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Molecular Epidemiological Analysis of Rabies Viruses Associated with the Population Structure of Bat Hosts

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MOLECULAR EPIDEMIOLOGICAL ANALYSIS OF RABIES VIRUSES ASSOCIATED WITH POPULATION STRUCTURE OF BAT HOSTS

A Thesis Submitted to the
School of Graduate Studies and Research

In Partial Fulfillment of the Requirements
for the Degree of
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Department of Biochemistry, Microbiology and Immunology
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ABSTRACT

To explore whether rabies viral variants co-localize with discrete bat host populations (sub-populations), both the host genome and rabies virus of *Eptesicus fuscus* (big brown bats), *Myotis lucifugus* (little brown bats) and other *Myotis* specimens, collected during 1989 to 2004 from diagnostic submissions from across the country, were genetically characterized.

Bat species population analysis was performed by nuclear DNA genotyping, scored by variation of several microsatellite loci, and through phylogenetic analysis of Cox-1 (cytochrome oxidase subunit I) gene sequences located on mitochondrial DNA. Microsatellites are relatively short DNA stretches consisting of tandem repeats of one to five nucleotides which exhibit high levels of allelic variation. Cox-1 gene sequence analysis provides accurate species level of identification for *Myotis lucifugus* specimens. Two hundred and ninety five DNA samples of *Eptesicus fuscus* were examined at 9 microsatellite loci, and 126 DNA samples of *Myotis lucifugus* were examined at 7 microsatellite loci, both datasets were analyzed by a series of genetic population analysis softwares. Phylogeny of Cox-1 gene sequences with 552 nucleotides by using 106 DNA samples of bats *Myotis lucifugus* was analyzed as an alternative strategy of the microsatellite gene marker for population structure determination of *Myotis lucifugus*. Consequently, two populations – East (group I) and West (group II) were determined for *Eptesicus fuscus* bats in Canada. No population structure was identified for *Myotis lucifugus* bats.

Rabies viral variants were identified by nucleotide sequencing of the central divergent portion of the P (phosphoprotein) gene – a region previously proved to be a sensitive target for molecular epidemiology analysis. Viral RNA, isolated from 231 samples of *Eptesicus fuscus* and *Myotis* species was sequenced over a 597 bp region. Phylogenetic

analysis of these data identified six rabies viral variants circulating in *Eptesicus fuscus* and one rabies viral variant circulating in *Myotis* species.

Based on the results of genetic characterization, the spatial distribution of the bat host subpopulations and their associated rabies virus variants were determined. Rabies variants I and II circulate in *Eptesicus fuscus* population I (East); rabies variants III, IV and V circulate in *Eptesicus fuscus* population II (West); and rabies variant VI circulates in both populations. A distinct rabies virus variant VII was associated with *Myotis* species. No population sub-structure would be identified for *Myotis lucifugus* bats.

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LIST OF ABBREVIATIONS

A	Adenine
ABLV	Australian Bat Lyssavirus
AMOVA	Analysis of Molecular Variance
bp	base pair
BSA	Bovine Serum Albumin
C	Cytosine
°C	Degree Celsius
CDC	Centers for Disease Control and Prevention
cDNA	Complementary Deoxyribonucleic Acid
CD+4 T cell	Helper T Lymphocyte
CD+8 T cell	Cytotoxic T Lymphocyte
CNS	Central Nervous System
CR	Control Region
Cox-1	Cytochrome Oxidase Subunit 1 Gene
CTAB	Hexadecyl Trimethyl Ammonium Bromide
CVS strain	Challenge Virus Standard strain
DEPC	Diethylpyrocarbonate
ddH_2O	double distilled water
dH_2O	distilled water
dN	nonsynonymous substitutions per nonsynonymous site
DNA	Deoxyribonucleic Acid
dS	synonymous substitutions per synonymous site
DTT	Dithiothreitol
DUVV	Duvenhage Virus
EBLV	European Bat Lyssavirus
EDTA	Ethylenediaminetetraacetic Acid
ESS	Effective Sample Size
FAT	Fluorescent Antibody Test
Fst	Wright's F-statistics
G	Guanine
G	Glycoprotein
kb	kilo bases
kDa	kilo Dalton
L	Large Protein (RNA Polymerase)
LBV	Lagos Bat Virus
Le	Leader Sequence

