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Phylogenetic relationships between rice dwarf phytoreovirus isolates from five countries

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Abstract

Rice dwarf virus isolates were collected from several locations in Japan, the Philippines, China, Nepal and Korea. Genomic dsRNA segment profiles in polyacrylamide gel electrophoresis differed among the isolates. There were less differences in the profiles between isolates from Japan and Korea than in those between these two Countries and others. Nucleic acid hybridization was used to examine the extent of genomic variation. Full-length cDNAs to all genomic segments encoding non-structural proteins (S4, S6, S9, S10, S11 and S12) were synthesized from two Japanese isolates, and were used for dot-blot hybridization. Hybridizations using probes generated from the full-length cDNA clones failed to differentiate isolates from different geographical areas. However, cDNA probes covering a variable region of S12 were able to distinguish Japanese and Korean isolates from those of other countries. Phylogenetic tree analysis based on the amino acid sequence of P12 encoded by S12 grouped Japanese and Korean isolates together. The Chinese isolates from two different locations (Yunnan and Fujian) were closely related to each other, and were the most distantly related to Japanese and Korean isolates.

Introduction

Rice dwarf (phytoreovirus) (RDV), a member of the family *Reoviridae* (Uyeda and Milne, 1995), has a genome composed of 12 segmented dsRNAs designated as S1 to S12 with an increasing order of mobility in polyacrylamide gel electrophoresis (PAGE). The virus is transmitted by leafhoppers. The rice dwarf disease was first reported in Japan (see Iida et al., 1972), and on several occasions has been reported to be a serious epidemic disease in rice. The disease has subsequently been reported in Korea, China and Nepal (Iida et al., 1972; Ou, 1985; Dahal et al., 1996), all in subtropical or temperate zones. More recently, it has also been isolated in Mindanao, Philippines (Cabauatan et al., 1993), a tropical area. The principal vector in Japan and Korea is *Nephotettix cincticeps*, and in Nepal and the Philippines it is *N.nigropictus* (Cabauatan et al., 1993; Dahal et al., 1996 a).

In order to understand how these viruses have evolved in different geographical areas, the extent of genomic variation was examined by hybridization analysis and amino acid sequence comparison of P12 encoded by S12 (Suzuki et al, 1992 b). Although the function of P12 has yet to be established, nucleotide sequence analysis and comparison of amino acid sequences between RDV, wound tumor virus, and rice gall dwarf virus showed that S12 is the most diverse gene within the genus *Phytoreovirus* (Uyeda et al., 1994). Due to its diversity, we used S12 for phylogenetic analysis of RDV isolates.

Materials and methods

Virus

Original culture of RDV-AN had two kinds of S12 resolved by polyacrylamide gel electrophoresis

Table 1. Rice dwarf virus isolates

Country	Prefecture or Province	City	Isolate abbreviation
Japan			RDV-H
	*Sa*itama	Kumagaya	RDV-Sa
	Ehime	Oosu-shi, *N*iiya	RDV-N
	Ehime	Oosu-shi, *W*ada	RDV-W
	Kochi	*A*ki-gun, *K*itagawa	RDV-AK
	Kochi	*A*ki-shi, *I*oki	RDV-AI
	Kochi	*A*ki-gun, *N*ahari	RDV-AN
	*Fu*kuoka	Fukuoka	RDV-Fu
	*N*agasaki	Nisisonogi	RDV-Ng
	*K*umamoto		RDV-K
	*Ka*goshima	*K*iiri	RDV-KaK
	*Ka*goshima	Kiiri-cho, *M*aenohama	RDV-KaM
*P*hilippines	Mindanao	North Cotabato	RDV-P
*N*epal	*I*madol		RDV-NEI
	*L*ubhu		RDV-NEL
*C*hina	Yunnan	*K*unming	RDV-CK
	*F*ujian		RDV-CFJ
*Kor*ea	Milyang		RDV-KOR

(Murao et al., 1994). After several passages by insect transmission, RDV-AN1, that had only one of the two S12s, was attained. RDV-H is the isolate that had been maintained at Hokkaido University. Their genomic dsRNAs were used for cDNA synthesis, RDV-P is the same isolate as that of Cabauatan et al. (1993). Isolates collected from several locations in Japan, the Philippines, China, Nepal and Korea. Individual naturally infected plants were numbered after isolate abbreviation listed in Table 1 and used for the comparison.

