the present European-Asian species and M. monax during the Pleistocene. The hypothesis implied that the European-Asian species of the Marmota-family has a close evolutionary relationship to M. monax and may likely be susceptible to WHV infection. In addition, it could be speculated that the ancestor of Marmota-species may carry WHV-like virus to the European-Asian population. Thus, it is reasonable to test Asian Marmota-species for their susceptibility to WHV and the presence of WHV-like viruses.

As HBV infection is highly prevalent in China, it is crucial to establish a useful and affordable animal for studies on HBV infection. Until now, the use of M. monax in China is very limited due to the high costs and safety issues. Therefore, it is rational to test Chinese Marmota-species for this purpose. There were previous Chinese reports about the possible existence of WHV-like viruses in Chinese marmots. These studies were performed by using commercially available diagnostic assays for serological and virological markers of HBV infection. No molecular biological method was applied in these studies. Therefore, no direct evidence was provided for the existence of a WHV-like virus. Jin et al. (1988) reported about 20 years ago that an experimental infection of a species of Chinese marmots, Marmota bobak Sibirica Rabbe, with WHV led to a detectable viraemia in one of seven animals. However, this study did not use specific assays that are now commonly accepted. Thus, it is difficult to interpret the obtained results in this study.

In the present study, we addressed the questions about the existence of WHV-like viruses in Chinese Marmota-species and the suitability of these species as an animal model for hepadnaviral infection. We collected and tested 653 samples of sera from Chinese marmots by specific serological assays for WHV infection and PCR amplification with conserved primers for hepadnaviruses. Finally, the susceptibility of three different Chinese Marmota-species to WHV was tested by experimental inoculation of naive animals.

**RESULTS**

**No evidence for the existence of a genetically closely related virus to WHV in Chinese marmots**

The early report about the possible existence of WHV-like viruses in Chinese marmots was mainly based on serological testing using assays designed to detect HBV surface and e antigens. In addition, virus-like particles were seen by electron microscopy. However, these findings were not confirmed by more specific serological assays and new molecular biological assays. Therefore, we attempted to find evidence for WHV-like viruses in samples from Chinese marmots by molecular methods including detection of anti-WHc by using a specific ELISA, spot blot hybridization with specific WHV and HBV probes and PCR amplification with conserved primers.

Firstly, 653 sera samples were collected from different regions in North-west China (Fig. 1). The majority of the samples were from two species Marmota baibacina and Marmota himalayana (Table 1). The specific serological assay for the detection of anti-WHc was applied to screening the samples for the infection of potentially existing WHV-like viruses. The samples collected from animals in the wild frequently tested positive for anti-WHc ELISA. However, such samples collected in the wilderness were often haemolytic and caused high unspecific signals in both the sandwich and competitive ELISA tests. Eighty-five serum samples from three Marmota-species were taken under laboratory conditions and were found to be negative for anti-WHc ELISA with only one exception.

The serum samples in group QT were subjected to spot blot hybridization with a full-length WHV or HBV genome as a probe, no specific signal was seen (data not shown). Further, PCR with different primers was performed with all samples that showed a positive signal for anti-WHc ELISA.
These primers are selected due to the conservation of these sequences among the mammalian hepadnaviruses. It is possible to amplify the corresponding region from the HBV and WHV genome using these primers. However, no positive results were generated by screening these serum samples of Chinese marmots (Supplementary Fig. S1, available in JGV Online).

Taken together, we did not find evidence supporting the hypothesis that WHV-like viruses exist in Chinese marmots. But the present negative evidence does not exclude the possible existence of hepadnaviruses genetically distant to WHV and HBV. Our results indicate that false-positive signals in ELISAs could be produced with sera from Chinese marmots due to inappropriate storage conditions of the samples.

