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Research paper

Inter-individual and intragenomic variations in the ITS region of *Clonorchis sinensis* (Trematoda: Opisthorchiidae) from Russia and Vietnam

Yulia V. Tatonova[a,*], Galina N. Chelomina[a], Hung Manh Nguyen[b]

[a] Federal Scientific Center of the East Asia Terrestrial Biodiversity, Far Eastern Branch, Russian Academy of Sciences, 100-letiya Street, 159, Vladivostok 690022, Russia
[b] Institute of Ecology and Biological Resources, Vietnam Academy of Science and Technology, 18 Hoang Quoc Viet Street, Cau Giay Dist., Hanoi, Viet Nam

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ABSTRACT

Here we examined the intraspecific genetic variability of *Clonorchis sinensis* from Russia and Vietnam using nuclear DNA sequences (the 5.8S gene and two internal transcribed spacers of the ribosomal cluster). Despite the low level of variability in the ITS1 region, this marker has revealed some features of *C. sinensis* across multiple geographic regions. The genetic diversity levels for the Russian and Vietnamese populations were similar (0.1 and 0.09%, respectively) but were significantly lower than the *C. sinensis* from China (0.31%). About half of the sequences of the Chinese (53%) and Korean (47%) populations and about a tenth of the Vietnamese (12%) and Russian (8%) sequences included a 5 bp insertion. No sequences with nucleotide substitutions both upstream and downstream of the 5 bp insertion were found within the whole data set. The population of northern China had both sequence variants (with substitutions either upstream or downstream of the insertion), while only one of these variants was presented at the other localities. The Vietnamese population had a higher frequency of intragenomic polymorphism than the Russian population (69% vs. 46% and 23% vs. 3% at the 114 bp and 339 bp positions, respectively). These data are discussed in connection with parasite origin and adaptation, and also its invasive capacity and drug-resistance.

1. Introduction

Clonorchis sinensis (Cobbold, 1875) is an epidemiologically important helminth that inhabits East and South-East Asia. Clonorchiasis cases are registered in many countries, including China, Korea, Vietnam, and Russia (WHO, 1995; Mas-Coma and Bargues, 1997; Chai et al., 2005; Bray et al., 2008; Keiser and Utzinger, 2009). More than 35 million people worldwide are infected by Chinese liver fluke (15 million of them in China), and over 200 million are at risk of infection (Lun et al., 2005; Hong and Fang, 2012). Definitive host infection by the causative clonorchiasis agent occurs by eating raw or insufficiently cooked fish. *C. sinensis* usually parasitizes in the liver bile ducts of humans and some carnivorous animals, and less frequently infests the gall bladder or pancreas (Komiya and Suzuki, 1964; Posokhov, 2004). Under experimental conditions, in cases of superinfection, this liver fluke can be detected in the duodenum (Besprozvannykh et al., 2013). A high infestation intensity of *C. sinensis* leads to disability and even to death, resulting from pathological changes in the liver and bile ducts. The parasite causes mechanical damage and influences the excretory products of the bile ducts and gall bladder (Kruglyakova et al., 1987; Posokhov et al., 1987; Mas-Coma and Bargues, 1997). Recently, this species has been listed as a biological carcinogen (Bouvard et al., 2009).

Investigation of parasite genetic diversity is important for understanding its biology and biogeography, as well as for the development of the basis for the treatment, control, and prognostic estimation of invasion spread. Internal transcribed rDNA spacers are often used in genetic studies of the Chinese liver fluke, despite the fact that the intraspecific variation of these molecular markers is low for trematodes (Hashimoto et al., 1997; Park, 2007). Park and Yong (2001) reported a high homology level for the 18S, ITS2 rDNA, and *cox1* sequences of *C. sinensis* from Korea and China. However, three years later, Lee and Huh (2004) detected intraspecific variability for Chinese liver fluke from these countries. In this study, the ITS1 rDNA nucleotide sequences were used in addition to those markers listed above. Park (2007) confirmed the variability for these genetic markers. Using complete ITS1 rDNA nucleotide sequences, Kang et al. (2008) increased the investigated sample set to 22 specimens and revealed seven genotypes for *C. sinensis* in China and Korea. At the same time, the nucleotide sequences of the ITS2 region and *cox1* gene were obtained for *C. sinensis* from Russia and Japan, and analysis of the intraspecific genetic polymorphisms showed a low level of variability for this parasite (comparing Russian, Japanese, Chinese, and Korean specimens), both for nuclear (0.9–1.8%) and

* Corresponding author.
E-mail address: ytatonova@gmail.com (Y.V. Tatonova).

