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**Short Communication**

# Genetic Structure of *Ascaris* Roundworm in Japan and Patterns of Its Geographical Variation

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**SUMMARY**: *Ascaris* roundworm isolates from Japan and central Europe were examined by sequencing analyses to better understand geographically induced nucleotide variation and genotype distribution according to host. Three well-supported clusters (denoted as A, B, C) were identified by generating *cox1* sequences of mtDNA from these regions. Among 5 pig isolates collected in eastern Honshu, Japan, in 2010, 3 carried DNA characteristics for cluster A and 2 corresponded with the characteristics of cluster B. The sequence of the human isolate JH1 from north-central Honshu, fixed in formalin since 1972, conformed to the characteristics of cluster A. Differential analysis of ribosomal ITS1 region revealed the JH1 isolate sequence profile of *Ascaris lumbricoides*. Cluster C, which was the most distinguish cluster, was formed by reference Slovak isolates and has been so far found almost exclusively in European pigs. A fluctuating prevailing distribution of A and B lineages in human and pig hosts in different territories of the world and the global distribution of several haplotypes indicate their establishment before secondary differentiation in a given region due to host affiliation. The protocol established for DNA isolation from formalin-fixed specimens using the modified procedure with the Qiagen extraction set can be used as a tool for retrospective studies in ascarid helminths when only archival specimens are available.

The giant roundworm *Ascaris* infects approximately 1.2 billion people worldwide and causes serious health consequences in 122 million cases per year (1). The human parasite *Ascaris lumbricoides* was the most common soil-transmitted helminth in Japan in the first half of the 20th century. During postwar redevelopment, a nationwide campaign involving an expansive public health center network, measures for maternal and child health care in schools, and environmental management was implemented. Following mass screening, treatment, and prevention measures for parasitic diseases, the egg detection rates of *Ascaris* in stool examinations decreased from 59.6% in 1950 to $<0.1\%$ in the 1980s (2). However, it should be emphasized that classical parasites such as *Ascaris* and hookworms do persist at a low level in the Japanese human population (3,4). Genetic studies of parasites are pivotal for categorizing the etiological agent and implementing effective control measures by identifying the source of infection. In the present report, *Ascaris* samples of human and pig origin from Japan and central Europe were examined by DNA sequencing analyses in relation to current knowledge of *Ascaris* genetics and epidemiology from a range of geographic locations.

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Many authors have emphasized the importance of archival specimens not originally preserved for genetic studies as sources of DNA for retrospective studies, and in recent years use of preserved specimens for genetic studies has been increasing (5,6). Formaldehyde (formalin) is by far the most widely used universal fixative as it preserves a wide variety of tissues and tissue components, although it may affect an array of downstream processes. Formalin-fixation of tissue leads to partial degradation of DNA, which may negatively affect the efficiency of PCR reactions (7). The fixation induces cross-linking with nucleic acids and proteins, thereby reducing the amount and quality of the extracted DNA. Furthermore, amplification inhibitors co-isolated with the extracted DNA may hamper PCR-based analyses of the organism. Establishing effective DNA-based protocols for use on archival material fixed in formalin is a particularly important task. Therefore, additional objective of the current study was to test the degree of DNA destruction and sample-related PCR inhibition in an archival Japanese human isolate, herein denoted JH1, which was recovered in 1972 in Ishikawa Prefecture (north-central Honshu). The sample has been fixed in formalin (10%, v/v) since 1972. The Japanese pig *Ascaris* specimens were collected in 2010 from slaughterhouses in Kanagawa Prefecture (isolates denoted as JP1, JP2), and Chiba Prefecture (JP3–JP5), both in eastern Honshu. The isolates were rinsed in normal saline and stored in 70% ethanol until they were used for DNA analysis. To gain a better understanding of the