**Experimental infection of Chinese marmots with WHV**

To answer the question whether Chinese *Marmota*-species are susceptible to WHV infection, wild-caught animals from different locations in China were kept under laboratory conditions and experimentally inoculated with WHV stocks. To accurately classify the animals included in the experiments, a genetic analysis based on the sequence of *cyt b* was performed. The *cyt b* sequences from wild animals that were caught were amplified and analysed by DNA sequencing. The *cyt b* sequences generated in this study were compared with known *cyt b* sequences from different *Marmota*-species using a online software CLUSTAL w, a bioinformatic tool to determine the phylogenetic relationship based on the sequence data. According to the analysis, three *Marmota*-species, *M. himalayana*, *M. baibacina* and a species between *M. baibacina* and *M. bobak* were caught (Fig. 2, Supplementary Table S3, available in JGV Online). The animals from the regions Tongren county, Huangnan county, Tongde county in the Qinghai province and Tangla mountain range belonged to the species *M. himalayana*. The marmots from Xingjiang were identified as *M. baibacina*. The animals from the

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<tr>
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<td><em>M. bobak-like</em></td>
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*The detection of viral DNA was based on spot hybridization or on specific PCR for hepadnaviral viruses.
†The determination of species was based on the morphology according to the zoological classification.
‡Sandwich ELISA by using HRP-SPA.
§Competitive ELISA by using mouse anti-WHc polyclonal antibody.
||Animals are maintained in an animal centre.
Guinan county in the Qinghai province were closely related to *M. baibacina* and *M. bobak*, but were more closely related to *M. bobak*, this species is tentatively indicated as *M. bobak* in this study.

Four of *M. himalayana* were inoculated with a dose of $10^8$ WHV genome equivalents (GE) and one animal served as an uninfected control (Fig. 3a, Table 2). All four inoculated animals developed anti-WHc. Three animals became positive for WHV DNA in week 2 post-inoculation (p.i.) and one additional in week 4 p.i. WHV DNA remained detectable until week 10 p.i. in all animals. One animal was sacrificed at week 10 p.i. and the WHV infection in this animal was demonstrated by detection of WHV replication intermediates in liver tissues by Southern blotting and by immunohistochemistry (IHC) staining of WHV proteins in liver sections (Fig. 3b). In liver sections of the WHV-infected *M. himalayana*, a typical liver inflammation with lymphocyte infiltration into the portal area could be observed (Fig. 3c). The expression of WHsAg and WHcAg in hepatocytes could be detected with specific anti-WHs and anti-WHc antibodies (Fig. 3c).

The susceptibility of *M. himalayana* to WHV infection was confirmed in a second experiment with eight animals (Fig. 4a, Table 2). Four of them developed typical acute WHV infection with viraemia, while four other animals developed only anti-WHc antibodies. One animal was sacrificed in week 12 p.i. for the preparation of liver samples. Another animal cleared WHV in peripheral blood in week 23 p.i. Two control animals were kept without inoculation and did not show any sign of WHV infection. These results indicated again that *M. himalayana* developed acute, self-limiting WHV infection like *M. monax*.

In contrast to *M. himalayana*, six *M. baibacina* did not show any sign of WHV infection after the inoculation with the same WHV stock. No WHV DNA, WHsAg and anti-WHc could be detected during a follow-up period of 35 weeks (Fig. 4b, Table 2). Additionally, two animals received treatment with cyclosporine for 4 weeks after the inoculation. However, these animals failed to show any sign of WHV infection. Thus, *M. baibacina* was not susceptible to WHV infection.

Five animals of the species *M. bobak* were inoculated with WHV. Three animals developed anti-WHc (Fig. 4c, Table 2). WHV DNA and WHsAg were detectable in two animals. Two other animals remained negative for all parameters of WHV infection. Our findings were consistent with the results of Jin et al. (1988). It was reported that an experimental infection of *M. bobak* Sibirica Rabbe with WHV led to detectable viraemia in only one of seven
Fig. 3. Experimental infection of *M. himalayana* with WHV. The animals (TR0302, 303, 304, 306 and control) were inoculated by intravenous injection of $10^8$ of WHV GE in a volume of 0.5 ml and sacrificed at weeks 10 or 12 p.i. WHV replication intermediates and WHV protein expression in liver tissues of infected *M. himalayana* were detected. (a) Detection of anti-WHc, WHsAg and WHV DNA. (b) WHV replication intermediates were detected by Southern blotting with a full-length WHV genome as probe. (c) WHsAg (left) and WHcAg (right) were detected in liver sections by IHC staining with a polyclonal antibody to WHsAg and mAb to WHcAg, respectively. MH−, Liver section from a naive *M. himalayana*; MH+, liver section from a WHV-infected *M. himalayana*; W, liver section from a WHV-infected woodchuck.
animals (Jin et al., 1988). Taken together, M. bobak seems to be only partially susceptible to WHV infection.

