1998). In contrast, three additional alleles detected in our previous study in Myanmar (TNFP-M1, M4 and M7) (Ubalee *et al.* 2001) were not observed in Vanuatu.

Table 2 shows the frequencies of the TNFP alleles on each island that were detected in the present study. The frequencies of the TNFP-A, B, D alleles ranged from 0.14 to 0.55 while the TNF-C allele was uniformly rare (0.00–0.03), thus our analysis of malarial selection focuses on the former three polymorphic alleles. To further examine the effect of malaria on the frequencies of both protective and susceptible allele frequencies, we also screened for multiple α-thalassaemias distinguished by their deletion length and also restriction site polymorphisms in the same population. As shown in Table 3, the $-\alpha^{3.7}$ deletion was more common than the $-\alpha^{4.2}$ deletion within the populations of Vanuatu examined as previously reported (Hill *et al.* 1985). The frequencies of α-thalassaemia alleles in the six islands ranged from 0.37 and 0.39 in MLK and PEN, the islands with highest parasite incidences, to 0.11 in ANT, the island with the lowest parasite incidence (Table 3). Subsequent analysis of $-\alpha^{3.7}$ subtypes ($-\alpha^{3.7}$ I, II and III) by *Apa*I restriction digestion revealed that the $-\alpha^{3.7}$ III was the most common haplotype background (>97%) in the population, consistent with previous studies of Vanuatu (Hill *et al.* 1985).

The relationship between parasite incidence determined in field surveys during 1985–90 (Kaneko *et al.* 1998) and the frequency of the three polymorphic TNFP alleles are shown in Figure 2a. The allele frequency of TNFP-D was significantly inversely correlated with parasite incidence ($r = -0.855$, $P = 0.03$) among the six Vanuatu islands studied. The highest frequency of TNFP-D (0.55) was observed in ANT, the island with the lowest parasite incidence whereas the lowest frequency (0.26) was observed in MLK, the island with the highest parasite incidence. In contrast, the TNFP-A and B frequencies showed no significant correlation with parasite incidence (TNFP-A: $r = 0.556$, $P = 0.252$; TNFP-B: $r = 0.587$, $P = 0.221$). The frequency of α-thalassaemia alleles and parasite incidence was statistically significant and is shown in Figure 2b.

The correlation between α-thalassaemia and TNFP alleles are shown in Figure 2c. TNFP-A and α-thalassaemia gene frequency showed significantly positive correlation ($r = 0.837$, $P = 0.038$) while TNFP-D and α-thalassaemia gene frequency showed significantly negative correlation ($r = -0.962$, $P = 0.002$). In contrast, the TNFP-B or TNFP-C gene frequency showed no significant correlation with α-thalassaemia (TNFP-B: $r = 0.426$, $P = 0.400$; TNFP-C: $r = -0.034$, $P = 0.108$).

Table 1 Alleles of TNF-α promoter region and those frequencies in Vanuatu. Four alleles of the 5′-flanking region of TNF-α detected in Vanuatu

	−238	−308	−857	−863	−1031
TNFP-A	G	G	C	C	T
TNFP-B	G	G	C	A	C
TNFP-C	A	G	C	C	C
TNFP-D	G	G	T	C	T

Discussion

A number of studies have indicated that polymorphisms in the TNF-α promoter region change the binding of various nuclear factors that up- or down-regulate transcription (Knight *et al.* 1999). Heel *et al.* (2002) demonstrated that the transcription factor OCT-1 binds the TNF-857T of the TNFP-D allele, but not the −857C of the TNFP-A, B and C alleles and interacts *in vitro* and *in vivo* with the proinflammatory NF-*k*B transcription factor p65 subunit at the adjacent binding site, −863C/A. Hohjoh and Tokunaga (2001) also showed that OCT-1 bound to alleles possessing either −857T (TNFP-D) or −863A, but not to alleles specifying −857C and −863C. Furthermore, Skoog *et al.* (1999) observed a difference in the binding of nuclear factors to −863C and −863A containing alleles by an electromobility shift assay that indicated the −863A allele was associated with lower transcriptional activity in a chloramphenicol acetyltransferase reporter gene study in human hepatoblastoma (HepG2) cells *in vitro* (Skoog *et al.* 1999). These observations specifically related to OCT-1 binding at the polymorphic site defining TNFP-D and TNFP-A strongly support our interpretation of TNFP-D allele susceptibility to CM via a mechanism of OCT-1 binding and TNF up-regulation in Vanuatu and TNFP-A being the most protective. Therefore, taken together with our previous finding in Myanmar, the TNFP-D allele is suggested to be continuously selected against by CM in malarious regions. In contrast to regionally inconsistent studies that associated HLA alleles and malarial resistance (Hill *et al.* 1991), we find the same TNFP-D allele implicated in malarial susceptibility by distinct analyses (case–control study and population analysis) in eight distinct populations in Myanmar, South-east Asia and Vanuatu, Melanesia. More detailed analyses of polymorphisms in the promoter region coupled with their effects on TNF transcriptional regulation in different cell types and with a wide range of stimuli will be required to fully understand the molecular basis of this disease association (Knight 2003; Knight *et al.* 2004).

Anthropological evidence suggests that the original settlers of Vanuatu migrated from Island South-east Asia

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Table 2 Alleles of TNF-α promoter region and those frequencies in Vanuatu. The frequencies of the alleles on the six islands in Vanuatu

Alleles	MLK (n = 180)	PEN (n = 107)	SAN (n = 176)	GAU (n = 177)	ERO (n = 149)	ANT (n = 285)
TNFP-A	0.43	0.42	0.47	0.50	0.35	0.25
TNFP-B	0.31	0.31	0.14	0.15	0.23	0.18
TNFP-C	0.00	0.00	0.03	0.00	0.01	0.02
TNFP-D	0.26	0.27	0.37	0.35	0.41	0.55

Table 3 The α-thalassaemia gene frequencies on six islands in Vanuatu

α-Thalassaemia	MLK	PEN	SAN	GAU	ERO	ANT
$-\alpha^{3.7}$	0.28	0.35	0.23	0.27	0.13	0.08
$-\alpha^{4.2}$	0.09	0.04	0.12	0.04	0.08	0.03
Total	0.37	0.39	0.34	0.31	0.22	0.11

about 3500 years ago (Diamond 1988). Numerous studies of neutral loci (Lum *et al.* 2002) and those associated with malarial resistance (Roberts-Thomson *et al.* 1996) have demonstrated that the current populations of Vanuatu are a composite of Island South-east Asian and New Guinean gene pools. Interestingly, if we calculate genetic distances among populations from TNFP allele frequencies, Vanuatu clusters with Japan (North-east Asia) rather than Myanmar (South-east Asia) because they share the same four alleles and both lack three others present in Myanmar. Thus, it appears that malarial selection has altered TNFP allele frequencies so that genetic relationships based on this single disease-selected locus reflect local malarial selection more strongly than population origins. Moreover, the haplotype analysis of the TNFP gene by Ackerman *et al.* (2003) in Africa, where malaria is endemic, also showed that the frequency of the TNFP-D was very low in the Gambia (0.05) and absent in Malawi, consistent with our finding that the parasite incidence is the factor driving allele frequencies in Asia and the Pacific.