host- and geographical region-related genetic structuring of *Ascaris*, European samples from Slovakia (21 isolates from humans, 24 from pigs) were also subjected to sequencing analyses. For the formalin-fixed JH1 isolate, genomic DNA was extracted using a DNA isolation protocol designed for a QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) (8), with several modifications. *Ascaris* tissue was placed into 1.5-ml microcentrifuge tubes containing 200 μl of buffer ATL (tissue lysis buffer) and manually homogenized with polypropylene pellet pestles without using motor-driven crushing. Then, 50 μl of proteinase K (20 mg/ml) was added, and the mixture was incubated at 55°C for 24 h. Next, an additional 30 μl of proteinase K and 95 μl of lysis buffer were added, and incubation was continued for another 24 h. If the tissue was not digested after 48 h, 30 μl of proteinase K was added for another 24 h. Once the tissue was dissolved, the extraction followed the manufacturer's recommendations, except that volumes of buffer AL and ethanol were increased from 100 μl to 200 μl. In addition, vortexing in the Qiagen protocol was replaced by gentle inversion of the tubes, thus minimizing the risk of additional DNA shearing.

The concentration and purity of the samples were determined by obtaining the 260/280 nm absorbance ratio using the NanoDrop® ND-1000 Spectrophotometer. The estimated DNA concentration for the JH1 isolate was 59.9 ng/μl, and the absorbance A260/A280 ratio reached a value of 1.74, indicating a relatively pure DNA sample free from substantial residual protein contamination. For the remaining Japanese isolates, the average DNA yield was 138.9 ng/μl. The primers used for amplifying the *cox1* fragment (384 bp) of mitochondrial (mt) DNA were AsCo1F (5′-TTTTTTGGTCAT CCTGAGGTTTAT-3′) and AsCo1R (5′-ACATAAT GAAAATGACTAACAAC-3′) (9). The ITS1 regions (312 bp) of nuclear ribosomal DNA were amplified using primers XZ5F (5′-TGATGTAATAGCAGTCGG CG-3′) and XZ1R (5′-GGAATGAACCCGATGGCGC AAT-3′) (10,11). PCR reactions were performed in 50 μl of master mix (Invitrogen, São Paulo, Brazil) containing 15 pmol of each primer. The PCR cyclic conditions were as follows: initial denaturation at 94°C for 5 min, followed by 35 cycles of 30 s at 95°C, 30 s at 55°C, 30 s at 72°C, followed by a final extension of 72°C for 5 min. Equal aliquots of the PCR products were run on a 1.6% agarose gel containing ethidium bromide and visualized under UV light. Amplicons were then purified using a NucleoSpin Extract II kit (Macherey-Nagel, Düren, Germany) and used as templates for direct sequencing with an automated DNA sequencer (ABI 3730XL; Applied Biosystems, Foster City, Calif., USA). Nucleotide sequences were aligned using ClustalW (12), and the nucleotide sequence homology was searched in the NCBI GenBank database using the BLAST program. For gathered sequences, MEGA5 software was used to estimate the best substitution model and to infer branching pattern (dendrogram) with the Neighbor-joining (NJ) method (13). A phylogenetic tree was also derived by Bayesian analysis using the HKY-I model with BEAST software (14). Bayesian analyses were run for $10^7$ generations and posterior probability values were inferred by discarding trees from the burn-in period ($2 \times 10^5$). The obtained sequences were deposited in the GenBank database under the accession numbers JN575625–JN575634.

The *cox1* sequences obtained from *Ascaris* samples from Japan and Slovakia were screened for their polymorphism along with sequences recently published from China (9), Japan (4), and Zanzibar islands, Tanzania (15) for the same gene target (variable nucleotide sites are shown in Fig. 1). To root the trees generated from these sequences, *Toxocara canis* (GbR AM411108) and *Heterakis isolonche* (GbR FJ009626) were used as outgroups. The trees obtained using the NJ and Bayesian approaches revealed the same topology, and three well-supported clusters, A (bootstrap, 60%; posterior probability, 90%), B (94%; 100%), and C (100%; 100%), were identified (Fig. 2). Cluster A was composed of 3 pig isolates from Japan (JP1, JP2, JP3) and the formalin-fixed JH1 isolate along with 6 human and 3 pig isolates previously examined in Japan by Arizono