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mitochondrial (0.3%) markers (Katokhin et al., 2008). Xiao et al. (2013), focusing on the ITS1 region, described the genetic variability of *C. sinensis* from different Chinese provinces. The parasites were obtained from different definitive hosts in each locality. In the intraspecific phylogenetic tree constructed by Xiao et al. (2013), clusters well subdivided the populations from the different localities. The ITS1 and ITS2 regions have also been used to compare modern and ancient *C. sinensis* samples (Liu et al., 2007). DNA was extracted from eggs obtained from a patient's feces and from an ancient corpse buried in 167 BCE. The ITS2 region was identical in both samples. There were 15 nucleotide substitutions in the modern ITS1 sequence compared to the ancient samples. However, the genetic difference between the modern and ancient samples is similar to that detected between *C. sinensis* sequences from different countries (Lee and Huh, 2004), as well as among populations within one country (Xiao et al., 2013).

In recent years, the multilocus approach has been more frequently used for intraspecific studies of the genetic diversity of *C. sinensis*. For example, Sun et al. (2013) combined sequences from four nDNA nucleotide sequences (non-coding ITS1 region and protein-coding genes: actin (*act*), *β*-tubulin (*tub*) and elongation factor (*ef-1a*)) and four protein-coding mtDNA (*cox1*, *cox3*, *nad4* and *nad5* genes). The total sequence length was 4896 bp. Combined sequence analysis did not detect intraspecific subdivisions associated with geographical distribution. Also, no connection between parasite and host was detected. In addition to the multilocus approach, a haplotype net for *C. sinensis* has been constructed based on sequences of the ITS1 region. This analysis found that the ancient expansion of *C. sinensis* probably originated from central China. It should be noted that the rate of evolution of mitochondrial and nuclear DNA is different (Vawter and Brown, 1986), as is the mutation rate in coding and non-coding regions. In a study by Sun et al. (2013), nucleotide variability was shown to be 3-times higher for mitochondrial than nuclear markers. Such mitochondrial and nuclear differences were ignored in the analysis of combined nucleotide sequences.

Russia and Vietnam are the northernmost and southernmost parts of the *C. sinensis* area, respectively. Previously, we compared the *cox1* gene mtDNA sequences of *C. sinensis* from these countries (Chelomina et al., 2014). A nuclear marker can provide additional data for understanding the features of parasite evolution. Here, we study the intraspecific variation in the ITS region of parasites from these same populations.

2. Materials and methods

Adult worms of *C. sinensis* were collected from three localities in the Southern Far East of Russia and from two localities in different provinces of Vietnam. In Russia, metacercariae were detected in Cyprinidae fish, which were fed to laboratory rats. Fish were caught by fish traps. We used two rats for each locality, and each of these animals consumed four to 17 fish according to their size and intensity of infection. Within 21 days after infection, the liver of the rats was examined for the presence of adult flukes. Euthanasia of laboratory animals was carried out in accordance with the Committee on the Ethics of Animal Experiments of the Federal Scientific Center of the East Asia Terrestrial Biodiversity, Russia (Permit Number: 3 of 02.06.2011). In Vietnam, adult flukes were extracted from naturally infected cats, and livers of these cats were bought at slaughterhouses. Information about sampling locations is given in Fig. 1. The HotSHOT technique (Truett et al., 2000) was used for DNA extraction from 65 flukes (13 worms from each locality). The complete ITS1–5.8S–ITS2 rDNA sequences were directly sequenced from the PCR products. The primers for amplification and sequencing, the composition of the reaction mixture, and PCR cycling conditions are reported in Tatonova et al. (2012). PCR reactions and sequencing reactions with each primer were independently repeated at least two times. The nucleotide sequences were assembled manually and aligned using the Clustal X option (Thompson et al., 1997) in MEGA version 5.03. The relevant sequences for *C. sinensis* from other regions were downloaded from GenBank. Sixty-eight complete (1116/1121 bp) ITS1-5.8S-ITS2 rDNA sequences, as well as 98 complete (657/662 bp) and 236 partial (451/456 bp) ITS1 rDNA sequences, were analyzed (Table 1). The levels of nucleotide diversity (*Pi*) were calculated in DnaSP version 5.10 (Librado and Rozas, 2009). Both for complete and partial sequences, we also analyzed the following features of the ITS1 region: (i) frequency of intragenomic polymorphism at the 114, 139, and 339 bp positions of the complete ITS1 sequences; (ii) frequencies of sequences with a 5 bp insertion in different countries; (iii) and substitutions upstream and downstream of this insertion.