**Passage of WHV in M. himalayana**

To test whether WHV could be passed in M. himalayana, serum samples were collected from WHV-infected animals during the acute phase and used for inoculation of naive individuals of M. himalayana. Two serum samples from M. himalayana contained high concentrations of WHV GE of $7.6 \times 10^8$ and $1 \times 10^9$. Inoculation of naive individuals of M. himalayana led to the development of acute WHV infection (data not shown). Two complete WHV genome clones from the WHV stock and sera of different infected animals were cloned and compared. In addition, eight clones of a partial polymerase gene (nt 1189–2675) from each sample were analysed. Sequencing analysis was performed with the WHV stocks passaged in M. himalayana and indicated that no accumulation of nucleotide substitutions in the WHV genome occurred during two passages.

**DISCUSSION**

In the present study, we found that different Chinese Marmota-species have different susceptibility to WHV infection. While M. himalayana was fully susceptible to WHV, M. baibacina showed no sign of WHV infection after inoculation with the same WHV stock. Another M. bobak species seems to show a limited susceptibility to WHV infection. Previously, Jin et al. (1988) described that only one of seven M. bobak developed acute WHV infection after experimental inoculation. Therefore, the bobak group of genus Marmota including M. bobak and M. baibacina is not useful as animal model for hepadnaviral infection due to their limited susceptibility to WHV infection. M himalayana appears to be suitable as an experimental animal model for hepadnaviral infection. Our results clearly showed that an inoculation of M. himalayana with WHV led to the development of acute WHV infection with viraemia, antigenemia, viral replication in liver and induction of specific antibodies to WHV proteins. The course of WHV infection in experimentally inoculated M. himalayana had the same characteristics like that of M. monax. Further, sera from infected M. himalayana were infectious, thus the WHV infection in the new host was productive. The nucleotide sequence of the WHV genome remained unchanged after two passages in M. himalayana. The close genetic relationship of M. himalayana to M. monax is of great advantage for the use of M. himalayana in studies on the immune responses to hepadnaviral infection. It was very time-consuming to generate the reagents and to establish immunological assays for the woodchuck model. Since many genes of interest from these two Marmota-species are nearly identical, such reagents generated for the woodchuck model will be useful for the new Marmota-model.

It is proposed that the Asian Marmota-species and M. monax are probably derived from a common ancestor and developed as species in the Pleistocene, later than the separation of the species homo and other members of the family Hominidae (Steppan et al., 1999). Based on the morphological criteria, Cardini (2003) also proposed that M. monax and Asian Marmota-species belong to the subgenera Marmota. The genetic and immunogenetic analysis indicated that the Chinese Marmota-species are extremely closely related to M. monax, consistent with the current model of the evolution of the genus Marmota. The genes of the immune system of the Marmota-species are nearly identical in their nucleotide sequences, as analysed so far. Besides the genes described in the present study, the alpha interferon (IFN-$\alpha$) system of M. himalayana was characterized by molecular cloning and sequence analysis (Lu et al., 2008). At least four IFN-$\alpha$ subtypes of M. himalayana have an identity over 99% with the counterpart of M. monax. To further examine the phylogenetic relationship between M. himalayana and M. monax, seven genes relevant for the immune responses and for housekeeping were characterized. The cDNA sequences analysed so far showed an extremely high identity between the Marmota-species. For example, the interleukin (IL)-10 sequences of M. himalayana and M. baibacina had an identity of 99.4 and 100% to the counterpart of M. monax, respectively. Other cDNAs of M. himalayana like IL-6, IL-15, stat-1, IRF-1, GAPDH and $\beta$-actin had high similarities from 99.4 to 99.8% to the corresponding sequences of M. monax (Lohrengel et al., 1998; Wang et al., 2005). These data suggest again the common origin and a short
Fig. 4. The course of experimental infection of *M. himalayana* (a), *M. baibacina* (b) and *M. bobak* (c) with WHV. The animals were inoculated by intravenous injection of $10^8$ of WHV GE in a volume of 0.5 ml. The WHV infection in the animals was monitored by detection of anti-WHc, WHsAg and WHV DNA. Control, Animals without inoculation with WHV.
evolutionary distance between the Asian Marmota-species and M. monax.

