# CHARACTERIZATION OF SRI LANKA RABIES VIRUS ISOLATES USING NUCLEOTIDE SEQUENCE ANALYSIS OF NUCLEOPROTEIN GENE

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**Summary.** – Thirty-four suspected rabid brain samples from 2 humans, 24 dogs, 4 cats, 2 mongooses, 1 jackal and 1 water buffalo were collected in 1995–1996 in Sri Lanka. Total RNA was extracted directly from brain suspensions and examined using a one-step reverse transcription–polymerase chain reaction (RT-PCR) for the rabies virus nucleoprotein (N) gene. Twenty-eight samples were found positive for the virus N gene by RT-PCR and also for the virus antigens by fluorescent antibody (FA) test. Rabies virus isolates obtained from different animal species in different regions of Sri Lanka were genetically homogenous. Sequences of 203 nucleotides (nt)-long RT-PCR products obtained from 16 of 27 samples were found identical. Sequences of 1350 nt of N genes of 14 RT-PCR products were determined. The Sri Lanka isolates under study formed a specific cluster that included also an earlier isolate from India but did not include the known isolates from China, Thailand, Malaysia, Israel, Iran, Oman, Saudi Arabia, Russia, Nepal, Philippines, Japan and from several other countries. These results suggest that one type of rabies virus is circulating among human, dog, cat, mongoose, jackal and water buffalo living near Colombo City and in other five remote regions in Sri Lanka.

**Key words:** rabies virus; isolates; Sri Lanka; nucleoprotein gene; nucleotide sequence; phylogenetic analysis; RT-PCR

## Introduction

Annually, 40,000–100,000 human deaths are caused by rabies. Most of them occur in developing countries. In 1995 and 1996, there have been a total of 272 human and 1934 animal rabies cases in Sri Lanka (RABNET, WHO). Several studies comparing a direct FA test and a nested PCR assay for rabies diagnosis have been reported (Kamolvarin *et al.*, 1993; McColl *et al.*, 1993). In addition, a rapid and sensitive hemi-nested RT-PCR assay has been used for detection of rabies virus and rabies-related viruses (Heaton *et al.*, 1997). That study has shown that the nested and hemi-nested RT-PCR assays were more sensitive than the FA test.

The rabies virus N gene, which is highly conserved, has been used as the target for RT-PCR analysis in several molecular epidemiological studies (Nadin-Davis *et al.*, 1993, 1994); Rupprecht and Smith, 1994; Smith *et al.*, 1995; De Mattos *et al.*, 1996, 1999; Bourhy *et al.*, 1999; Ito *et al.*, 1999; David *et al.*, 2000). N gene contains two large variable regions at the 3' and 5' ends that have been targeted for analysis (Smith *et al.*, 1992, 1995; McColl *et al.*, 1993;

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**Abbreviations:** FA = fluorescent antibody; FITC = fluorescein isothiocyanate; nt = nucleotide; N = nucleoprotein; NS = non-structural; RT-PCR = reverse transcription–polymerase chain reaction

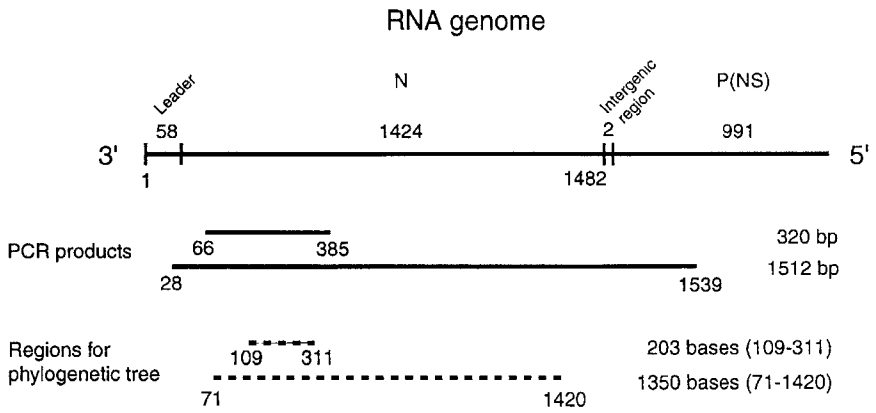


**Fig. 1**
**Scheme of amplified and sequenced regions on the genome of rabies virus**
The rabies virus RNA genome consists of a leader of 58 nt, N gene of1424 nt, an intergenic region of 2 nt and phosphoprotein (P) gene of 991 nt The amplified RT-PCR products consisted of 320 and 1512 bp. The sequenced regions consisting of 203 and 1350 nt were used for phylogenetic analysis

