Hepatitis delta virus (HDV) is a unique, viroid-like human pathogen that needs to be associated with hepatitis B virus infection (Monjardino, 1996). It was first detected in the liver cell nuclei of patients with hepatitis B surface antigen (HBsAg)-positive chronic liver disease (Rizzetto et al., 1977). HDV has a single-stranded, negative-sense, circular RNA genome of 1.7 kb in length (Wang et al., 1986, 1987; Makino et al., 1987; Saldanha et al., 1990; Imazeki et al., 1991; Casey et al., 1993; Lee et al., 1996; Wu et al., 1998). The RNA genome encodes two proteins, 24 and 27 kDa, referred to as the small hepatitis delta antigen (S-HDAg) and the large hepatitis delta antigen (L-HDAg), respectively (Bonino et al., 1986; Bergmann & Gerin, 1986). S- and L-HDAg share a unique RNA-editing event. The two forms of HDAg have distinct and opposing functions: S-HDAg is required for hepatitis delta virus replication, whereas L-HDAg inhibits HDV replication and is required for virion formation (Gerin et al., 2001).

Based on geographical origin and genomic divergence, HDV strains are divided into at least three genotypes: I, II and III (Casey et al., 1993; Lai, 1995). Recently, Wu et al. (1998) reported a novel strain of genotype II in Taiwan and proposed genotypes Ila and Iib. The Miyako Islands, which are located in the southernmost part of Japan, about 390 km east of Taiwan, are one of the areas in Japan that is endemic for HDV (Sakugawa et al., 1995, 1997a, b, 1999; Nakasone et al., 1998; Arakawa et al., 2000). The majority of patients with chronic HDV-related liver disease in the Miyako Islands exhibit a relatively mild course of infection that evolves to a quiescent cirrhotic condition (Sakugawa et al., 1997a). Sakugawa et al. (1999) sequenced the 645 nt of HDAg (position 940–1584; numbering according to Wang et al., 1987) of six strains of genotype II, collected from patients with HDV-related chronic liver disease in the Miyako Islands, and reported the presence of genotype Iib. However, the entire genome sequence of the Miyako isolate has not been reported. Hence, we describe the entire nucleotide sequence of HDV isolated in the Miyako Islands.

The serum sample L215 was chosen from a panel of sera collected in 1992 from cirrhotic patients and stored at the University of the Ryukyus, Okinawa, Japan. L215 serum was positive for HBsAg and negative for hepatitis B e antigen (HBeAg), while it was positive for anti-HBe antibodies and HDV RNA. RNA was extracted from the serum using the acid guanidinium/phenol extraction method (Chomczynski & Sacchi, 1987) and was dissolved in 30 μl DEPC-treated distilled water. The RNA was denatured at 94 °C for 2 min and then reverse-transcribed at 42 °C for 1 h in a mixture containing RNA template, 50 mM Tris/HCl (pH 8.3), 75 mM KCl, 3 mM MgCl₂, 10 mM DTT, 250 nM each of four dNTPs, 100 pmol antigenomic-sense primer (Fig. 1), 10 units RNaseout (GibcoBRL) and 100 units RiverTraAce reverse transcriptase (Toyobo). PCR was carried out in a buffer containing 10 mM Tris/HCl (pH 8.9), 0.1 M KCl, 1.5 mM MgCl₂, 250 nM each of four dNTPs, 100 pmol genomic-sense primer and 4 units Tth DNA polymerase (Boehringer Mannheim), and performed for 40 cycles; each

The complete genomic sequence of hepatitis delta virus genotype Iib prevalent in Okinawa, Japan

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The Miyako Islands, located in the southernmost part of Japan, have been reported to be endemic for hepatitis delta virus (HDV). The majority of HDV patients in this area exhibit a relatively mild course of infection that evolves into a quiescent cirrhotic condition. The entire nucleotide sequence of the Miyako isolate (L215) of HDV obtained from a cirrhotic patient infected with HDV was determined. This isolate, L215, comprises 1682 nt and encodes 213 aa of the hepatitis delta antigen. Phylogenetic analysis showed that L215 is closely related to the Taiwanese genotype IIb. However, the entire genome sequence of the Miyako isolate has not been reported. Hence, we describe the entire nucleotide sequence of HDV isolated in the Miyako Islands.