Analysis of α-globin gene deletions in Vanuatu suggests that selection via parasite incidence is likely responsible for the high frequencies of α-thalassaemia alleles (Flint *et al.*

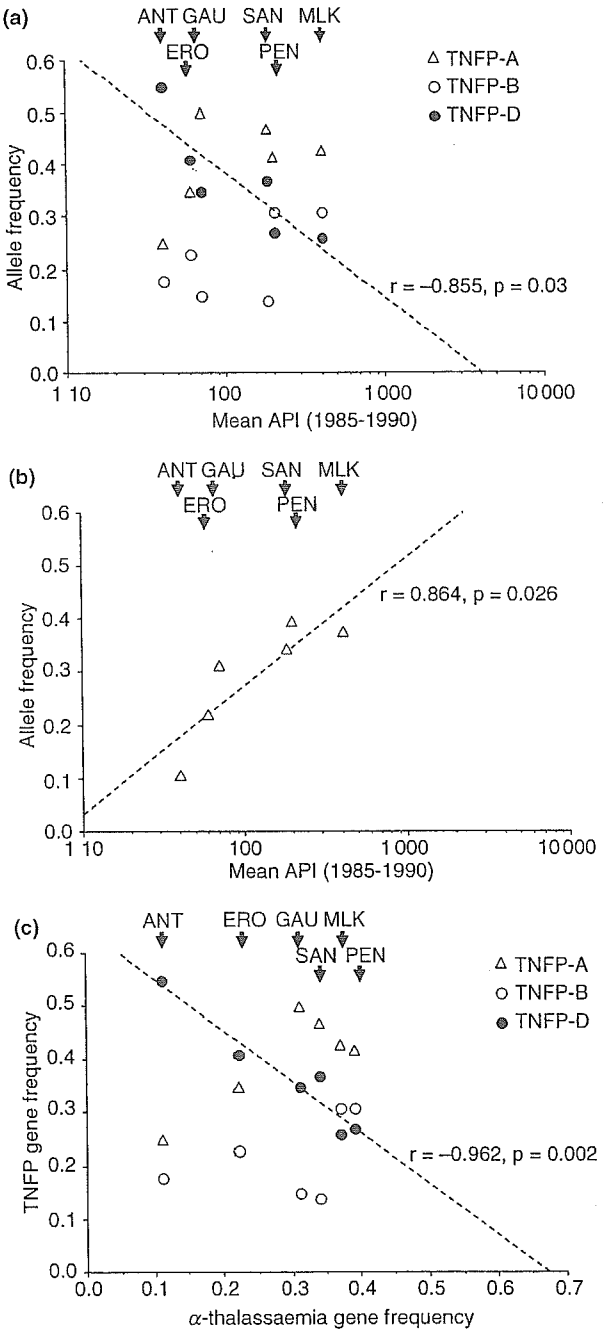

Figure 2 (a) Correlation between TNFP-D allele frequency and parasite incidence. Kaneko *et al.* had already reported each island's parasite incidence based on the Annual Parasite Incidence per thousand inhabitants (API) by passive case detection activities at local health facilities from 1985 to 1990. Mean API were 400 in Malakula (MLK), 200 in Pentecost (PEN), 180 in Espiritu Santo (SAN), 70 in Gaua (GAU), 60 in Erromango (ERO), and 40 in Aneityum (ANT). *Plasmodium falciparum* accounted for about 60% of total *Plasmodium* infections (Kaneko *et al.* 1998). (b) Correlation between α-thalassaemia gene frequency and parasite incidence. (c) Correlation between TNFP and α-thalassaemia gene frequency.

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1986). The predominance of the $-\alpha^{3.7}$ III allele, believed to have been introduced into Melanesia with South-east Asian colonists 3500 years ago (Hill *et al.* 1985), indicates that for this malaria-selected locus, population origins are still detectable. Analyses of both loci reveal that the frequencies of the putatively susceptible TNFP-D allele and protective α-thalassaemia alleles are correlated with parasite incidence in the expected directions, consistent with previous surveys of G6PD-deficiency alleles (Kaneko *et al.* 1998). The observation that there was a significant correlation ($P = 0.002$) between the gene frequencies of α-thalassaemia and TNFP-D alleles as shown in Figure 2c, indicated that those gene frequencies might be more reliable indicators than the present API for the cumulative malaria pressure since the original population migrated to each island.

Recently, lymphotoxin-α or TNF-α, a related member of the TNF family, have been suggested to be involved in CM in a murine model (Rudin *et al.* 1997; Engwerda *et al.* 2002). It is possible that the association we observed between TNFP-D and parasite incidence resulted from linkage disequilibrium with some polymorphisms of TNF-α located near TNF-α. Although there was no significant association between severe malaria and the polymorphism of TNF-α in Sri Lanka patients (Wattavidanage *et al.* 1999), further analyses of the extended haplotype are necessary to explore this possibility.

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Altered expression of goblet cell- and mucin glycosylation-related genes in the intestinal epithelium during infection with the nematode *Nippostrongylus brasiliensis* in rat.

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Running head: Mucin response in nematode infection

Summary

Intestinal nematode infection induces marked goblet cell hyperplasia and mucus secretion, but the mechanisms of regulation of the changes still remain to be elucidated. In the present study, epithelial cells were isolated from the rat small intestine at various times after *Nippostrongylus brasiliensis* infection, and the levels of expression of goblet cell- and mucin glycosylation-related genes were estimated by semi-quantitative reverse transcription (RT)-PCR. Among the genes investigated, mucin core peptide (MUC) 2, sialyltransferase (Siat) 4c and trefoil factor family (TFF) 3 were upregulated as early as 2-4 days post-infection, suggesting that they are associated with an early innate protective response. Seven days post-infection and thereafter, when the nematodes reached maturity, significant upregulation of MUC3, MUC4, resistin-like molecule β (Relmβ) and 3O-sulfotransferase (3ST)1 was observed, while 3ST2 expression levels increased after the majority of the worms were expelled from the intestine. Similar alterations of glycosylation-related gene expression were also observed in mast-cell-deficient *Ws/Ws* rats, suggesting that mast cells in the epithelium are not relevant to the upregulation of these genes. The present finding that the expression level of each goblet cell- or glycosylation-related gene was altered differently during the time course of infection indicates the progression of sequential qualitative changes in the mucus layer after infection.

Key words: mucin; glycosylation; goblet cell; intestine; *Nippostrongylus*

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Introduction

Intestinal nematode parasites such as ascaris and hookworms infect more than 1.3 billion people in the world. Although they cause relatively little mortality, infections result in high levels of morbidity that can result in developmental retardation in infected children (1). Although it has been clarified that Th2 cytokines and/or Th2 cytokine-dependent responses such as mastocytosis and eosinophilia might have crucial roles in protective immunity against certain nematode infections (2), further clarification of factors or mechanisms that are responsible for colonization of worms in and/or rejection of worms from the intestine is an issue of importance for developing more effective measures to combat intestinal parasites. Among factors of potential importance, particularly interesting factors are produced in and secreted from goblet cells, including mucins and

other secretory peptides. In fact, intestinal nematode infection induces marked goblet cell hyperplasia and mucus secretion, which may be induced directly via the local release of bioactive factors or indirectly via the activation of host immune cells (3,4).

The nematode *Nippostrongylus brasiliensis*, a lumen-dwelling parasite, is a suitable model for studying human and other clinically relevant hookworms because of the similarities of habitat and life cycle (5). Interestingly, the mucus response which is induced by infection with *N. brasiliensis* has been suggested to be responsible for the rejection of nematodes from the intestine, whereas mast cells are not essential for expulsion of this parasite (3,4). It has been shown that alterations in the terminal sugars of goblet cell mucins are associated with *N. brasiliensis* worm expulsion from the intestine by studies of lectin-binding as well as by biochemical analyses (6-10). Recently, Knight *et al.* (11) and Pemberton *et al.* (12) reported that the expression levels of a variety of goblet cell- and/or mucin-related genes were altered during infection with *Trichinella spiralis* in mice. The results indicated that molecular changes that are induced in the epithelial and/or mucus layer during nematode infection are a complicated series of changes whose mechanisms of regulation are virtually unknown, as are the roles of each molecule in mucosal protection.

We therefore attempted to determine by semi-quantitative reverse transcription (RT)-PCR whether there were alterations of the expression of some goblet cell-related genes in the small intestine during the time-course of *N. brasiliensis* infection in rats. The genes examined included those of mucin core peptides MUC2, MUC3 and MUC4 (13-16) and goblet cell-specific secretory peptides such as Relmβ (17) and intestinal trefoil factor (TFF3)(18,19). Because of the possible importance of the terminal structure of oligosaccharide chains of mucins and/or membrane glycoproteins for colonization and/or rejection of worms, we also examined the gene expression of sialyltransferase, sulfotransferase, fucosyltransferase and some histo-blood group transferases, which might modulate the terminal sugar chains of mucin and/or membrane glycoproteins (20-26).

MATERIALS AND METHODS

Animals, nematode infection and autopsy

Specific-pathogen-free male Brown Norway/Sea (BN) rats and male Fischer (F)-344 rats were purchased from SLC Inc. (Shizuoka, Japan). SPF male and female mast cell-deficient *Ws/Ws* rats were produced in our laboratory as described previously (27). Animals at 8 weeks of age were injected subcutaneously with 2,000 *N. brasiliensis* infective-stage (L3) larvae as described elsewhere (27). The animals were allowed to feed *ad libitum* throughout the experiment.