PAGE

Genomic dsRNAs were directly extracted (Murao et al., 1994) from infected leaves, and 200–400 ng of the genomic dsRNAs were subjected to 9% PAGE at 300 V constant for 40–60 h in 40 mM Tris-acetate, 1 mM EDTA buffer using a 20 × 40 × 0.08 cm gel.

cDNA cloning

Full-length cDNA clones were synthesized by the polymerase chain reaction coupled with reverse transcription as previously described by Murao et al. (1994). cDNAs to S6, S9, S10, S11 and S12 was made from genomic RNAs of RDV-AN1 and S4 from that of RDV-H. The PCR products were cloned into plasmid vector pUC 119. The integrity of several of the apparent fun-length clones has been confirmed by analysis of the terminal nucleotide sequences.

Synthesis of randomly labeled probes from cDNA

cDNAs to RDV genomic segments were cleaved by appropriate restriction enzymes from the plasmid vector and purified by 1% agarose gel electrophoresis in TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 7.8). Eluted DNAs were labeled for at least 3 h at 37 °C with [α -^{32}P] dCTP using a random primer and Klenow fragment of *E. coli* DNA polymerase I.

Dot blot hybridization

Heat-denatured dsRNA of RDV isolates was spotted to a nylon membrane Hybond^{TM}-N^{+} (Amersham). The spotted membranes were dried and cross-linked by a GS Gene Linker^{TM} (Bio-Rad). Prehybridization was performed at 42 °C for at least 2 h in 50% formamide, 5 × SSC, 50 mM sodium phosphate (pH 6.7), 500 μg/ml denatured salmon sperm DNA, 0.1% SDS and 5 × Denhardt's solution. Hybridization was done at 52 °C overnight, with more than 10^6 cpm/ml of probes. After hybridization, the membrane was washed 3 times for l0 min at room temperature in 2 × SSC, 0.1% SDS followed by 3 washings for 20 min in 0.l × SSC and 0.1% SDS at60 °C.The membranes were dried and signals were analyzed with a BAS 1000 image analyzer (FUJIX).

Sequencing

The cDNAs to S12 were sequenced by the dideoxynucleotide chain termination method (Sanger et al, 1977) using a Sequenase version 2.0 DNA sequencing kit (United States Biochemical) or Thermo Sequenase fluorescent labeled primer cycle sequencing kit with 7-deaza-dGTP (Amersham). The entire sequence was analyzed in both directions. Deduced nucleotide and amino acid sequences were assembled and analyzed using the computer program DNASIS (Hitachi Software Engineering). The nucleotide sequence of S12 of an isolate from Fujian, China (RDV-CFJ) was taken from Li et al. (1994).

Results

Differentiation of RDV isolates by electrophoresis of dsRNA

When the extracted dsRNAs were compared using 9% PAGE, the migration patterns differed among most