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cycle consisted of 95 °C for 1 min, 42 °C for 1·5 min and 72 °C for 1 min. The PCR product was analysed by electrophoresis in 1·5 % agarose gels and visualized by staining with ethidium bromide. The specific PCR products were cut out from the agarose gels and cleaned using the QIAquick Gel Extraction kit (Qiagen). Cleaned products were then cloned into the pCR2.1 TA cloning vector (Invitrogen), according to the manufacturer’s instructions. Nucleotide sequencing was performed using the Dye Terminator Cycle Sequencing FS Ready Reaction kit (Applied Biosystems), according to the manufacturer’s instructions. Three clones of each PCR product were sequenced. Sequencing data were analysed using DNASIS, version 3.6 (Hitachi Software Engineering).

Five overlapping cDNA clones covered the entire viral genome (Fig. 1). The entire viral genome of the L215 strain contained 1682 nt. The RNA-editing event of HDV is highly specific for both of the sequences that neighbour position 1012 and the base-paired context of position 1012 within the unbranched rod structure of HDV RNA (Casey et al., 1992, 1993). The nucleotide substitution at position 1014 also affects the rod structure of HDV RNA (Casey et al., 1992; Yang et al., 1995). This substitution at position 1014 on
HDV genotype II appears in all sequences published (Sakugawa et al., 1999). HDV genotype I isolates with a mutation at position 1014 (U→C) were reported to be associated with less severe liver diseases (Yang et al., 1995). Sakugawa et al. (1999) reported that all six Miyako isolates showed substitutions at position 1014 (U→C). The L215 strain also had a C residue at position 1014.

Phylogenetic analysis was performed using the UPGMA method, both on the entire HDV nucleotide sequences of L215 and five previously reported strains, including three genotypes (Fig. 2a). The L215 strain showed the highest homology (80–9 %) to the Taiwan genotype IIb strain (TWD62), while it was distantly related (69–2 %) to genotype Ia and more distantly related to genotypes I and III (65–5 and 54–8 %, respectively). A more inclusive phylogenetic analysis using the available six partial HDV sequences of Miyako isolates (nt 940–1584) was performed also (Fig. 2b). Results demonstrated that all of the Miyako isolates clustered into genotype IIb.

The complete cDNA sequence and the deduced amino acid sequence indicated that the L215 strain encoded 213 aa. It was reported that amino- and carboxyl-termini of the HDAg of genotype IIb showed a higher homology to those of genotype Ia, while the middle region showed a higher homology to genotype I (Wu et al., 1998). Our results also agreed with this finding. It was speculated that genotype IIb might have arisen from the recombination of HDV genotype I and II genomes during replication (Wu et al., 1998).

The RNA-editing event is an important control point in the HDV life cycle because it results both in packaging of viral RNA and in inhibiting HDV replication (Casey et al., 1992). Thus, the predicted secondary structures of the nucleotide pairs between positions 578 and 583, and 1022 and 1017 (nucleotide positions are according to the L215 strain) of three different genotypes were compared. There were at least three Watson–Crick pairs on either side of the RNA-editing site within genotype I and III sequences, although the secondary structure of this region was significantly different between these two strains (Fig. 3). Our predicted secondary structures also agreed with previous reports (Casey et al., 1992; Nakano et al., 2001). The published genotype II sequences have four conserved Watson–Crick pairs on the right-hand side of the RNA-editing site. However, there were only two G–C pairs on the left-hand side. Iviushina et al. (2001) reported that genotype II(a) isolates from Yakutia, Russia, have two conserved G–C pairs on the left-hand side of the RNA-editing site. However, the L215 strain (genotype IIb) had only one G–C pair in this position, which might form a less strong secondary structure. Hsu et al. (2002) reported that the editing efficiency of HDV genotype I is higher than that of genotype II. They reported also that the nucleotide and structural changes surrounding the RNA-editing site might be responsible for the lower RNA-editing efficiency of genotype II strains. Further study is needed to evaluate the RNA-editing efficiency of the Miyako isolate L215 strain.

![Fig. 3. Comparison of the predicted editing targets in the anti-genomic HDV RNA of genotypes I, Ila, IIb and III. Numbering is based on the sequence of each strain. Asterisks indicate the editing site corresponding to nt 1012. Watson–Crick pairs are indicated by solid lines.](http://vir.sgmjournals.org)

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