Preparation of intestinal epithelial cells

The animals were sacrificed with an overdose of ether after overnight fasting with free access to water. The separation of intestinal epithelial cells was carried out at 4°C in EDTA-Hanks' solution (Ca^{2+}, Mg^{2+}-free Hanks' balanced salt solution supplemented with 10 mM HEPES, pH 7.3, 1 mM DTT and 1.0 mM EDTA) as described elsewhere (28) with slight modifications. In brief, a piece of jejunum 18-22 cm from the pyloric ring and a piece of ileum 10-14 cm from the ileocecal junction, or a 4-cm-long segment of the proximal colon, were removed, opened longitudinally and cut into segments 1 cm in length. After a brief wash in PBS, 4 pieces of tissue were put into a 15-ml tube containing 4 ml of EDTA-Hanks' solution, and debris attached to the mucosal surface

was removed by vigorously shaking the tubes 15 times by hand. The tissues were then transferred into another tube containing EDTA-Hanks' solution. After 75 min on ice with occasional agitation of the tissues by inverting the tubes, the epithelial cells were separated by 60 strokes of vigorous shaking of the tube by hand. After discarding the tissue, detached epithelial cells were collected by centrifugation at 600 xg for 3 min at 4°C, washed once with EDTA-Hanks' solution, and the cell pellets were stored at -80°C until use. Giemsa staining of the separated epithelial fractions showed not only epithelial cells, but also a small number of mononuclear cells, the majority of which we considered might have been intraepithelial lymphocytes. Histological examination of the tissue after collection of the epithelia showed that villus epithelium was separated completely, whereas the epithelial lining cells in the lower part of crypts were still attached to the tissue in approximately half of the crypts. In uninfected rats, the basal lamina of the epithelium was intact and lamina propria cells were retained in the tissue, but in animals after 10 days of infection, the basal lamina was partly obscured, indicating that some propria mucosal cells contaminated the epithelial fractions. Concerning the stomach, a mucosal scrape specimen of the glandular stomach was prepared by scraping the mucosa with the edge of a glass slide and stored at -80°C until use.

Extraction of total RNA, cDNA synthesis, RT-PCR and relative quantification

Total RNA was extracted using TRIZOL Reagent (Life Technologies, Rockville, Maryland). Two-microgram aliquots of RNA were reverse transcribed in 20 μl of reverse transcription buffer containing 5 mM $MgCl_2$, 1 mM dNTP mixture, 1U/μl RNase inhibitor, 0.25 U/μl AMV reverse transcriptase and 0.125 μM oligo dT-adaptor primer (Takara RNA LA PCR kit, Takara Biomedicals, Osaka, Japan) at 42°C for 50 min. One-microliter aliquots of the synthesized cDNA were mixed with Sybr Green PCR master mix (Applied Biosystems, Foster City, CA) with appropriate primers and amplified using a real-time PCR system 7300 (Applied Biosystems, Foster, CA, U.S.A.). The sense and antisense primers used were:
5'-CGGATCCAATGGAACAGTGG-3' and 5'-TGCCACTGGTAGGATGATTG-3' for MUC2; 5'-GTTTCAACTCGACTGCCACC-3' and 5'-ATAGCTGCAGTTCTTGGAGG-3' for MUC3; 5'-GCGGAAGAGGAGTGGAGAAG-3' and 5'-AGATGGCCAGTAGCAAGAGG-3' for MUC4; 5'-TTCCTTCTCTCGCTGATGGT-3' and 5'-GCAGTGGCAAGTAGTTCCAT-3' for Relmβ; 5'-ATGGAGACCAGAGCCTTCTG-3' and 5'-TGGGATGCTGGAGTCAAAACA-3' for Tff3; 5'-CTACACCTCTGCGACTTGGT-3' and 5'-GGTTCTTGACAGCTCCCATC-3' for Siat4c; 5'-CCCTTTCCCTGAGATCCAGA-3' and 5'-CCGGCCTTTGGACTCATGTA-3' for 3ST1; 5'-CCCAGATCCACTTCGTCAGT-3' and 5'-AAAATTCCCGGAGCTGGTCT-3' for 3ST2; 5'-AGCAATGGCATGAGATGGTG-3' and 5'-TCTGGAAGGGTGAAGTTAGC-3' for FUT1; 5'-GGTGCCGGGAGAACATTAAT-3' and 5'-GAGAATCCGGAAGGGTGTAG-3' for FUT2; 5'-GATTTCCCTAGTGCTGCCTC-3' and 5'-GTTGTGGATACTCTTGGGCT-3' for FUT4; 5'-ATGTACAAGTGGCCAGCCTA-3' and 5'-GAATCTTCCCTTCCCCAGAG-3' for Lew 1.
Abbreviated terms for each gene are listed in Table 1. The specificity of each amplified product was confirmed by dissociation analyses giving a single sharp dissociation peak, the absence of the amplified product without reverse transcription, and the appearance of a band of the expected size on electrophoresis of the amplified product. For the amplification of β-actin, Actb primers (Rn00667869, Applied Biosystems) and TaqMan PCR master mix (Applied Biosystems) were used. For relative quantification, standard curves of the threshold cycle (Ct) of amplification of each target against log ng total RNA were created using cDNA samples which showed the lowest Ct value in preliminary runs, and relative quantification was performed for each sample. All quantified values were normalized to those of β-actin (quantified value for a certain target/quantified value for β-actin).

Tissue preparation for histology and goblet cell count

A segment of the jejunum 22-26 cm distal to the pyloric ring and a segment of ileum 6 -10 cm from the ileocecal junction were removed, opened longitudinally, fixed in 4% buffered formalin overnight and embedded in paraffin in such a position that histological sections could be cut perpendicular to the luminar surface. Five-micrometer sections were cut and the periodic acid-Schiff (PAS) reaction with hematoxylin nuclear staining was carried out. Ten villi, which were cut as nearly perpendicularly as possible, were selected per animal, and the numbers of goblet cells and numbers of epithelial nuclei in each villus were counted under a microscope. Goblet cell number/100 epithelial cells was calculated as [Number of goblet cells/number of epithelial nuclei] x 100. The average number of goblet cells/100 epithelial cells in 10 villi was used as the representative value in a given animal, and means and SE of 4 animals were calculated.

Worm counts

After removing the jejunal and ileal segments for separation of the epithelium and tissue section preparation, the numbers of worms in other parts of the small intestine were determined by the saline incubation method.

Statistical analysis

Student's *t*-test (2-tailed) was employed for statistical analysis; a P value of less than 0.05 was considered significant.

RESULTS AND DISCUSSION

Expression of goblet cell- and glycosylation-related genes along the gastrointestinal tract

The relative expression levels of goblet cell- and mucin glycosylation-related genes in the normal BN rat gastrointestinal tract were examined by semi-quantitative RT-PCR (Table 1). Except for Lewl and 3ST2, the goblet cell- and mucin synthesis-related genes examined were expressed at significantly higher levels in the colon than in the jejunum. This may partly reflect the greater abundance of goblet cells in the colon than in the small intestine, in which absorptive cells are the major epithelial constituent, and is consistent with previous reports that MUC2 and MUC4 gene/protein expression levels were higher in the colon than in the small intestine (14,16). In the stomach, MUC2, MUC3 and MUC4 expression was undetectable, while abundant expression of glycosylation-related genes was observed. This is consistent with the fact that MUC5AC and MUC6 are the major mucin core peptides in the stomach (29).

Goblet cell hyperplasia during the course of **Nippostrongylus brasiliensis** *infection in the small intestine*

N. brasiliensis larvae reach the small intestine as early as 2-3 days after cutaneous infection, develop to sexual maturity, and begin to lay eggs by 7 days post-infection (PI). However, infection does not continue for a long time: in the normal rat, the majority of adult worms are rejected from the small intestine around 14 days PI by a T-cell dependent mechanism, leaving only a small number of residual worms in the intestine (5, 30). To examine the kinetics of goblet cell response during the time course of infection, BN rats were infected with 2,000 L3 larvae of *N. brasiliensis* and autopsied 7, 14 and 21 days PI. The numbers of worms recovered from the intestine excluding the intestinal segments used for tissue preparation and epithelial separation were 345.8 $\pm$ 180.4, 40.0

± 17.1 and 42.2 ± 32.9 (average ± SD) after 7, 14 and 21 days of infection, respectively, showing that the majority of worms were rejected from the intestine by 14 days PI, but a small number of worms escaped the rejection and continued to parasitize the rat at least until 21 days PI. PAS staining of the jejunal and ileal tissue sections revealed goblet-cell hyperplasia 7 and 14 days PI, while goblet cell numbers decreased to preinfection levels by 21 days PI (Table 2).