M	Matrix Protein
M	Molar
Mabs	Monoclonal Antibodies
mg	microgram
ml	millilitre
ML	Maximum Likelihood
MOKV	Mokola Virus
MP	Maximum Parsimony
Msat	microsatellite
mtDNA	Mitochondrial DNA
N	Nucleoprotein
NJ	Neighbor-Joining
ORF	Open Reading Frame
P	Phosphoprotein
PAGE	Polyacrylamide Gel Electrophoresis
PCR	Polymerase Chain Reaction
PV Strain	Pasteur Virus Strain
RNA	Ribonucleic Acid
RNP	Ribonucleoprotein
T	Thymine
tRNA	Transfer Ribonucleic Acid
RABV	Classical Rabies Virus
rpm	revolutions per minute
RRVs	Rabies Related Viruses
RT	Reverse Transcriptase
RT-PCR	Reverse Transcription Polymerase Chain Reaction
SMM	Stepwise Mutation Model
STR	Short Tandem Repeats
TRS	Tandemly Repetitive Sequences
TE	Tris-EDTA Buffer
TLE	Tris Low-EDTA Buffer
Tr	Trailer Sequence
VNA	Virus Neutralizing Antibody
V-RG	Vaccinia-Rabies Glycoprotein
VSV	Vesicular Stomatitis Virus
µl	Microliter
UTM	Universal Transverse Mercator

UV	Ultraviolet
v/v	volume per volume
WHO	World Health Organization
w/v	weight per volume

INTRODUCTION

Rabies, an infectious disease causing fatal encephalopathy in mammals including humans, is caused by the lyssaviruses. The disease is most often transmitted by the bite of a clinically rabid animal through infectious saliva. The prior transmission of rabies virus via corneal transplants was confirmed (Javadi et al., 1996). In addition, human to human transmission of infection has also been reported in organ transplantations from rabid individuals recently (Jenwitheesuk, 2005; Roos, 2005; Srinivasan et al., 2005).

The disease of rabies in dogs was known in ancient times, as early as 2300 BC. However, rabies was first found to be of infectious origin as "causative seeds" in 1808, and was recognized to be of infectious etiology by Pasteur in the 1880's. Pasteur's work on the serial passage of rabies virus in rabbits eventually succeeded in producing preparations of varying infectivity which were used to treat patients bitten by rabid dogs. The understanding of the contagious nature of the disease, its pathogenesis and Pasteur's post exposure prophylaxis to prevent rabies marked the most significant developments in rabies research of the nineteenth century. Despite extensive technological development and research over more than one hundred years to restrain this notorious disease, unfortunately, rabies is still a significant threat to public health (Madhusudana, 2005). World-wide deaths of humans due to rabies are estimated to be over 55,000 per year by the World Health Organization (World Health Organization, 2005). Cases occur mainly in developing countries due to unavailable and unaffordable pre- and post- exposure vaccines (Wilde et al., 1999).

Rabies Virus Taxonomy

Rabies virus is the prototype species of the *Lyssavirus* genus belonging to the *Rhabdoviridae* family, one of four families of the Mononegavirales order (Wunner et al., 1995). The *Rhabdoviridae* family comprises six genera designated as: *Vesiculovirus*,

Lyssavirus, *Ephemerovirus*, *Novirhabdovirus*, *Cytorhabdovirus*, *Nucleorhabdovirus*, and a large number of other unclassified agents. The Rhabdoviruses have broad host ranges including humans, other mammals, fishes, insects and plants, whilst lyssaviruses are restricted to infecting mammals (Buchen-Osmond, 2005).

The lyssaviruses all share similar characteristics of biology, physiology and biochemistry. Seroneutralization and monoclonal antibody studies of the nucleocapsid protein and the glycoprotein inferred four lyssavirus serotypes (Flamand et al., 1980a; Flamand et al., 1980b; Schneider et al., 1973). Subsequent molecular and phylogenetic analysis of the viral N gene indicated that lyssaviruses could be divided into seven genotypes.

Rabies virus (RABV, classical rabies virus) is designated as genotype 1 and serotype 1 and it includes the majority of field viruses recovered from terrestrial mammals globally and Chiropteran specimens of the Americas (World Health Organization, 1994). Other lyssaviruses termed as rabies-related viruses (RRVs), cause a clinical disease very similar to rabies; these viruses are more restricted in their host range and geographical distribution (Tordo et al., 1998), and their general information is described in Table 1 (Tordo et al., 2004).