In the present study, three different Chinese Marmota-species were tested for their susceptibility. The difference in the susceptibility to WHV is not yet understood. It will be interesting to investigate the factors determining the susceptibility to WHV infection. An immunosuppression with cyclosporine did not facilitate the WHV infection in M. baibacina, in contrast to early studies in M. monax (Cote et al., 1991, 1992). The block of WHV infection in M. baibacina may have other different reasons. It would be informative to test whether cultured hepatocytes from M. baibacina could be infected with WHV. Further, it will be of great interest to find out why only part of the M. bobak species was susceptible to WHV infection.

In this study, we screened a great number of serum samples from different Marmota-species, but failed to find any indication for the existence of WHV-like viruses. The early reports about the possible existence of such WHV-like viruses were based on the commercial serological assays for HBV and may not be reliable. A number of sera from Chinese marmots showed high background signals caused by haemolysis. Sera samples with haemolysis were often provided by hunters since they needed days to bring the blood over a long distance to the laboratories for processing. Such samples led to false-positive results in commercial assays and may be interpreted as evidence for the existence of WHV-like viruses in China. A careful examination of such samples showed that no WHV-like viruses could be detected.

There are two closely related hepadnaviruses identified in animals belonging to the squirrel family: WHV, ground squirrel hepatitis virus (GSHV) and arctic ground squirrel hepatitis virus (Marion et al., 1980; Summers et al., 1978; Testut et al., 1996). These viruses have a high identity over 90 % to each other. Thus, it is not surprising that the antigens of these viruses showed cross-reactivity in serological assays. Thus, it was reasonable to assume that the serological assays will detect the serological markers for the infection with a WHV-like virus in Chinese marmots. However, no positive signal was detected with sera from animals if the samples were prepared properly, for example, from 85 animals kept in the laboratories. To detect WHV-like viruses in serum samples from Chinese Marmota-species, PCR primers were designed according to the conserved regions of hepadnaviral genomes. The PCRs using these primers were successfully performed for amplification of the sequences of HBV, WHV and GSHV. However, we failed to amplify any DNA sequences related to a WHV-like virus. In summary, we did not find any evidence for the existence of WHV-like viruses in Chinese marmots.

**METHODS**

Animals. Adult animals were caught in different areas of North-west China: Ulumuqi, Xingjiang province; Tongren county, Thangla county and Guinan county, Qinghai province (Supplementary Table S1). Serum samples were collected from three Marmota-species from different areas of Xinjiang and Qinghai province, as indicated in Fig. 1. Serum samples (568) of groups XJ, QH and QT were collected by hunters from animals in the wild. Another 85 serum samples were taken from animals kept in the laboratory. The study protocol complies with the institution’s guidelines.

**Infection of Chinese Marmota-species with a WHV stock.** The animals were infected by intravenous injection of 500 µl serum from a chronically WHV-infected woodchuck via Vene saphane. The WHV stock contained 10^8 WHV GE ml⁻¹. Blood was taken from infected animals and tested for serological and virological markers of WHV infection. Animals were sacrificed and liver samples were preserved for extraction of DNA and RNAs and detection of WHV proteins.

**Serology.** Antibodies to WHV core antigen (anti-WHc) were detected by two different ELISA formats, the sandwich and competitive ELISA, as indicated in Table 2. Recombinant WHcAg particles were produced in Escherichia coli and purified by a combined protocol with precipitation with 30 % saturation of ammonium sulfate and chromatographic separation though a Superose 6 column (Zhang et al., 2006). The microtitre plate was coated with 10 µg purified WHcAg ml⁻¹. After blocking with 5 % FCS, 100 µl woodchuck sera of dilution 1:10 were added and incubated for 1 h at 37°C. In the sandwich format, bound woodchuck anti-WHc antibodies were directly detected by HRP-conjugated protein-A. For the competitive ELISA, biotin-labelled woodchuck anti-WHc were dispensed into the wells and incubated for 1 h at 37°C and the bound biotin-anti-WHc was detected by avidin-HRP conjugates. Tetramethylbenzidine was added for the colour development and the optical density (OD) values were measured at 450 nm. The inhibition of binding of biotin-anti-WHc was calculated with the formula 100 × (1-OD sample/OD negative control). The positivity of a sample for anti-WHc was defined if the inhibition was greater than 50 %.