Alterations of goblet cell- and glycosylation-related gene expression during the course of* Nippostrongylus brasiliensis *infection in the small intestine

At least 3 types of mucins are expressed in the small intestine: MUC2, which is restricted to goblet cells, MUC3, which is expressed in both columnar and goblet cells, and MUC4, which is expressed in columnar cells (13-16). RT-PCR analyses of gene expression in isolated epithelial cells of BN rats showed upregulation of MUC2, MUC3 and MUC4 in the jejunum, but not in the ileum after infection (Fig. 1), indicating that upregulation of mucin core peptide genes occurred mainly in the local mucosa, which was parasitized by large numbers of worms. In F-344 rats, similar kinetics of gene expression were observed after nematode infection (data not shown). It has been reported that MUC2 and MUC3 mRNA expression was increased in the small intestinal epithelium after infection with the epithelium-invading nematode *T. spiralis* in mice (31). The present findings that not only MUC2 and MUC3, but also MUC4 mRNA expression was upregulated after infection suggest that the mucin response to nematode infection might have occurred not only in goblet cells, but also in columnar cells.

The gene expression of goblet cell-specific non-mucin peptide Relmβ, whose expression is dependent on Th2 cytokines and has a potentially important role in protective immunity (11,17), was also upregulated after infection (Fig. 1), consistent with previous reports showing that Relmβ expression was induced after infection of mice with *Trichuris muris, T. spiralis and N. brasiliensis* (11,17). The finding that strong upregulation of Relmβ occurred not only in the jejunum, but also in the ileum, where scarcely any parasites were found, may reflect the systemic effect of Th2 cytokines. TFF3, which might protect mucous epithelia from a range of insults and is known to contribute to mucosal repair (18,19), was also upregulated, consistent with its upregulation in *T. spiralis*-infected mice (32). In a previous report, TFF3 gene expression was not altered in mice infected with *N. brasiliensis* (33); the discrepancy may possibly be due to the difference of host species.

Not only the production and secretion of mucins and goblet cell-specific peptides, but also the glycosylation status of the terminal sugar chains of mucins and/or membrane glycoproteins might have an important role in the colonization of nematodes in and/or rejection of nematodes from the intestine. Although a variety of glycosyltransferases may be involved in the modulation of the terminal sugar chains of mucins and membrane glycoproteins, little is known about the dynamic changes of these genes after infection, except that the upregulation of Siat 4c, FUT2 or A-type transferase has been reported in mice or rats after infection with *T. spiralis* or *N. brasiliensis* (9-11). The present results showed that the gene expression of Siat 4c, 3ST1, 3ST2, FUT2 and Lewl was upregulated after infection (Fig. 1), showing for the first time that not only sialyltransferase and fucosyltransferase but also sulfotransferase gene expression changed during nematode infection. The slight upregulation of the FUT2 and Lewl genes after infection suggests that some changes also occurred in the histo-blood antigens after infection. Although the upregulation of these genes was transient and the levels of the mRNAs returned to steady state levels by 21 days PI, 3ST2 expression was

instead upregulated 21 days PI, suggesting that the expression of the two sulfotransferases is regulated by different mechanisms.

Several types of glycosylation-related genes, especially O-sulfotransferase genes, are considered to be specifically expressed in mast cells, which synthesize sulfated glycosaminoglycans such as heparin and/or chondroitin sulfate (34). It is well established that the number of mast cells increases not only in the propria mucosa, but also in the epithelium, after *N. brasiliensis* infection (35). Thus, intraepithelial mast cells contaminating the epithelial fraction might be responsible for the upregulation of 3ST1 and/or 3ST2 after infection. To clarify this point, we examined the gene expression in the epithelial fraction of *Ws/Ws* rats, which have a small deletion of the c-kit gene and consequently lack mast cells (35). As shown in Fig. 2, the expression of Relmβ, Siat4c and 3ST1 was upregulated 7 days PI, and that of 3ST2 was upregulated 21 days PI, indicating that mast cells are not relevant to the expression of these genes. These results indicate that the glycosylation status of mucins and/or membrane glycoproteins was modified markedly during the course of infection. Although the precise roles of these changes in inflammatory conditions still remain to be elucidated, some reports have indicated a crucial role of the sialylation and sulfation levels of mucins in *Strongyloides venezuelensis*-infected mice and rats, with the sulfation levels in goblet cells affecting the establishment and distribution of the nematodes in the intestine (36, 37).

Since *N. brasiliensis* larvae reach the small intestine as early as 2-3 days PI, early responses of goblet cell- and mucin glycosylation-related genes after infection were examined. As shown in Fig. 3, MUC2, TFF3 and Siat 4c expression in the jejunal epithelium was upregulated as early as 2-4 days after infection.

Taken together, the present findings show that the expression of each goblet cell- and glycosylation-related gene was altered differently during the course of infection (Fig. 4). The early onset of Siat4c, MUC2 and TFF3 gene upregulation might be associated with innate immunity to pathogens or may be related to an acute phase reaction. MUC3, MUC4, Relmβ and 3ST1 gene upregulation, which occurred in the adult-worm colonization and rejection stages, might be regulated by different mechanisms from those of early activated genes, possibly through T-cell- or Th2 cytokine-dependent acquired immunity. Moreover, the upregulation of sialyl- and sulfo-transferase genes suggests that the sialylation and sulfation status of the mucins, membrane glycoproteins and/or histo-blood group antigens might be altered dramatically during nematode infections. The results imply that goblet cell and/or mucin responses to infection are a far more complicated series of changes than previously thought, involving the sequential expression of many factors that can individually or coordinately affect the colonization or rejection of pathogens. Further elucidation of the roles and mechanisms regulating the expression of each mucin and non-mucin secretory peptide and regulating the glycosylation status would lead to development of new strategies for the treatment or prevention of not only intestinal nematode infection but also infection with other pathogens.

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TABLE 1. *Relative expression levels of goblet cell- and mucin glycosylation-related genes in the gut mucosa of normal BN rats. Total RNA was extracted from epithelium separated from the jejunum, ileum or proximal colon, or was extracted from the glandular stomach mucosal scrape, and RT-PCR was performed. Levels of each gene expression were normalized to that of β-actin. In the Table levels in the jejunum were arbitrary expressed as 1.00.*

	Stomach	Jejunum	Ileum	Colon
MUC2 (mucin core peptide 2)	UD	1.0 ± 0.2	4.5 ± 1.8	153.2 ± 49.8*
MUC3 (mucin core peptide 3)	UD	1.0 ± 0.4	1.5 ± 0.3	7.5 ± 3.0*
MUC4 (mucin core peptide 4)	UD	1.0 ± 0.3	0.7 ± 0.1	41.0 ± 13.1*
Relmβ (resistin-like molecule β)	2.8 ± 1.7	1.0 ± 0.4	0.4 ± 0.1	47.3 ± 21.1*
TFF3 (intestinal trefoil factor)	0.2 ± 0.1	1.0 ± 0.4	2.7 ± 0.5*	3.9 ± 0.4*
Siat 4c (α-2,3-sialyltransferase IV)	3.6 ± 1.0*	1.0 ± 0.2	11.7 ± 4.1*	7.0 ± 3.0*
3ST1 (3-0 sulfotransferase-1)	12.7 ± 2.2*	1.0 ± 0.2	1.3 ± 0.2	15.4 ± 3.0*
3ST2 (3-0 sulfotransferase-2)	1.9 ± 0.7	1.0 ± 0.4	1.1 ± 0.3	3.3 ± 1.6
FUT1 (α-1,2-fucosyltransferase 1)	1.0 ± 0.2	1.0 ± 0.2	1.5 ± 0.1	13.8 ± 0.5*
FUT2 (α-1,2-fucosyltransferase 2)	3.6 ± 0.6*	1.0 ± 0.1	1.5 ± 0.3	14.3 ± 2.8*
FUT4 (α-1,3-fucosyltransferase 4)	4.7 ± 0.9*	1.0 ± 0.1	0.7 ± 0.1*	3.0 ± 0.2*
Lew 1 (Lewis type 1 antigen synthase: β1,3-N-acetylglucosaminyltransferase 5)	0.1 ± 0.0*	1.0 ± 0.3	0.3 ± 0.0	2.9 ± 0.9

Data shown are mean ± SE of 4 rats. *Significantly different from the levels in the jejunum ($p<0.05$). UD: undetectable.