Rupprecht and Smith, 1994; De Mattos *et al.*, 1996, 1999; David *et al.*, 2000; Kissi *et al.*, 1995; Arai *et al.*, 1997; Bourhy *et al.*, 1999). Earlier we have reported partition of rabies virus isolates into 9 patterns (A to I) by the GCG program using RT-PCR analysis of the rabies N gene (Arai *et al.*, 1997).

In this study, RT-PCR was applied to 34 suspected rabid brain samples from Sri Lanka. We determined partial N gene sequences of 27 and complete N gene sequences of 14 out of 28 rabies virus isolates. We compared them with available sequences of N gene from the database to analyze phylogenetic relationships among rabies virus isolates circulating worldwide. An identical rabies virus type was present among a variety of animal species living near Colombo City and in 5 remote regions. The Sri Lanka rabies virus type was determined to be similar to an earlier rabies virus isolate from India but different from isolates from China, Thailand, Malaysia, Israel, Iran, Oman, Saudi Arabia, Russia, Nepal, Philippines, Japan and several other countries.

## Materials and Methods

*Virus isolates.* Thirty-four suspected rabid brain samples were obtained from 2 humans, 24 dogs, 4 cats, 2 mongooses, 1 jackal and 1 water buffalo in Sri Lanka in 1995–1996.

*FA test.* Acetone-fixed brain smear slides were prepared in Sri Lanka. A direct FA test for detection of rabies virus antigen was carried out with a fluorescein isothioocyanate (FITC)-labeled antibody as described by Arai and Montalban (1993). The FITC-labeled W502 was provided by Dr. C.E. Rupprecht (Centers for Disease Control and Prevention, Atlanta, USA) (Fraser *et al.*, 1996).

*Viral RNA extraction and RT-PCR.* Total RNA was extracted from brain suspensions with Isogen (Nippon Gene Co., Tokyo, Japan). A single tube RT-PCR assay of rabies virus N gene was performed as described by Arai *et al.* (1997). A reaction mixture underwent 1 cycle at 48°C for 60 mins, 1 cycle at 94°C for 2 mins, 40 cycles at 94°C for 30 secs, at 48°C for 20 secs and at 68°C for 2 mins, and 1 cycle at 68°C for 7 mins in a DNA thermal cycler. The reaction was completed within 3 hrs in a single tube to avoid contamination. The amplified sequences are shown in Fig. 1.

*Assay of rabies virus by RT-PCR.* Two rabies virus N gene regions were assayed by RT-PCR: a part (320 nt) of N gene (nt 59–1482) and a complete (1512 nt) including N gene (nt 59–1482) by use of specific primers. For the 320 nt sequence we employed a sense primer RNS-66 (5'-CTACAATGGATCCGAC-3', nt 66–82) and an antisense primer RNC-365 (5'-TGGGGTGATCTT-3', nt 365–385). For the 1512 nt sequence we used a sense primer RHN-1 (5'-ACAGACAGCGTCAATTGCAAAGC-3', nt 28–50) and an antisense primer RHNS-3 (CTAGGATTGACAAAGATTTTGCTC-3', nt 1516–1539).