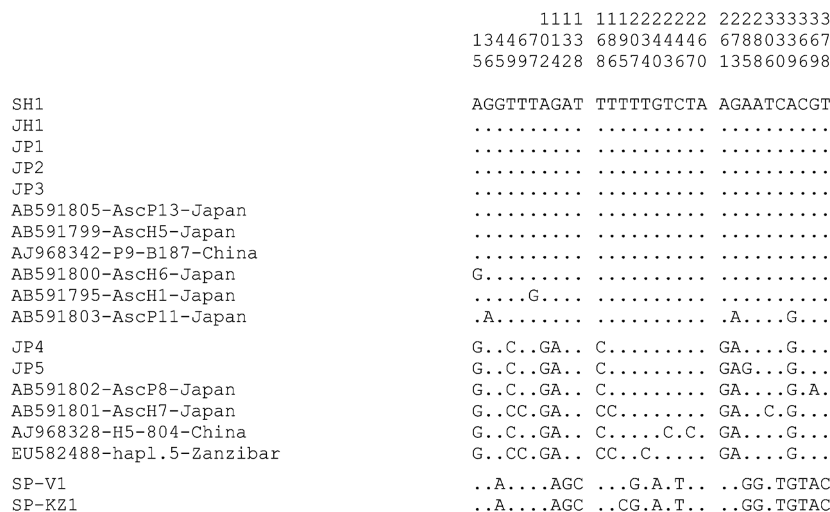


Fig. 1. Polymorphic sites in *cox1* sequences (384 bp) of *Ascaris* isolates. JP1–JP5, pig isolates from Japan; JH1, human isolate from Japan; SH1, human isolate from Slovakia; SP-V1 and SP-KZ1, pig isolates from Slovakia. Remaining sequences are from selected isolates deposited in GenBank. Dots indicate identity with reference sequences.