TABLE 2. *Number of goblet cells in the jejunum and ileum after* N. brasiliensis *infection.*

Days after infection	Jejunum	Ileum
0	13.1 ± 0.7	12.3 ± 0.5
7	19.1 ± 1.2*	14.9 ± 1.2
14	21.5 ± 1.2*	20.0 ± 0.9*
21	11.3 ± 1.0	11.3 ± 1.3

Each measurement was performed on paraffin-embedded tissue sections. Figures in the table represent numbers of goblet cells/100 villus epithelial cells. All data are mean ± SE of 4 rats. *Significantly different from day 0 ($P<0.05$).

Figure legends

Fig 1. Expression of goblet cell- and mucin glycosylation-related genes in the jejunal (closed columns) and ileal (open columns) villus epithelium of BN rats after infection with the nematode *N. brasiliensis*. Total RNA was extracted from the epithelial fraction, reverse transcribed, and relative quantification was carried out by RT-PCR. The quantified value for each sample was normalized with respect to that for β-action. The data are means + SE of 4 animals. The vertical axis shows the expression levels, with day-0 average levels expressed as 1.0. *indicates values significantly different from the day-0 level ($P<0.05$).

Fig. 2. Expression of mucin glycosylation-related genes in the jejunal villus epithelium of mast cell-deficient *Ws/Ws* rats after infection with the nematode *N. brasiliensis*. Total RNA was extracted from the epithelial fraction and semi-quantitative RT-PCR was performed as described in Fig. 1. The data are means + SE of 4 animals. The vertical axis shows the expression levels, with day-0 average levels expressed as 1.0. *indicates values significantly different from the day-0 level ($P<0.05$).

Fig. 3. Expression of goblet cell- and mucin glycosylation-related genes in the jejunal villus epithelium of BN rats in the early period after infection with the nematode *N. brasiliensis*. Total RNA was extracted from the epithelial fraction and semi-quantitative RT-PCR was performed as described in Fig. 1. The data are means + SE of 4 animals. The vertical axis shows the expression levels, with day-0 average levels expressed as 1.0. *indicates values significantly different from the day-0 level ($P<0.05$).

Fig. 4. Successive upregulation of goblet cell- and mucin glycosylation-related genes in the intestinal epithelial cells during the course of *N. brasiliensis* infection.

Fig. 1

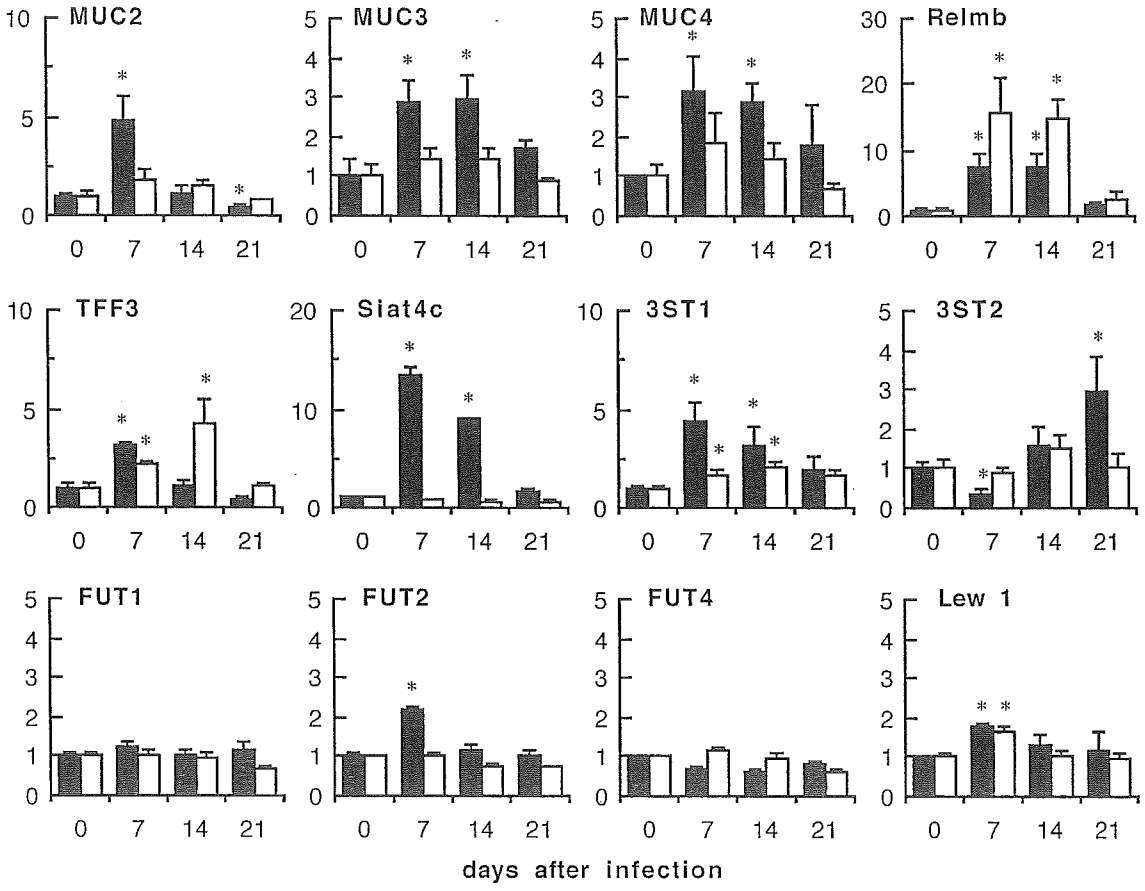

MUC2
MUC3
MUC4
Relmb
TFF3
Siat4c
3ST1
3ST2
FUT1
FUT2
FUT4
Lew 1
0 7 14 21
days after infection

Fig. 2.

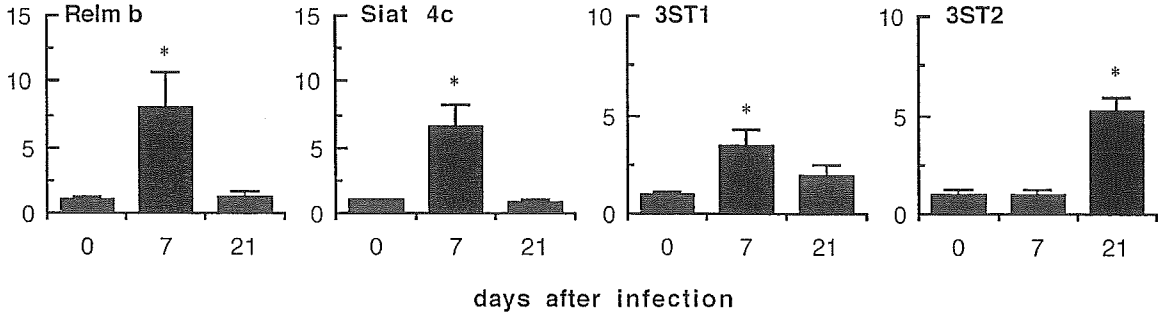

Relm b
Siat 4c
3ST1
3ST2
0 7 21
days after infection

Fig. 3.