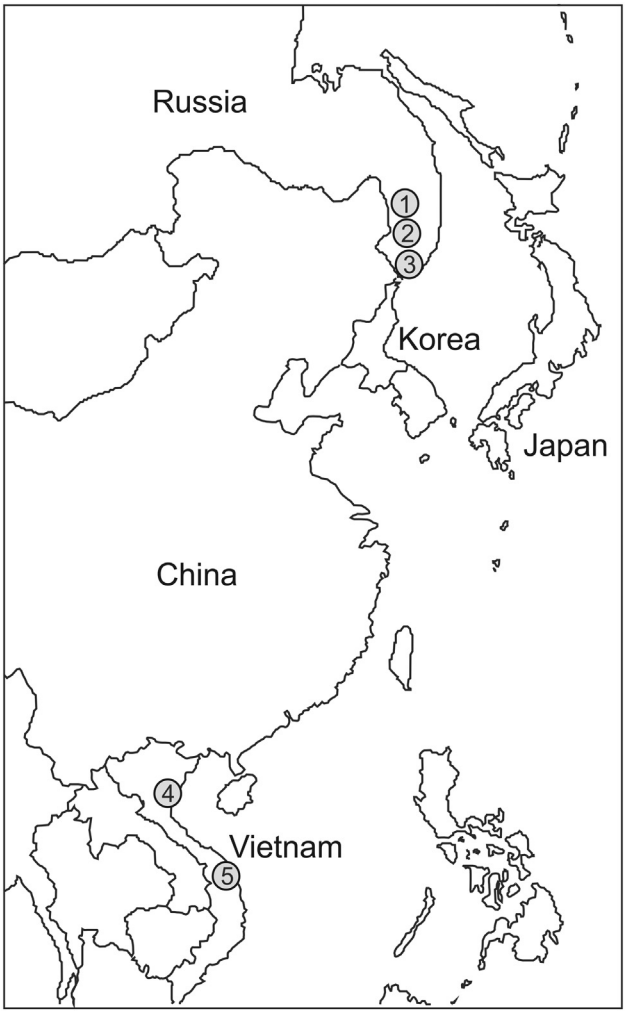

Fig. 1. Sampling locations for *Clonorchis sinensis*. Russia: 1 – Magdikovoe lake; 2 – unnamed storage reservoir (Kronshtadtka); 3 – the Komarovka river (Kondratenovka). Vietnam: 4 – Thai Binh; 5 – Danang.

3. Results

3.1. Analysis of the ITS1-5.8S-ITS2 rDNA sequences

The 5.8S rDNA sequences obtained in this study and downloaded from GenBank were identical. Also, except for the unique ITS2 nucleotide sequence from Danang (Vietnam), which carries an intragenomic polymorphism (double peak, G ↔ T transversions) at its 145 bp position, all sequences of this region were identical.

The length of the complete ITS1 sequences was 657 or 662 bp, and the partial region was 451 or 456 bp. The first position of the partial ITS1 sequence corresponds to the 152nd nucleotide of the complete