**Detection of viral DNA.** Spot-blot hybridizations with a full-length WHV8 or a HBV genome as probes were routinely performed to detect WHV-like viral DNA in sera. For PCR detection of WHV-like DNA in sera, nucleic acids were extracted from sera by using a blood kit (Qiagen). Serum samples from WHV chronic-infected woodchucks (13 636 and 13 637) were used as positive controls. WHV primers Cp1/cw3a and WB1/WB2 were designed according to the conserved sequences on the hepadnaviral genomes (Supplementary Table S2, available in JGV Online). The PCR was run for 30 cycles with 94 °C for 1 min, 50 °C for 1 min and 72 °C for 1.5 min. WHV DNA in woodchuck sera was quantified by real-time PCR with light cycler DNA master SYBR green kit (Roche). The primers used for the PCR were QP1 and QP2 (Supplementary Table S2). The reactions were run in light cycler (Roche) with 95 °C for 0 s, 53 °C for 10 s and 72 °C for 12 s. A plasmid containing the full-length WHV genome was diluted and served as a standard. The detection limit of this assay was at 10^7 WHV GE per reaction.

**Analysis of WHV replication intermediates.** Total DNA from liver samples of WHV-infected woodchucks and Chinese marmots were extracted using the QIAamp Tissue kit (Qiagen) according to the manufacturer’s instructions. Briefly, about 25 mg frozen liver samples were ground to a powder in liquid nitrogen by using pestle and mortar, lysed in 180 µl lysis buffer and digested with protease K. Samples were then mixed with 210 µl ethanol and applied to a QIAamp spin column. DNA was bound to the column, washed twice and eluted by buffers supplied with the kit. WHV replication intermediates were analysed by Southern blot hybridization with a full-length WHV8 genome as probe as described previously (Lu et al., 2001; Nakamura et al., 2001).
IHC. Polyclonal antibodies to WHSAg were generated by immunization of rabbits with WHSAg. Staining for WHAg was performed using a monoclonal mouse antibody as described previously (Zhang et al., 2006). Liver tissues from WHV-infected animals were collected after the animals were sacrificed. The liver sections were prepared according to standard procedures followed by fixing in 10 % formalin, embedding in paraffin and cut into 4 μm. Sections were then stained with antibodies to WHSAg and WHAg or control antibodies according to the manufacturer's instructions. Briefly, the sections were treated with standard procedures to remove paraffin and then incubated with 0.3 % H2O2 for 30 min to eliminate the endogenous peroxidase activity. After washing with PBS, the sections were incubated with 10 % of normal goat serum for 30 min at room temperature. Antibodies to WHSAg and WHAg or unrelated control antibodies at a dilution of 1:500, 1:5000 and 1:1000, respectively, were added for overnight incubation at 4 °C. The bound antibodies were detected with Dako Envision kit.

Analysis of cyt b sequences from Marmota-species. For the phylogenetic analysis of Chinese Marmota-species, total DNA was extracted from peripheral blood lymphocytes or liver tissues of the animals and subjected to PCR amplification of the cyt b sequences. The PCR primers cytB-1 and cytB-2 were designed according to the cyt b sequence of M. himalayana (Supplementary Table S2). PCR was performed over 30 cycles of 1 min at 94 °C, 1 min at 55 °C and 2 min at 72 °C. The PCR generated DNA fragments of a length of 1140 bp. The PCR fragments were purified and subjected to direct sequencing using internal primers cytB-3 and cytB-4 from both orientations. The analysis of the sequence data was performed online with the software CLUSTAL W at the website of European Bioinformatics Institute (http://www.ebi.ac.uk). Only the part between nt 233 and 654 of the cyt b sequences was included for the analysis since the direct sequencing only generated reliable data for the middle part of PCR fragments. The analysed data were compared with the published phylogenetic data about the Marmota-species of Steppan et al. (1999).

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