*Nucleotide sequence analysis* was performed on two regions of rabies virus N gene with conserved sequences flanking the most variable region of N gene: a 203 nt region (nt 109–311) and a 1350 nt region (nt 71–1420). For this purpose the 320 bp and 1512 bp RT-PCR products were excised from gel and purified using a commercial kit (QIAquick PCR Purification Kit, Qiagen). The abovementioned sequences were determined on the purified DNAs using PRIMS Ready Reaction Dyedeoxy Terminator Cycle Sequencing Kit (Applied Biosystems, USA). RT-PCR primers for the 203 nt and 1350 nt regions were designed according to the Pasteur strain of rabies virus (Tordo *et al.*, 1986). The 203 nt were sequenced using the sense primer RNS-66 and the antisense primer RNC-365. For the 1350 nt sequence the following primers were used: the sense primers RHN-1 (5'-ACAGACAGCGTCAATTGCAAAGC-3' (Ito *et al.*, 1999)), SRLN-S1 (5'-CTCCAGATTCTCTTGTGGAG-3'), SRLN-S2 (5'-CTGGAGTACCATACCGAACT-3'), and SRLN-S3

(5'-ATGCAGTCGGCCACGTGTTTAAT-3'), and the anti-sense primers RHNS-3 (5'-CTAGGATTGACAAAGATTTTGCTC-3' (Ito *et al.*, 1999)), SRLN-C1 (5'-GCCTCGTATTCTTGGAGTTC-3'), SRLN-C2 (5'-CCTCCTCAAAGTTCTTGTGG-3'), and SRLN-C3 (5'-GCTCTATCCTATCTGCAATG-3'). The names of the isolates with respective accession numbers of the sequences of the 1350 nt region of N gene deposited in the DDBJ database are as follows: SRL 1032 (AB041964), SRL 1036 (AB041965), SRL 1060 (AB041966), SRL11077 (AB041967), SRL 1143(AB041968), and SRL1145 (AB 041969).

*Phylogenetic analysis.* Multiple sequence alignment of the data was generated by Clustal W (Higgins *et al.*, 1992; Thompson *et al.*, 1994) or Clustal X (version 1.81) program (Thompson *et al.*, 1997) based on the 203 and 1350 nt sequences of N gene. A phylogenetic tree was constructed using the neighbor-joining method (Satou and Nei, 1987). Distances (percent divergence) between pairs of sequences were calculated from multiple alignment and Kimura's (1980) correction for multiple substitutions was used. The bootstrap probabilities of each node were calculated using 100 replicates. Bootstrap values over 70% were regarded as evidence for phylogenetic grouping (Hillis and Bull, 1993). The TREEVIEW program was used to produce graphic output (Page, 1996).

## Results and Discussion

*Rabies virus assay by FA test and RT-PCR*

Out of 34 suspected rabid brain samples 28 were positive for rabies virus by both FA test and RT-PCR, while 6 samples were negative. FA test-negative samples were examined using a monoclonal antibody W502 for detection of another lyssavirus, but they yielded a negative result (data not shown). Fig. 2 demonstrates a representative experiment showing RT-PCR products of 320 bp and 1512 bp. The latter corresponds to the complete coding region of N gene.

*Analysis of the 203 nt sequences of N gene in rabies virus isolates*

Twenty-two rabies isolates originated from 15 dogs, 3 cats, 1 jackal, 1 human, 1 mongoose, and 1 water buffalo from the vicinity of Colombo City. Additional 6 isolates were obtained from a mongoose from Anuradapura, a human from Galle, and 4 dogs from Watareka, Madampe, Negambo, and Mathugama (Fig. 3, Table 1). One positive human isolate (sample #1049) was not sequenced because of insufficient quantity. The isolates exhibited 9 patterns of nucleotide sequence (A to I). Sixteen isolates from 12 dogs, 1 cat, 1 human, 1 mongoose and 1 jackal had nucleotide sequence of pattern A. Patterns B (3 dogs), C (1 mongoose), D (1 dog and 1 cat), E (1 cat), F (1 water buffalo), G (1 dog), H (1 dog) and I (1 dog) were also recorded. The pattern E found in one isolate (sample #1143) had arginine in the pattern A substituted by lysine.