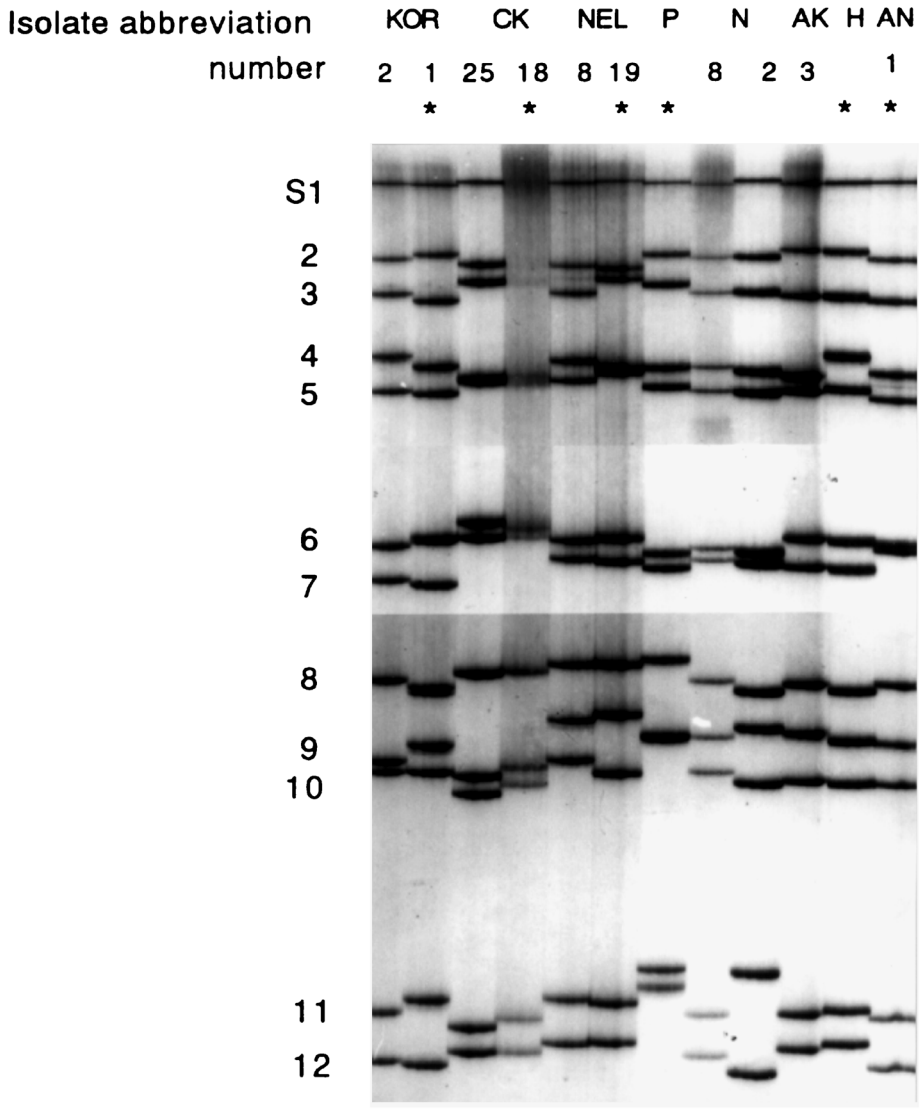

Figure 1. Comparison of the genomic dsRNA migration profiles of RDV isolates obtained from differenet geographical areas. The dsRNAs directly extracted from infected rice plants were loaded on 9% polyacrylamide gels and silver-stained. The isolates used are indicated above by abbreviations (see Table 1). The number refers to naturally infected plants within the same field. Segments are numbered on the left. The isolates used for sequence analyses of S12s are indicated as asterisks.

samples, and the isolates could be differentiated by using these patterns. Figure 1 shows a PAGE comparison of selected isolates from locations listed in Table 1. Between the Japanese and Korean isolates, mobility differences were not as great as that between isolates from Japan and Nepal, from Japan and China, and from Japan and the Philippines. S12 of RDV-P migrated slower than S11 because of rearrangment as reported by Murao et al. (1996).

Differentiation of RDV isolates by dot-blot hybridization of dsRNAs

In order to discern genome homology among the RDV isolates, the full-length cDNAs to S4, S6, S9, S10, S11 and S12 coding nonstructural proteins were used as probes. When full-length cDNA probes of S4, S6, S9, S10 and S11 were used, significant differences in the signals among RDV isolates were not detected. Using a full-length cDNA probe to S12, isolates from foreign countries showed slightly weaker signals than those