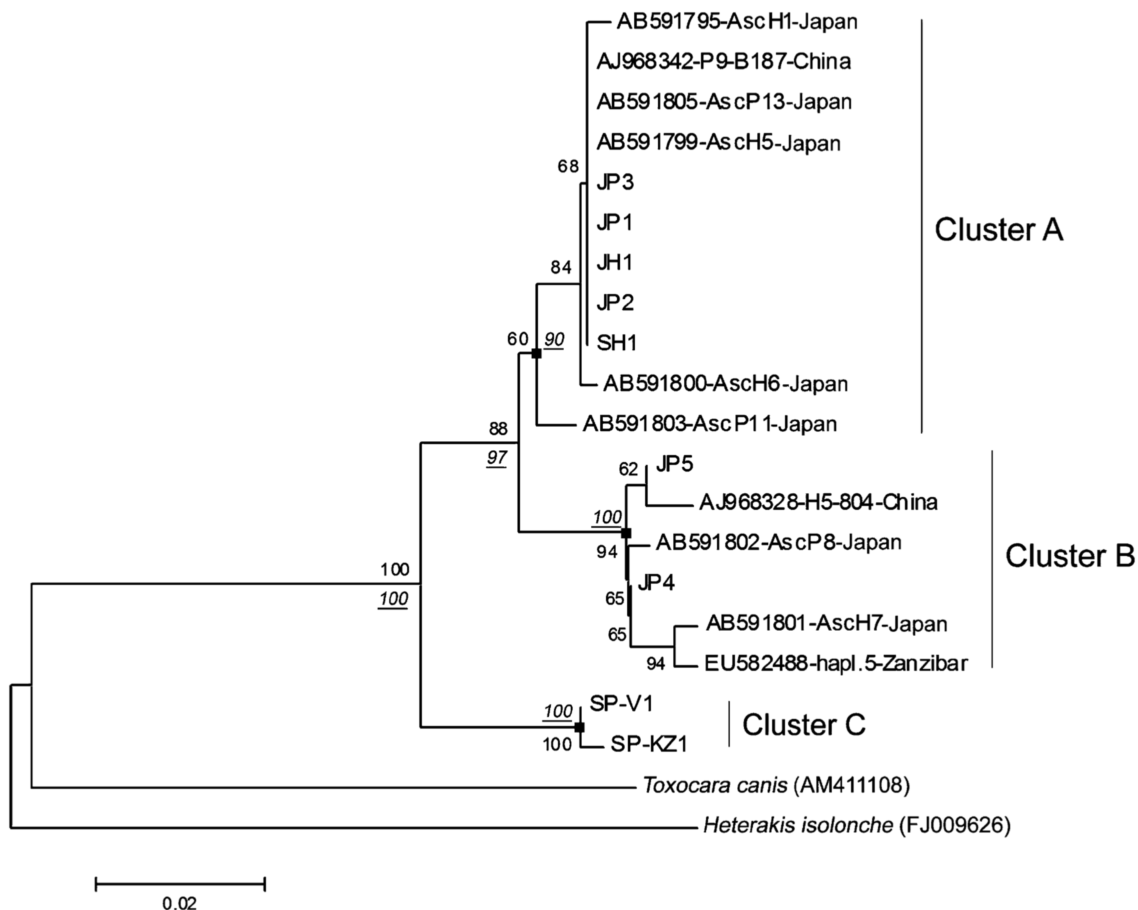


Fig. 2. Dendrogram constructed by the Neighbor-joining and Bayesian analyses (run for $10^7$ generations) from *cox1* sequences (384 bp) in examined *Ascaris* isolates from Japan (JH1, JP1–JP5) and Slovakia (SH1, SP-V1, SP-KZ1), compared to isolates from other world regions using GenBank data (H, human host; P, pig host). Numbers at the nodes of branches indicate bootstrap values (1,000 pseudoreplications) and posterior probability values (underlined) obtained by discarding trees from the burn-in period ($2 \times 10^5$).

et al. (human isolate AscH5 together with 3 additional human isolates [AscH1, AscH6, and AscP11] and 1 pig isolate [AscP13] with identical structures) (4), 7 of 10 pig haplotypes from China (representative isolate presented in Figs. 1 and 2 with the P9-B187 code) detected by Peng et al. (9), and all human-derived samples from Slovakia (21 isolates carrying patterns corresponding to the reference human isolate SH1, Figs. 1 and 2) examined herein. Hybridization events between lineages or incomplete lineage sorting are probably responsible for the peculiar pattern seen in AscP13, with the 2 nucleotide substitutions (273G/A, 339A/G) relative to cluster A and identical to those seen in cluster B, but with greater overall affinity to cluster A. All isolates allocated to cluster B differed from the isolates grouped in cluster A in 8 fixed positions (2.33% of bases) and, except for the JP4 isolates, also carried some additional base differences. Cluster B was formed by the 2 pig isolates from Japan (JP4, JP5) studied here, 1 human and 1 pig isolate (AscH7 and AscP8, respectively) from the Japanese report by Arizono et al. (4), the reference sample (isolate H5–804 in Figs. 1 and 2) from the Chinese report by Peng et al. (9), and the reference sample (haplotype 5) from the Zanzibar report by Betson et al. (15). Cluster C comprised pig-derived isolates from Slovakia and constituted the most distinguished group among the 3 clusters, differing in 14 fixed positions from cluster A (3.64% of bases). Two haplotypes mutually differing in a single substitution (195C/T) were recorded in this cluster, with 15 specimens bearing the nucleotide composition recorded in the SP-V1 reference isolate, and 9 isolates conforming to the SP-KZ1 isolate (Figs. 1, 2). Pairwise genetic differentiation between regions estimated by measuring GammaST ($\gamma$ST) values (16) was the lowest between Japanese and Chinese samples (0.023) and highest between samples from Japan and Zanzibar (0.202), while a value of 0.093 was obtained between isolates from Japan and Slovakia.