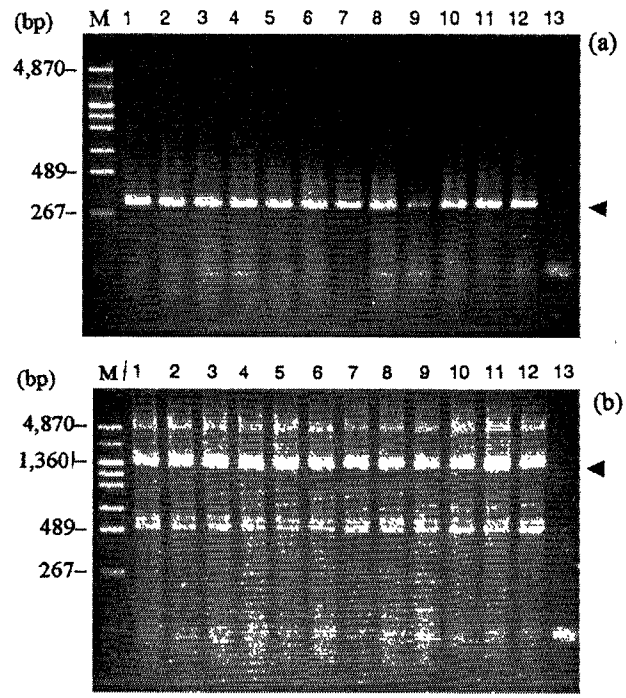


**Fig. 2**
**Agarose gel electrophoresis of amplified RT-PCR products of 320 bp and 1512 bp**
(a) Molecular size marker (lane M). Isolates (samples ) 1032, 1060, 1077, 1094, 1097, 1127, 1134, 1138, 1139, 1141, 1142, and 1153 (lanes 1–10, respectively). FA test-negative control (lane 11). The arrow indicates the RT-PCR product of 320 bp.
(b) Molecular size marker (lane M). Isolates (samples) 1032, 1060, 1077, 1094, 1097, 1127, 1134, 1138, 1139, 1141, 1142, and 1153 (lanes 1–10, respectively). Reagent control without template (lane 11). The arrow indicates the RT-PCR product of1512 bp.

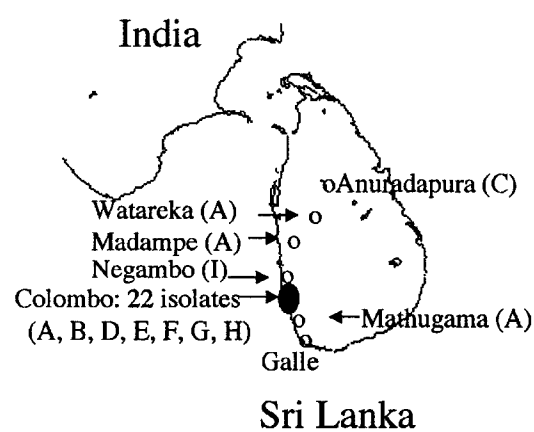


**Fig. 3**
**Regions of sample collection in Sri Lanka and designated nucleotide sequence patterns of rabies virus isolates**
Nucleotide sequence patterns A-I refer to the 203 nt-long region of N gene.

**Table 1. Regions, animal species and nucleotide sequence patterns of 28 rabies virus isolates from Sri Lanka**

| Sample No. | Sequence pattern | Species | Region |
|---|---|---|---|
| 1032 | A | Jackal | Pannipitiya |
| 1036 | A | Human | Ragama |
| 1040 | A[a] | Dog | Mathugama |
| 1049 | ND[a] | Human | Galle |
| 1060 | B | Dog | Seeduwa |
| 1061 | C[a] | Mongoose | Anuradapura |
| 1077 | A | Mongoose | Naranhenpita |
| 1094 | A | Cat | Moratuwa |
| 1095 | A | Dog | Gampaha |
| 1097 | A | Dog | Periyagoda |
| 1124 | A | Dog | Colombo |
| 1125 | I[a] | Dog | Negambo |
| 1127 | A | Dog | Colombo |
| 1132 | D | Cat | Colombo |
| 1134 | A | Dog | Panadura |
| 1136 | A[a] | Dog | Watareka |
| 1138 | A | Dog | Talawathugoda |
| 1139 | A[a] | Dog | Madampe |
| 1140 | B | Dog | Kotte |
| 1141 | A | Dog | Bandaragama |
| 1142 | A | Dog | Hendala |
| 1143 | E | Cat | Waskaduwa |
| 1144 | D | Dog | Ja-Ela |
| 1145 | F | Water buffalo | Maharagama |
| 1150 | B | Dog | Rajagiriya |
| 1151 | G | Dog | Gowinna |
| 1153 | A | Dog | Kiribathgoda |
| 1162 | H | Dog | Panadura |