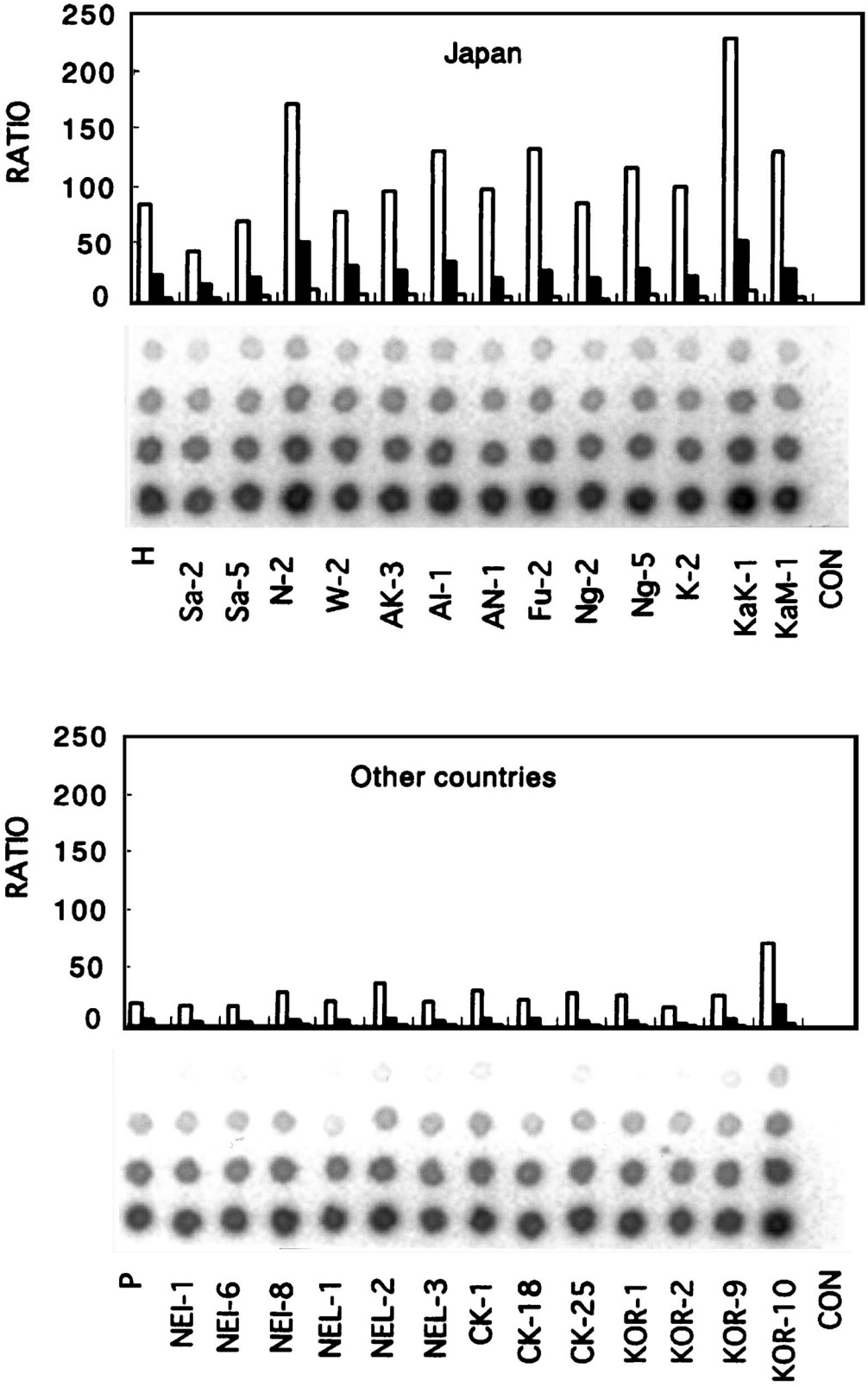

Figure 2. Dot-bolt hybridization with ^{32}P-labeled genome segment 12. Total genome was dotted at dilution series (50, 10, 2 and 0.4 ng). The numbers at left show relative signal intensities when the intensity of RDV-AN11 is taken as 100, using a BAS1000 image analysis. The isolates used are indicated below by abbreviations (see Table 1). Control (CON) is a background of the signal.