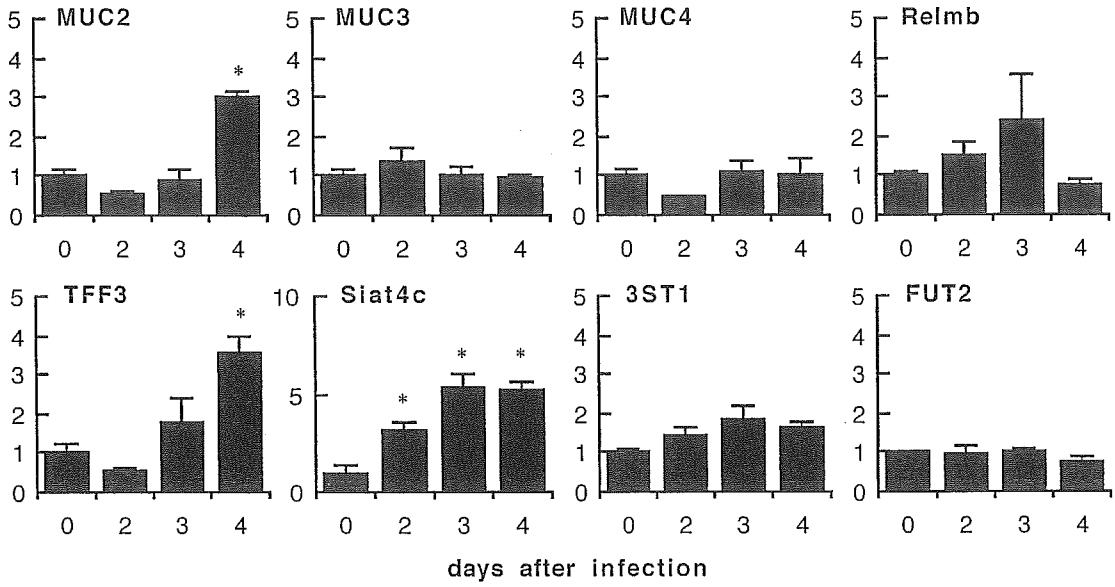
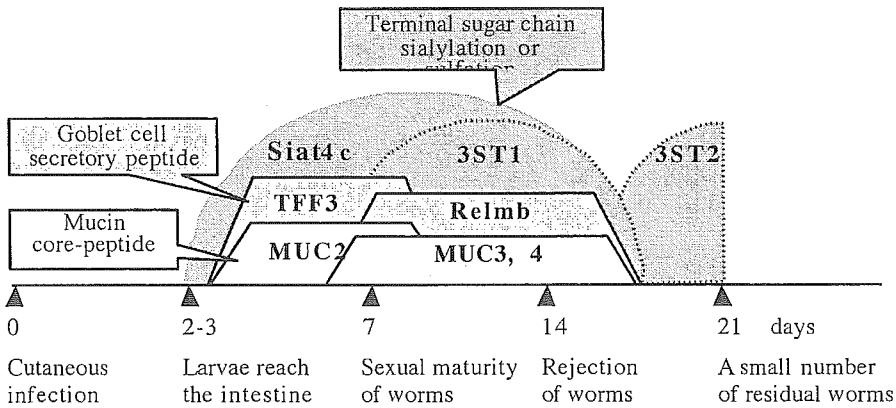

MUC2
MUC3
MUC4
Relmb
TFF3
Siat4c
3ST1
FUT2
days after infection

Fig. 4


Terminal sugar chain sialylation or
Goblet cell secretory peptide
Mucin core-peptide
Siat4c
3ST1
3ST2
TFF3
Relmb
MUC2
MUC3, 4
0
2-3
7
14
21 days
Cutaneous infection
Larvae reach the intestine
Sexual maturity of worms
Rejection of worms
A small number of residual worms

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Molecular phylogenetic relationships among seven Japanese species of *Cercopithifilaria*☆

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Abstract

DNA sequences from a portion of the mitochondrial *COI* gene were used to clarify phylogenetic relationships among Japanese species in the genus *Cercopithifilaria*. Sequences were determined from seven Japanese species, five (*C. shohoi*, *C. multicauda*, *C. minuta*, *C. tumidicervicata* and *C. bulboidea*) from the serow (*Capricornis crispus* F. Bovidae) and two (*C. longa* and *C. crassa*) from the sika deer (*Cervus nippon nippon* F. Cervidae). No base substitutions were observed between *C. bulboidea* and *C. longa*, suggesting that recent host switching of a lineage of *C. bulboidea* between bovid and cervid hosts gave rise to *C. longa*. In phylogenetic trees inferred using a variety of methods, the morphologically ancestral type, *C. bulboidea*, appeared as a derived species. *C. multicauda* was found to be basal in the analyses. It seems therefore that *C. multicauda* is the most primitive out of the seven species.
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Keywords: *Cercopithifilaria*; CO1; Host switching; Molecular phylogeny

1. Introduction

Eberhard [1] discovered a small filaria in a baboon (*Papio anubis*) in Africa, and created a new subgenus *Cercopithifilaria* in the genus *Dipetalonema*. Later, Bain et al. [2] raised *Cercopithifilaria* to generic status in the subfamily Onchocercinae. Morphological characteristics of this genus are a preesophageal cuticular ring at the mouth and an undivided esophagus [2]. There are 28 species in the genus *Cercopithifilaria* worldwide [3]. The final hosts are marsupials, rodents, primates and ruminants. Because of the large number of species, it is considered that this genus might have originated in Africa [4]. In Japan, *C. japonica* was discovered in the Japanese bear [5]. Later, five species (*C. shohoi* Uni, Suzuki and Katsumi 1998, *C. multicauda* Uni and Bain 2001, *C. minuta* Uni and Bain 2001, *C. tumidicervicata* Uni and Bain 2001, *C. bulboidea* Uni and Bain 2001) were discovered from the Japanese serow (*Capricornis crispus*, F. Bovidae).

Recently, Uni et al. [6] examined filariae from 17 Japanese sika deer (*Cervus nippon nippon*, F. Cervidae) captured on Mt. Sobo in Oita Prefecture, and discovered two new species of *Cercopithifilaria*, *C. longa* Uni, Bain and Takaoka 2002 and *C. crassa* Uni, Bain and Takaoka 2002.

☆ Nucleotide sequences data reported in this paper are available in the EMBL, GenBank and DDJB data bases under the accession numbers: AB178834, AB178835, AB178836, AB178837, AB178838, AB178839, AB178840, AB178841, AB178842, AB178843, AB178844, AB178845, AB178846, AB178847, AB178848, AB178849, AB178850, AB178851, AB178852, AB178853.

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In the present study, DNA sequences from a portion of the mitochondrial *COI* gene were determined from all of these seven species, and their phylogenetic relationships were analyzed using these sequences.

2. Materials and methods

2.1. Parasites

Table 1 shows collection time and localities of the seven species examined in this study. Sequences from *Dirofilaria immitis*, *Brugia malayi*, *Wuchereria bancrofti* and *Onchocerca volvulus* were used as outgroups for the phylogenetic analysis.

2.2. PCR and sequencing

Genomic DNA was extracted from whole worms. The worms were incubated in extraction buffer (Invitrogen extraction kit) containing SDS and proteinase K. The solubilized samples were treated with phenol and chloroform. A single worm was used in each case. The PCR conditions and amplification reactions have been described elsewhere [7]. Purified PCR products were precipitated with ethanol and suspended in distilled water, and aliquots were sequenced using the PRISM kit (ABI). PCR primers were used as sequencing primers. The purified reactions were applied to an ABI sequencer. Primers used were *COI*intF (5′-TGA TTG GTG GTT TTG GTA A-3′) and *COI*intR (5′-ATA AGT ACG AGT ATC AAT ATC-3′). Analyses for multiple sequence alignments were done using the programs CLUSTAL V and GENETYXMAC (ver. 6.0). Phylogenetic analysis was performed using distance (neighbor joining (NJ)) and parsimony methods in MEGA (ver. 2.1) and PAUP (ver. 3.1.1), respectively.

3. Results

3.1. Intra-specific variation

The size of the *COI* region of mtDNA of the 7 species of the genus *Cercopithifilaria* was 621 bp. There was one base pair substitution among 6 individuals (5 females and 1 male) of *C. bulboidea*, and between two individuals (2 females) of *C. multicauda*. Similarly, there were two base pair substitutions in two individuals (2 females) of *C. tumidicervicata* and in two individuals (2 females) of *C. minuta*. On the other hand, there were no intra-specific variations in *C. shohoi* (2 females), *C. longa* (4 females) or *C. crassa* (2 females).