A to I indicates the designed patterns of sequence of the 203 nt region.
ND = not done.
[a]Regions remote of Colombo City.

Furthermore, similar nucleotide sequences of two Sri Lanka mongoose isolates (samples #1061 and #1077) and two African mongoose isolates (Acc. Nos. U22628 and U22861) suggested that mongoose rabies virus isolates are not unique to this animal species or to a specific area of the world (data not shown).

Based on the nucleotide sequence of the 203 nt-long region of N gene, 27 of the 28 Sri Lanka isolates, in addition to Mokola isolate (Acc. No. U22843), were subjected to phylogenetic analysis together with another 24 rabies virus isolates deposited in the database (Fig. 4). The small diversity between the 27 isolates and the earlier Sri Lanka isolate 9425SRL (Acc. No.U22917, Kissi *et al.*, 1995) suggested that a genetic rabies virus variation might occur in Sri Lanka (Fig. 2). All the Sri Lanka isolates grouped in the same branch in the phylogenetic tree (bootstrap probability of 93%). Furthermore, the Sri Lanka isolates were similar to the Indian isolate (Acc. No. AF374721), but are distinctly different from the isolates from China (CTN, Acc. No. AF367863 and N7/dog, Acc. No. AB010549), Malaysia (8677MAL/human, Acc. No. U22916), Thailand (THA1009/dog, Acc. No. D85383, THA1013/dog, Acc. No. D85384, THA1017/dog, Acc. No. D85385, THA-Abha/dog, Acc. No. D85386, and 8738THA/human, Acc. No. U22653), Israel (9329ISR/dog, Acc. No. U22850), Iran (8702IRA/wolf, Acc. No. U22483), Oman (9135OMA/red fox, Acc. No. U22480), Saudi Arabia (8706ARS/red fox, Acc. No. U22481), Russia (9141RUS/Arctic fox, Acc. No. U22656), Nepal (9426NEP/dog, Acc. No. U22918), Philippines (Phi152-14, Acc. No. AB070772, Phi151-13, Acc. No. AB070771, Phi147-12, Acc. No. AB070770, Mdn130/51, Acc. No. AB070800, Mdn127/48, Acc. No. AB070798, and Mdn132/53*, Acc. No. AB070801), and Japan (KMG/dog).

*Analysis of the 1350 nt sequences of N gene in rabies virus isolates*

Nucleotide sequencing of the 1350 nt-long region of N gene was performed for 14 Sri Lanka isolates originating from various hosts including human, dog, cat, mongoose, jackal, and water buffalo (#1032, #1036, #1040, #1060, #1077, #1094, #1097, #1125, #1132, #1140, #1143, #1144, #1145 and #1162) (data not shown). The 14 isolates showed 99% or greater homology. For example, the cat isolate (sample #1143) exhibited only one amino acid difference (lysine substituted by arginine) Thus, a similar type of rabies virus appeared to be present in different animal species. The 99% homology suggested that a canine virus might have been transmitted to other species in the environment around Colombo resulting in an overlap of urban and sylvatic rabies. In addition, the Sri Lanka isolates showed a 94% homology with the Indian isolate (Acc. No. AF374721). Since Sri Lanka is an island, the similarity of the Sri Lanka and Indian isolates suggested that this virus "crossed" the Park Channel between the two countries.

Based on the sequence of the 1350 nt-long region of N gene, the Sri Lanka isolates showed 88% or less homology with 96 isolates from other countries (Fig. 5).