Rabies Virus Structure

Rabies virus has a quite simple structure. Its bullet shaped virus particle has a length of approximately 180 nm and cross-sectional diameter of around 75 nm (Hummeler et al., 1967; Vernon et al., 1972). Its central core is composed of ribonucleoprotein (RNP) generated by a helical viral genome of single-stranded, negative-sense, and unsegmented RNA, encapsulated by nucleoprotein (N), and associated with RNA polymerase (L) and phosphoprotein (P). A lipid-bilayer protein envelope is arranged tightly around the RNP

Table 1. Listing of Established Groups of Rabies Related Viruses of the *Lyssavirus* Genus

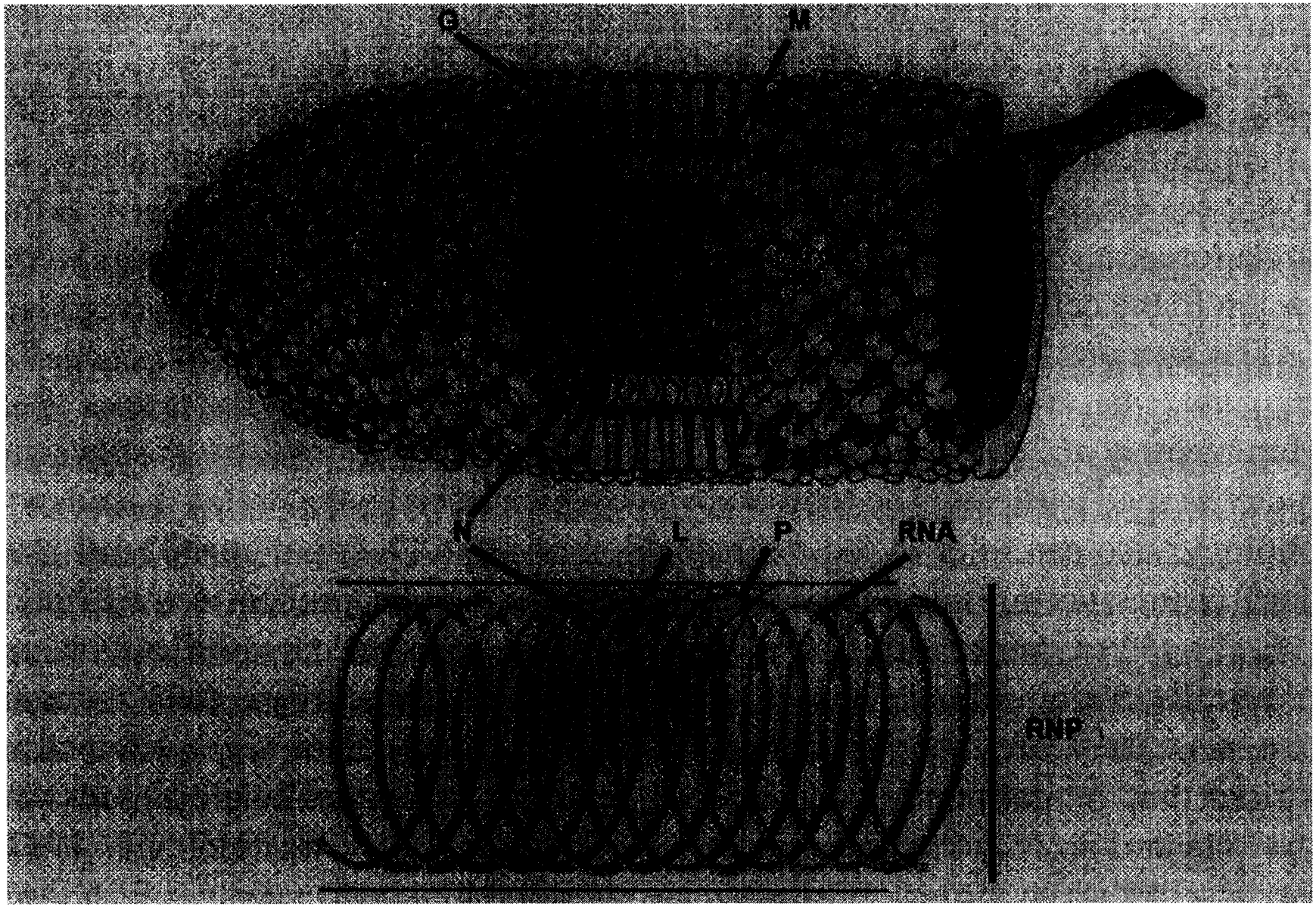
Name	Type		Reservoir Host	Known Range
	Geno-	Sero-		
Lagos bat virus (LBV)	2	2	Fruigivorous bats	Central, South Africa
Mokola virus (MOKV)	3	3	Unknown. Isolated from shrews, cats, humans	Central, West Africa
Duvenhage virus (DUVV)	4	4	Bats (*Miniopterus sp.*)	South Africa
European bat lyssavirus type 1 (EBLV-1)	5		Bats (*Eptesicus serotinus*)	Europe
European bat lyssavirus type 2 (EBLV-2)	6		Bats (*Myotis sp.*)	Europe
Australian bat lyssavirus (ABLV)	7		Bats (*Pteropus sp.*, Insectivorous bats)	Australia

core. The matrix protein (M) is located between the envelope and RNP core. Glycoprotein (G) spikes, anchored evenly over the entire envelope surface, protrude outwards (Figure 1).