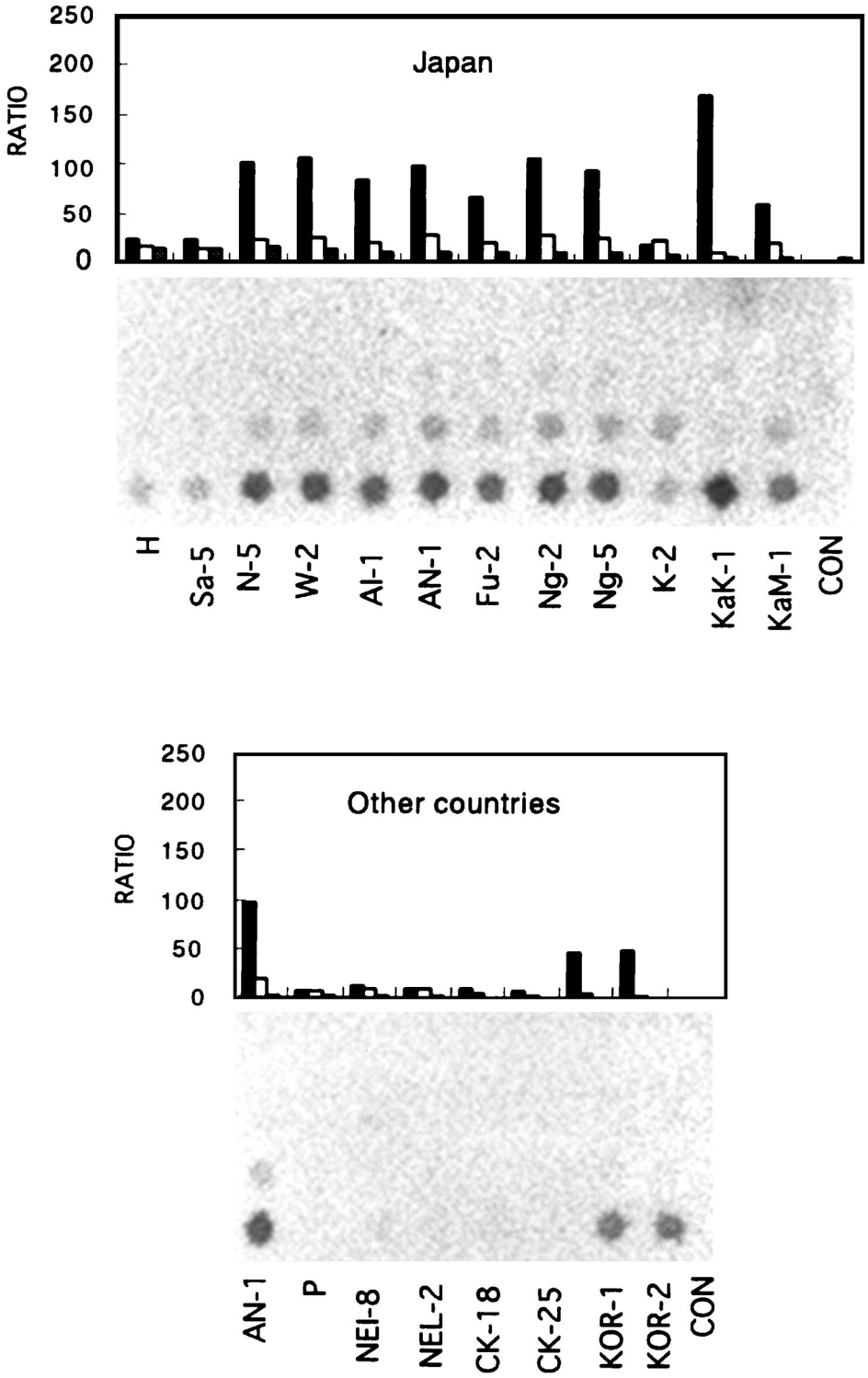

Figure 3. Dot-bolt hybridization with genome segment 12 partial cDNA probe. The partial cDNA probe was made from 283 nt to 381 nt of a variable region of S12 of RDV-AN1. Hybridization was performed as in Figure 2.

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H      283  CCGGAAACTTTGGGTCCTCTGGACCAACTAATGCTCACTAC
AN1    283  ..........................T.........TACT
KOR1   283  ..........................T.........TAC.
CK18   283  ..........C.A.......................A...CT
NEL19  283  .........C......T.....G..T.T.......T...CT
P      283  ......................C....T.....T.T.T.C.

H      324  AATCAGAACGCTAATGGCGATCGATTGGCTGAAAATCTTTT
AN1    324  ........................................
KOR1   324  ........................................
CK18   324  ............G..............T.........C.
NEL19  324  ............T.T........................A.
P      324  ......................A.................C.