3.2. Inter-species variation

Table 2 shows the numbers of pair-wise base pair and amino acid substitutions of the 7 species of the genus *Cercopithifilaria*. The largest number of nucleotide sub-

Table 1
Collection sites and dates of seven species of *Cercopithifilaria* and accession number for outgroup species from four genera

Species	Strains	Sex	Collection site	Collection date	Final host	Host no.	Accession no.
C. bulboidea	Gifu-111	♀	Hida Highlands, Furukawa, Gifu	10.Jul.99	Japanese serow	Gifu #1	AB178834
	Gifu-133	♀	Hida Highlands, Furukawa, Gifu	10.Jul.99	Japanese serow	Gifu #1	AB178835
	SW3-FL7	♀	Mt. Ena, Iwamura, Gifu	12.Nov.99	Japanese serow	Gufu #3	AB178836
	SW3-FL8	♀	Mt. Ena, Iwamura, Gifu	12.Nov.99	Japanese serow	Gufu #3	AB178837
	SW3-MB1	♂	Mt. Ena, Iwamura, Gifu	12.Nov.99	Japanese serow	Gufu #3	AB178838
	SW3-UA1	♀	Mt. Ena, Iwamura, Gifu	12.Nov.99	Japanese serow	Gufu #3	AB178839
C. crassa	S15-97	♀	Taketa, Oita	22.Dec.99	Japanese deer	S15	AB178840
	S15-101	♀	Taketa, Oita	22.Dec.99	Japanese deer	S15	AB178841
C. longa	S32-2	♀	Ogata, Oita	5.Feb.01	Japanese deer	S32	AB178842
	S32-4	♀	Ogata, Oita	5.Feb.01	Japanese deer	S32	AB178843
	S33-4	♀	Ogata, Oita	10.Feb.01	Japanese deer	S33	AB178844
	S33-6	♀	Ogata, Oita	10.Feb.01	Japanese deer	S33	AB178845
C. minuta	SW3-FL3	♀	Mt. Ena, Iwamura, Gifu	12.Nov.99	Japanese serow	Gufu #3	AB178846
	SW3-FL12	♀	Mt. Ena, Iwamura, Gifu	12.Nov.99	Japanese serow	Gufu #3	AB178847
C. multicauda	Gifu-39T	♀	Hida Highlands, Furukawa, Gifu	10.Jul.99	Japanese serow	Gifu #1	AB178848
	Gifu-49C	♀	Hida Highlands, Furukawa, Gifu	10.Jul.99	Japanese serow	Gifu #1	AB178849
C. shohoi	Gifu-7	♀	Mt. Ontake, Asahi, Gifu	29.Jul.99	Japanese serow	Gifu #2	AB178850
	Gifu-14	♀	Mt. Ontake, Asahi, Gifu	29.Jul.99	Japanese serow	Gifu #2	AB178851
C. tumidicervicata	Gifu-91	♀	Hida Highlands, Furukawa, Gifu	10.Jul.99	Japanese serow	Gifu #1	AB178852
	Gifu-132	♀	Hida Highlands, Furukawa, Gifu	10.Jul.99	Japanese serow	Gifu #1	AB178853

Outgroup species	Accession No.
B.malayi	AJ271610
D.immitis	AJ271613
O.volvulus	AF015193
W.bancrofti	AJ271612

Table 2
Inter-specific variations in CO1 region of mitochondrial DNA within the genus *Cercopithifilaria* as well as among five different genera

	[1]	[2]	[3]	[4]	[5]	[6]	[7]	[8]	[9]	[10]	[11]	[12]	[13]	[14]	[15]	[16]	[17]
[1] C.b.FL7		1/0	1/0	1/0	1/0	61/8	53/6	63/7	62/7	67/7	67/7	61/6	62/6	87/17	89/16	73/16	78/14
[2] C.b.G.111	*		0/0	0/0	0/0	62/8	54/6	64/7	63/7	68/7	68/7	62/6	63/6	88/17	90/16	74/16	79/14
[3] C.b.G.133	*	*		0/0	0/0	62/8	54/6	64/7	63/7	68/7	68/7	62/6	63/6	88/17	90/16	74/16	79/14
[4] C.b.UA-1	*	*	*		0/0	62/8	54/6	64/7	63/7	68/7	68/7	62/6	63/6	88/17	90/16	74/16	79/14
[5] C.l.S33-4	*	*	*	*		62/8	54/6	64/7	63/7	68/7	68/7	62/6	63/6	88/17	90/16	74/16	79/14
[6] C.c.S15101	1.259	1.259	1.296	1.296	1.296		64/10	68/11	67/11	81/11	81/11	67/10	68/10	85/20	89/20	79/20	81/17
[7] C.mi.FL3	1.409	1.455	1.455	1.455	1.455	1.207		66/6	65/6	68/5	68/5	45/2	46/2	89/16	82/13	71/13	79/13
[8] C.mu.G.39T	0.909	0.939	0.939	0.939	0.939	1.000	0.886		1/0	69/7	69/7	66/6	67/6	79/14	90/14	81/14	76/13
[9] C.mu.G.49C	0.938	0.969	0.969	0.969	0.969	1.030	0.912	*		69/7	69/7	65/6	66/6	78/14	90/14	81/14	75/13
[10] C.s.G.7	2.722	2.778	2.778	2.778	2.778	1.793	1.833	1.760	1.833		0/0	70/5	71/5	84/16	94/16	87/16	96/15
[11] C.s.G.14	2.722	2.778	2.778	2.778	2.778	1.793	1.833	1.760	1.833	*		70/5	71/5	84/16	94/16	87/16	96/15
[12] C.t.G.91	1.346	1.385	1.385	1.385	1.385	1.030	2.214	0.886	0.912	1.692	1.692		2/0	81/16	83/13	83/13	79/13
[13] C.t.G.132	1.385	1.423	1.423	1.423	1.423	1.061	2.286	0.914	0.941	1.731	1.731	*		83/16	83/13	84/13	78/13
[14] D.i.	0.977	1.000	1.000	1.000	1.000	0.977	0.935	0.837	0.857	1.471	1.471	0.929	0.976		97/14	86/14	70/7
[15] B.m.	1.225	1.250	1.250	1.250	1.250	1.171	1.278	1.093	1.119	1.474	1.474	1.075	1.075	1.205		60/3	86/8
[16] W.b.	1.355	1.387	1.387	1.387	1.387	1.633	1.029	1.025	1.051	2.222	2.222	1.128	1.154	1.606	1.609		76/9
[17] O.v.	0.950	0.975	0.975	0.975	0.975	1.189	0.975	1.054	1.083	1.400	1.400	0.975	0.950	0.944	1.389	1.303	

Values above the diagonal are pairwise numbers of nucleotide substitutions/amino acid substitutions. Those below are transition/transversion ratios.
[1] C.b.FL7: *C. bulboidea* (SW3-FL7) [2] C.b.G.111: *C. bulboidea* (Gifu-111) [3] C.b.G.133: *C. bulboidea* (Gifu-133) [4] C.b.UA-1: *C. bulboidea* (SW3-UA-1) [5] C.l.S33-4: *C.longa* (S33-4) [6] C.c.S15101: *C. crassa* (S15-101) [7] C.mi.FL3: *C. minuta* (SW3-FL3) [8] C.mu.G.39T: *C. multicauda* (Gifu-39T) [9] C.mu.G.49C: *C.multicauda* (Gifu-49C) [10] C.s.G.7: *C. shohoi* (Gifu-7) [11] C.s.G.14: *C. shohoi* (Gifu-14) [12] C.t.G.91: *C. tumidicervicata* (Gifu-91) [13] C.t.G.132: *C. tumidicervicata* (Gifu-132) [14] D.i.: *D. immitis* [15] B.m.: *B. malayi* [16] W.b.: *W. bancrofti* [17] O.v.: *O. volvulus*.

stitutions (81) was observed between *C. crassa* and *C. shohoi*, and the largest numbers of amino acid substitutions (11) were observed between *C. crassa* and *C. multicauda* as well as *C. crassa* and *C. shohoi*. On the other hand, sequences from *C. longa* were not distinguishable from those from *C. bulboidea*.

The smallest value (0.886) of the transition/transversion ratio was observed between *C. minuta* and *C. multicauda* as well as *C. tumidicervicata* and *C. multicauda*. Similarly, the largest value (2.778) was observed between *C. shohoi* and *C. bulboidea* as well as *C. shohoi* and *C. longa*. There were no transversions within species or between *C. bulboidea* and *C. longa*.

3.3. Phylogenetic analysis

3.3.1. Trees constructed using nucleotide sequences

Three main clusters were formed by the NJ method. As shown in Fig. 1a, *C. multicauda* was basal within *Cercopithifilaria*, and *C. shohoi* emerged next. The third cluster contained *C. minuta* and *C. tumidicervicata*, and most derived cluster consisted of *C. crassa*, *C. bulboidea* and *C. longa*. In this cluster, *C. crassa* was sister to the remaining two species.

In trees inferred using the parsimony method (Fig. 1b), *C. crassa* was basal within the genus. Next to emerge is a cluster consisting of *C. minuta* and *C. tumidicervicata*. *Cercopithifilaria multicauda/C. shohoi* and *C. bulboidea/C. longa* constitute the two most derived clades in this tree.