Rabies Virus Genome and Its Encoding Proteins

The rabies virus genome is approximately 12,000 nucleotides long with a molecular weight of approximately 4.6 x 10^{-6} kDa. The first 58 nucleotides at the 3' end of the genome is a noncoding leader sequence (Le). The Le sequence of rabies virus and vesicular stomatitis virus (VSV) acts as a gene promoter for initiation of genome RNA transcription (Tordo et al., 1986a; Calain and Roux, 1995; Conzelmann and Schnell, 1994; Wertz et al., 1994; Whelan and Wertz, 1999). Following immediately after this region are the five structural genes (N, P, M, G and L in 3' to 5' orientation) that encode respectively five proteins:

Figure 1. The Schematic Structure of Rabies Virus
The viral bulletin shape, genome RNA, RNP organization and five viral proteins (Nucleoprotein, Large protein (RNA polymerase), Phosphoprotein, Glycoprotein, and Matrix protein) are presented. (*Reprinted from Fig.1 in RABIES of page 29, edited by Alan C. Jackson and William H.Wunner, copyright © 2002 Elsevier Science (USA), with permission from Elsevier* (Appendix 2)).


G
M
N
L
P
RNA
RNP

nucleoprotein, phosphoprotein, matrix protein, glycoprotein, and RNA polymerase (large protein). A region of 70 nucleotides at the 5' end of the genome is a noncoding trailer (Tr) sequence. The five coding genes are separated by short sequences, around 2 to 423 nucleotides, forming intergenic regions of the virus genome (Tordo et al., 1986b) (Figure 2).

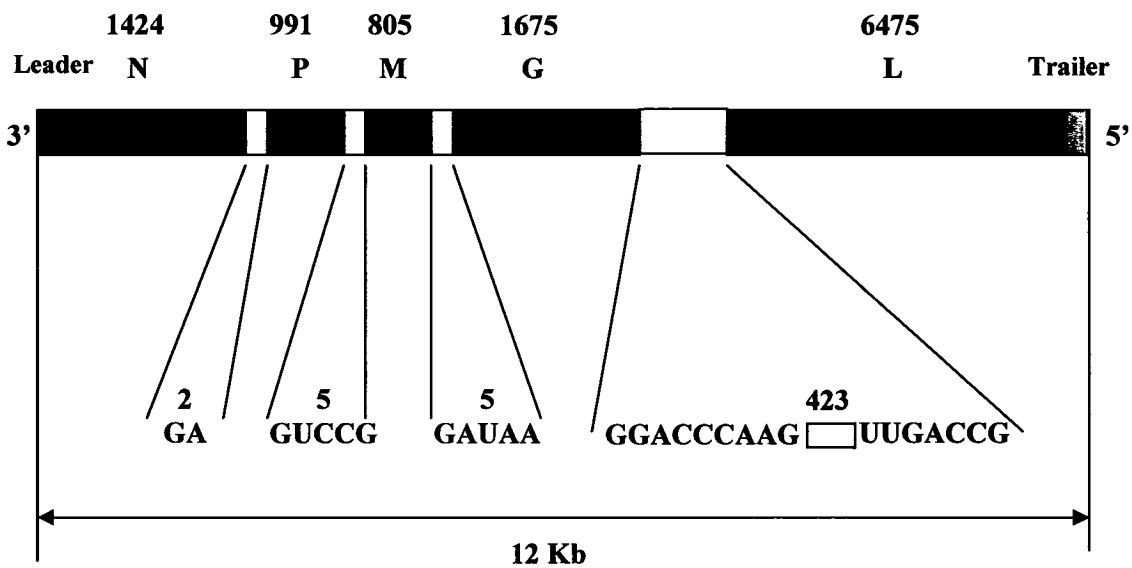
Nucleoprotein, RNA polymerase and phosphoprotein which associate with RNA, work together to initiate, participate in, and regulate viral transcription and replication events.

The nucleoprotein is the most conserved component of the virus in terms of its amino acid sequence similarity, regulating and modulating virus RNA transcription and replication by promoting read-through of the termination signals (Patton et al., 1984; Yang et al., 1998; Yang et al., 1999). It also presents the virus group-specific core antigen (Kissi et al., 1995; Schneider et al., 1973), a target for antibodies used to differentiate viral serotypes and strains (Dietzschold et al., 1987; Flamand et al., 1980a; Smith, 1989).