H      365  TTGCTTAAGGAATCGTC
AN1    365  .................
KOR1   365  .................
CK18   365  C................
NEL19  365  A................
P      365  C................
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Figure 4. Comparison of nucleotide sequences of cDNAs to S12 from 283 nt to 318 nt of RDV-H, -AN1, -KOR1, -CK18, -NEL19 and P. Only differences from the RDV-H sequence are shown. RDV-H and -AN1 represent the two groups reported by Murao et al. (1994).

of Japanese isolates (Figure 2). Significant differences in the signals among isolates, however, were detected using a partial cDNA probe of variable regions from 283 nt to 381 nt of S12. Japanese and Korean isolates showed strong signals, while the Philippines, Chinese and Nepalese isolates did not show any detectable signals (Figure 3).

Comparison of nucleotide sequences of S12

The exact genomic relationship between the isolates was examined by comparing the nucleotide sequence of S12s. Figure 4 shows variations in S12 sequences of RDV-H, AN1, KOR1, CK18, NEL19 and P from 283 nt to 381 nt. RDV-CK18, NEL19 and P isolates were found to have greater variation than RDV-KOR1, in comparison with RDV-H and AN1. This result was consistent with the groupings based on signals in dot-blot hybridization. Phylogenetic analysis of amino add sequence deduced from the nucleotide sequence of S12 showed that isolates appear to fall into two groups. Isolates CK and CFJ were closely related to each other, and separated from other isolates. Isolates N, SUZ, AN1, KOR1, W1, AK, AN2 and H formed a cluster, and then isolates NEL19 and P were grouped in order. This showed that the homology of Japanese and Korean isolates is very high, in comparison with that of Chinese, Nepalese and Philippine isolates (Figure 5).

Discussion

In order to investigate differences among RDV isolates from several prefectures in Japan and other countries, PAGE and dot-blot hybridization was initially performed. S12 of Japanese isolates could be divided into two groups by PAGE and sequence analysis (Murao et al., 1994). Sequence comparison in the present study showed that the specific region of S12 varied greatly between the two Japanese groups and other countries. A cDNA probe to this region clearly distinguished RDV isolates from different geographical areas. In this respect, a partial cDNA probe of S12 could be a useful tool for distinguishing RDV isolates.

Phylogenetic analysis of S12 by deduced amino acid sequence showed that Chinese isolates were the least related to Japanese isolates. A comparison of isolates from the Philippines, Korea and Nepal showed that Korean and Japanese isolates are closely related. This is probably because the virus has evolved together with the geographical isolation of the vector insect, i.e., Korean and Japanese isolates share a common pool of the insect vector. The main vector of RDV is *Nephotettix cincticeps* in Japan, Korea (Iida et al., 1972) and China (Virus Research Team, Plant Protection Institute, Zejian Academy of Agricultural Sciences, 1985), and *N.nigropictus* in the Philippines (Cabauatan PQ et al., 1993) and Nepal (Dahal et al., 1996 a). The finding that Chinese isolates are less related to Japanese and Korean than are Nepalese and the Philippine isolates is unexpected, based on the type of the vector insect. Further nucleotide sequence analysis of S12 of RDV isolates in areas between Japan and China should provide a clearer picture of the evolutionary relationships of different RDV isolates.

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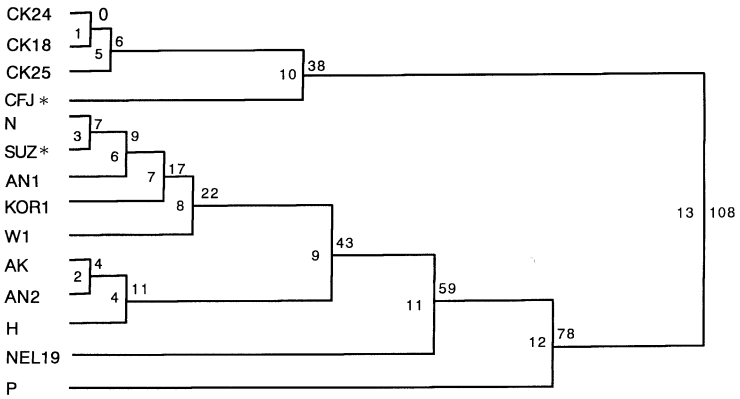

Figure 5. Phylogenetic analysis of P12 deduced from the nucleotide sequence of S12. The analysis was performed by a UPGMA method installed in a program of GeneWorks (IntelliGenetics). * RDV-CFJ: Li et al. (1994), RDV-SUZ: Suzuki et al. (1992).

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