The unrooted and rooted phylogenetic trees showed that the 104 rabies virus isolates under comparison could be separated into 8 clusters: (1) North America: New York, Florida, Pennsylvania, raccoon; (2) Asia: Thailand and China; (3) Asia: Sri Lanka and India; (4) Africa: Chad, Ivory Coast, Guinea, Burkina, Mauritania, Niger, Benin, Cameroon; (5) South America: Guyana, Brazil; (6) Rep. South Africa, yellow mongoose; (7) North America: Arctic, Canada, Russia; (8) Africa: Algeria, Ethiopia, Gabon, Morocco, Tanzania, Nigeria, Namibia, Zaire, Rep. Central Africa, Rep. South Africa, Mozambique; Middle East: Oman, Israel, Iran; Europe: Germany, France, Estonia, Poland, Yugoslavia; Africa: Egypt, vaccine strain (SAD, PV) and Mexico (Fig 5). Thus, the Sri Lanka isolates and the

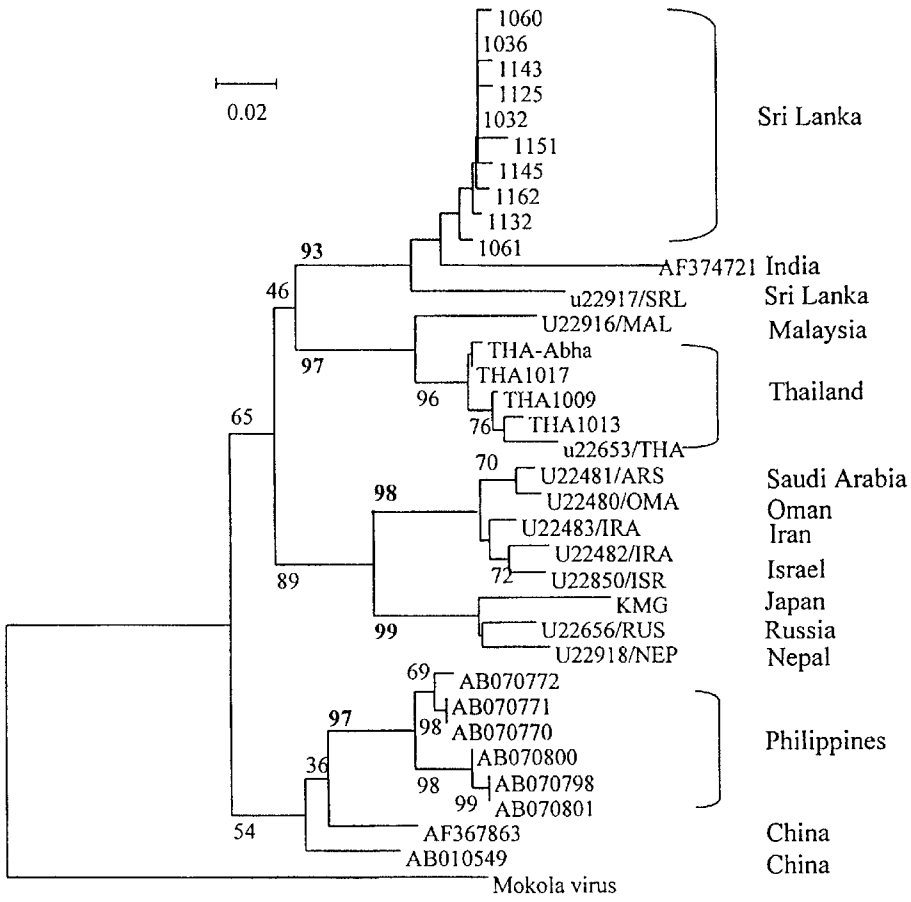


**Fig. 4**

**A rooted phylogenetic tree indicating genetic relationships among rabies virus isolates from various countries**

Phylogenetic relationships were determined by comparing the 203 nt sequences of N gene of rabies virus isolates from Thailand (Kissi *et al.*, 1995; Arai *et al.*, 1997), China (Ito *et al.*, 1999; Gao *et al.*, 2001), Malaysia, Israel, Oman, Saudi Arabia, Iran, Russia, Nepal (Kissi *et al.*, 1995), Philippines (Nishizono *et al.*, 2001), Japan (Arai *et al.*, 1997; Ito *et al.*, 1999), India (Jayakumar, 2001), and Sri Lanka by the neighbor-joining method. using Mokola isolate as an outgroup.