3.3.2. Tree constructed using amino acid sequences

NJ and parsimony methods yielded almost identical topologies. In the NJ tree (Fig. 2a), *C. multicauda* was basal within the genus and next to emerge were *C. tumidicervicata/C. minuta/C. shohoi*. *Cercopithifilaria crassa*, *C. longa* and *C. bulboidea* formed the most derived clade. The parsimony tree (Fig. 2b) differed only in that the branch separating *C. multicauda* from the rest of the genus was collapsed.

In general, the trees produced by the NJ method using either nucleotide sequences or amino acid sequences were both very similar to those produced by the parsimony method using amino acid sequences. However, in the trees produced by the parsimony method using nucleotide sequences, *C. crassa* was placed at the base of the genus.

4. Discussion

In the morphological study of the filaria, any complicated characteristics are considered to be primitive [8]. This idea is based on the hypothesis that complicated characteristics gradually degenerate over evolutionary time. Although apparently valid for the genus *Onchocerca*, if this also applies to *Cercopithifilaria*, we would be obliged to recognize *C. bulboidea* as a plesiomorphic form. Many characteristics of this species are complex: for example, the arrangement of the caudal papillae of males and the number of these papillae [9]. On the other hand, *C. multicauda*, *C. minuta* and *C. tumidicervicata* seem to be relatively simplified in their morphology. Further, *C. shohoi* from serows has intermediate characteristics, and seems similar to *C. longa* from deer in the morphology of the precloacal papillae (having 4 pairs) and in the direction of the cuticular striations in the lateral midbody of females. *Cercopithifilaria crassa* seems quite different in morphology from any

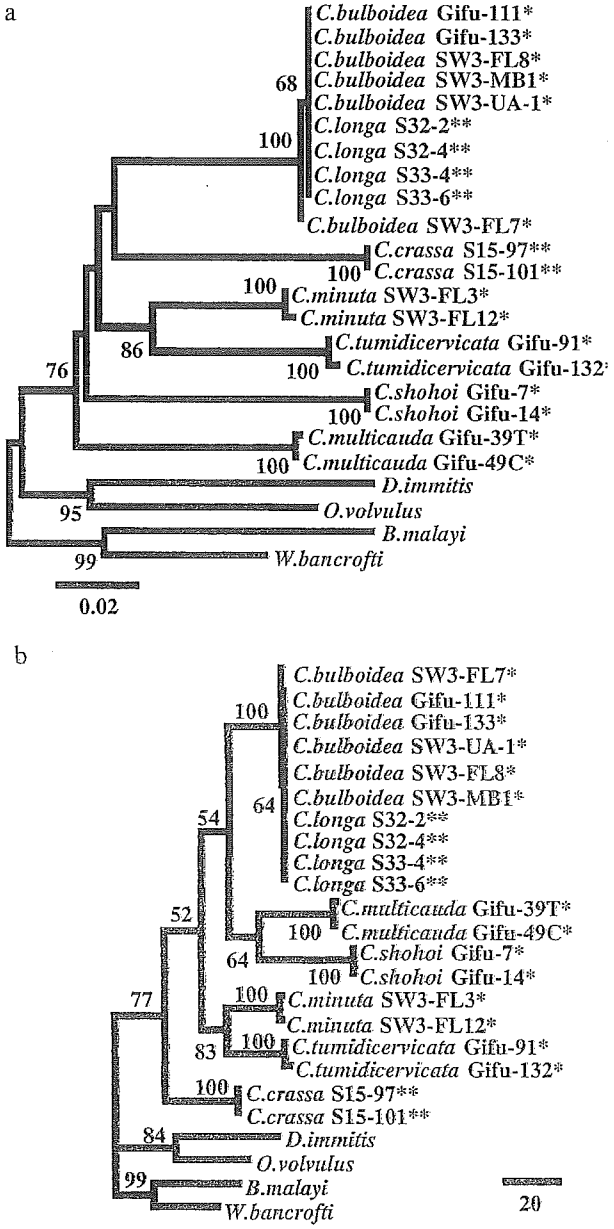

Fig. 1. a. CO1 tree constructed by the neighbor joining method (1000 bootstrap) using the Mega program. Nucleotide sequences were used in this tree. Scale bar indicates the number of changes inferred as having occurred along each branch. *: *Capricornis crispus*; **: *Cervus nippon nippon*. b. CO1 tree constructed by the parsimony method (heuristic: 1000 bootstrap) using the Paup program. Nucleotide sequences were used in this tree. Scale bar indicates the number of changes inferred as having occurred along each branch. *: *Capricornis crispus*; **: *Cervus nippon nippon*.

other species of the genus, although its features, such as the arrangement of the caudal papillae and direction of the cuticular striations in the lateral midbody of females, seem similar to those of *C. multicauda*, *C. minuta* and *C. tumidicervicata* [6]. On the basis of morphology of parasites, Uni et al. [6] considered that *C. bulboidea* (including *C. longa*) is plesiomorphic within the genus *Cercopithifilaria*. Further, *C. shohoi* may have arisen from this ancestral form, and *C. multicauda*, *C. minuta*, *C. tumidicervicata* and *C. crassa* may have appeared later. However, the results of the present DNA analysis differed from the results of morphological analysis. To begin with, *C. bulboidea* and *C. longa* both occupied derived positions within the trees

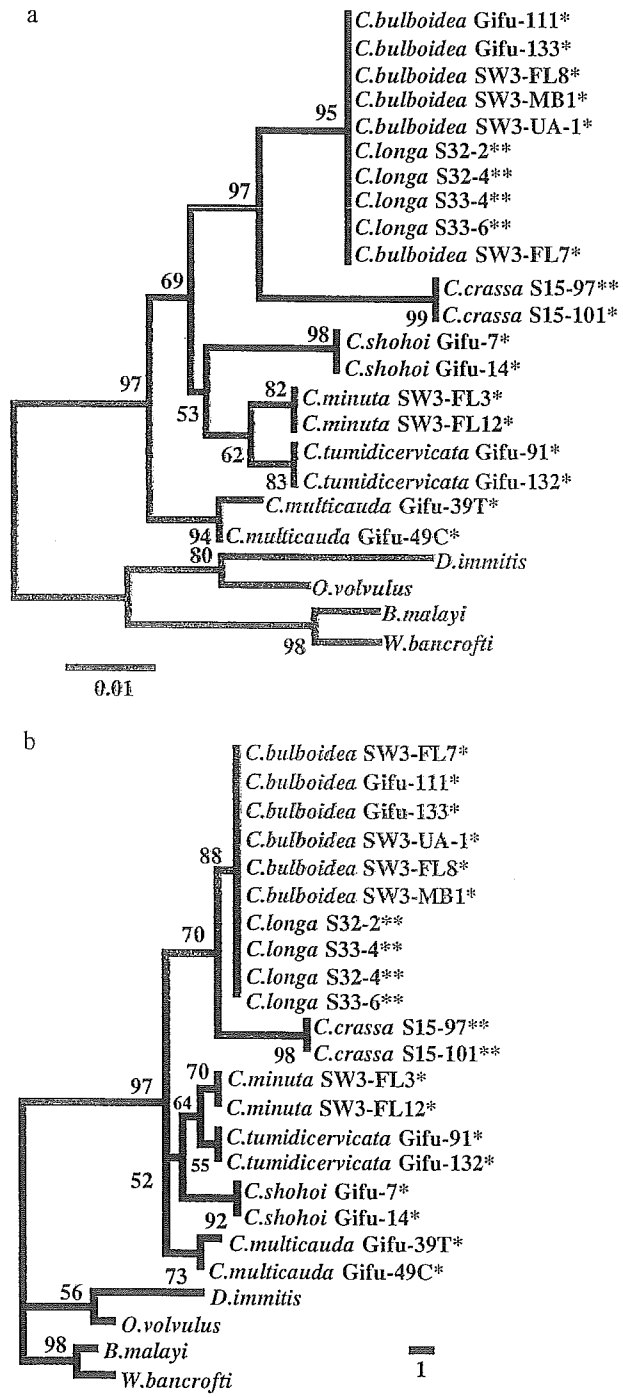

Fig. 2. a. CO1 tree constructed by the neighbor joining method (1000 bootstrap) using the Mega program. Amino acid sequences were used in this tree. Scale bar indicates the number of changes inferred as having occurred along each branch. *: *Capricornis crispus*; **: *Cervus nippon nippon*. b. CO1 tree constructed by the parsimony method (heuristic: 1000 bootstrap) using the Paup program. Amino acid sequences were used in this tree. Scale bar indicates the number of changes inferred as having occurred along each branch.*: *Capricornis crispus*; **: *Cervus nippon nippon*.