When processing the data in terms of host affiliation on a worldwide scale, distinct patterns were observed in different geographical regions. All human-derived parasites from Slovakia analyzed in the present study were allocated to cluster A. Conversely, human haplotypes from Zanzibar and China clustered more frequently in cluster B than in cluster A, with 12 haplotypes in cluster B and 4 haplotypes in cluster A (15), and 7 haplotypes in cluster B with 3 haplotypes in cluster A (9), respectively. For Japan, the present data coupled with haplotype patterns taken from the recent genetic report by Arizono et al. (4) indicated that cluster B is mostly encountered in pig-derived parasites (3 pigs, 1 human), whereas cluster

A exhibits a more balanced host distribution (7 humans, 5 pigs). Additional sampling is needed to draw a more accurate picture of *Ascaris* host affiliation in Japan.

Our results provided a similar tree topology and partitioning of genetic differences between clusters as those reported by Anderson and Jaenike in a large-scale *Ascaris* study in which mitochondrial sequences of the *nad4* gene (355 bp), a non-coding region (117 bp), and the *cox1* gene (161 bp) were screened (17). These authors identified *Ascaris* allocated to cluster B as predominating in humans from the New World, and cluster C was composed of isolates obtained from pigs in Scotland, Switzerland, the Philippines, and a single human-derived parasite from Guatemala. In the frame of our data, cluster C was confined solely to the Slovak pig population. However, no explicit comparison of the distribution of isolates across clusters was possible between data of Anderson and Jaenike (17) and our data given the partially different mitochondrial regions under examination (only a partial *cox1* region was shared between the studies), and the absence of sequences in GenBank from the above study (17). The present report corroborated a fluctuating distribution of major clusters according to hosts in different regions of the world and a global distribution of several mitochondrial haplotypes, suggesting that 3 different lineages of mtDNA were already present in ancestral populations prior to host affiliation. Their existence is most likely explained by retention of ancestral polymorphism, as previously proposed (17,18).

By examining the ITS1 region previously established as the putative genetic marker for differentiation of *A. suum* (pig parasite) and *A. lumbricoides* (human parasite) (19), a temporally changing epidemiological pattern towards that characteristic for non-endemic countries, where cross-infection with pig parasites occurs proportionally more frequently than in endemic countries, was derived for Japan (4). In our study, transversal substitution A/T in the 312-bp region identified the preserved Japanese human JH1 isolate recovered in 1972 as *A. lumbricoides*. Unlike human isolates with unequivocal chromatogram profiles, pig samples from both Japan and central Europe displayed several ambiguous nucleotide positions that led to genotype misclassification due to ITS1 intra-individual variability. A similar phenomenon in helminths resulting from variable copy numbers of repeats was also recorded in *Ascaris* eggs from Brazil and in the cervid strain of *Echinococcus granulosus* (11,20). For our samples, the sequence patterns were indicative of interbreeding events between individuals bearing different genotypes or historical introgression.

Formalin fixation has been commonly reported to cause amplification failure along with degradation of DNA; these findings are mainly reported in fragments longer than 300–500 bp, in which changes in base structure are more likely to arise (21,22). In the present study, PCR was employed for mitochondrial and nuclear fragments with lengths of 384 bp and 312 bp, which may have also contributed to the satisfactory DNA yield for sequencing reactions. The protocol established for formalin-fixed specimens included slight modifications in the amount of the reagents and timing of protocol steps, and vortexing was replaced with gentle inversion of tubes to minimize the risk of DNA rupture. The protocol has so far been tested for short gene fragments (up to 400 bp), and has the potential to be used for molecular typing studies in *Ascaris* and related helminths under conditions where only archival collections are available.

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**Conflict of interest** None to declare.

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