Indian isolate formed a cluster that was distinct from other rabies virus isolates worldwide.

Our results indicate that the rabies virus isolated from different animal species from different areas of Sri Lanka are genetically homogeneous and similar to the rabies virus isolated in India. The results also confirm earlier studies (Smith *et al.*, 1992; Kissi *et al.*, 1995; Arai *et al.*, 1997) indicating that nucleotide sequence analysis of selected regions of rabies virus N gene is an excellent technique for determining the areas of the world in which exposure to rabies virus had occurred.

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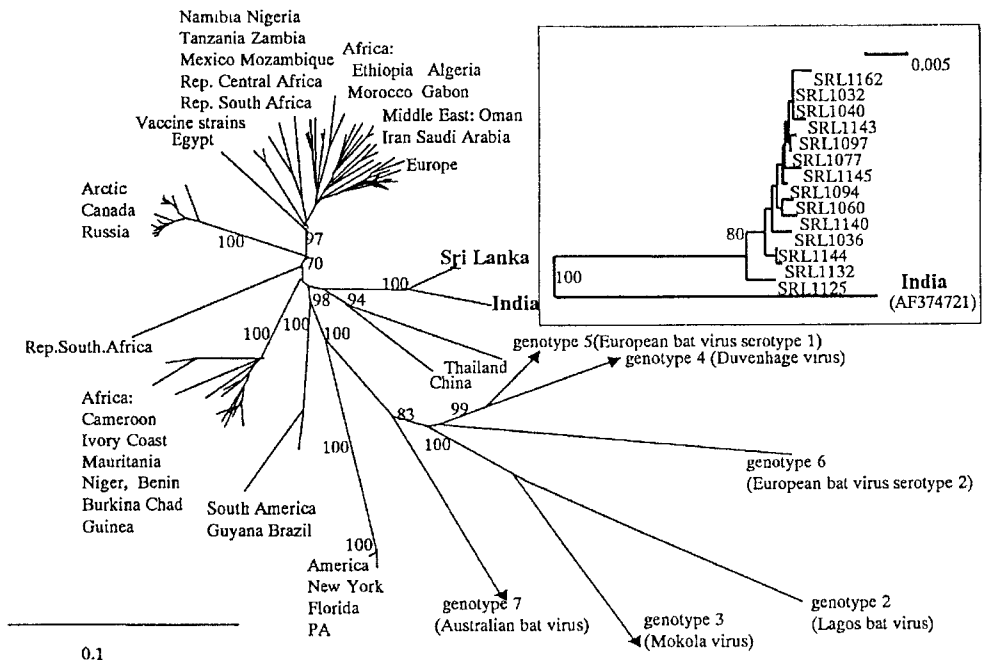


**Fig. 5**

**An unrooted phylogenetic tree showing relationships among 110 rabies virus isolates from various hosts and various countries**

Phylogenetic relationships were determined by comparing the 1350 nt sequences of N gene by the neighbor-joining method. The sequences originated from Conzelman *et al.*, 1993; Kissi *et al.*, 1995; Mannen *et al.*, 1991; Nadi-Davis *et al*, 1993, 1994, 1995; Poch *et al*, 1993; Summner *et al.*, 1993; Tordo *et al.*, 1993, 1999; Gao *et al.*, 2001; Jayakumar, 2001. The insert shows 14 Sri Lanka isolates and an Indian isolate. The sequences reported here were deposited in the DDBJ database under the following Acc. Nos.: SRL 1032 (AB041964), SRL 1036 (AB041965), SRL 1060 (AB041966), SRL 11077 (AB041967), SRL 1143 (AB041968), and SRL 1145 (AB041969).

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