The virion-associated RNA polymerase or large protein is the catalytic component of the polymerase complex. Several conserved and variable domains of the RNA polymerase confer its multifunctional properties including all enzymatic activities involving the RNA transcription and replication processes of the virus (Poch et al., 1989; Poch et al., 1990; Tordo et al., 1988).

The glycoprotein is a surface fusion protein which binds to surface receptors on the host cells and thus relies on its conformational changes to mediate and finish the process of virus entry into host cells during virus infection (Bussereau and Perrin, 1982). It also is a main antigenic component of the virus and stimulates the host immune system to produce neutralizing antibodies (Flamand et al., 1993), and stimulates T cells (Macfarlan et al., 1984).

Figure 2. Rabies Virus RNA Genome
Rabies viral genome is a nonsegmented, negative sense, single-stranded RNA including N, P, M, G and L genes. The sequential order of five genes and nucleotide intergenic regions is presented.


1424
991
805
1675
6475
Leader
N
P
M
G
L
Trailer
3'
5'
2
GA
5
GUCCG
5
GAUAA
423
GGACCCAAG
UUGACCG
12 Kb

Mutations at position 333 of the glycoprotein reduced the virulence of some rabies variants by decreasing the rate of virus spread from the entry site to the central nervous system (Dietzschold et al., 1983; Kucera et al., 1985; Seif et al., 1985; Tuffereau et al., 1989).

The matrix protein, the smallest rabies virus protein of around 25 KDa, is also a multifunctional protein which is associated with virus assembly, RNA synthesis, and RNA transcription regulation (Ahmed and Lyles, 1998; Ferran and Lucas-Lenard, 1997; Ito et al., 1996; Mebatsion et al., 1999). M protein acts as a structural protein which forms a sheath around the RNP core and to produce the virion skeleton (Bourhy et al., 1993; Conzelmann et al., 1990; Gould et al., 1998; Hiramatsu et al., 1993; Mebatsion et al., 1999; Rayssiguier et al., 1986).

Phosphoprotein of Rabies Virus - Another Multifunctional Protein

The phosphoprotein of rabies virus plays critical multifunctional roles in viral transcription, replication and pathogenesis through interaction with viral proteins - nucleoprotein (N) and RNA polymerase (L), and a host protein - dynein light chain (LC_8).

The phosphoprotein is important in transcription and replication of the viral genome RNA through its interaction with newly synthesized nucleoprotein and genome associated nucleoprotein. The phosphoprotein functions as a molecular chaperone to direct nucleoprotein encapsidation (Masters and Banerjee, 1988; Yang et al., 1998). It also binds with the RNP template in different phosphorylation states to differentiate transcription activities (Barik and Banerjee, 1992a; Barik and Banerjee, 1992b; Gao and Lenard, 1995; Toriumi and Kawai, 2004). Some studies have identified two N protein binding domains located in N-terminal (between amino acids 69 and 177) and C-terminal (within 30 amino acids) regions of the phosphoprotein (Chenik et al., 1994; Fu et al., 1994). The N-terminal domain of P protein is probably used when P interacts with nascent N protein, while the C-

terminal domain is involved in N-P binding after N protein encapsidates the viral RNA (Toriumi et al., 2002). A study using combined two hybrid assays has more accurately mapped the C-terminal binding site for N protein to a short lysine-rich motif FSKKYKF at 209 to 215 amino acid residues of the P protein (Jacob et al., 2001).

The phosphoprotein binds to RNA polymerase as a non-catalytic cofactor to regulate the viral genome replication and transcription (Emerson and Wagner, 1972; Masters and Banerjee, 1988; Takacs and Banerjee, 1995). The interacting domain was mapped to the N-terminal lysine-rich 19 residues of the phosphoprotein (Chenik et al., 1998; Nadin-Davis et al., 2002), while the carboxy-terminal part of the RNA polymerase large protein mediates this P-L complex formation.

The interaction of the phosphoprotein and the host cell dynein light chain (LC_8) was identified by a yeast 2-hybrid system. Since LC_8 is involved in microtubule associated movement in the cytoplasm of neuronal cells, it has been proposed that this association may form the basis for movement of rabies viral components along neuronal cell axons (Raux et al., 2000). A molecular technique mapped its binding site at the amino acid residues around 138 to 172 at the center of phosphoprotein (Jacob et al., 2000; Jacob et al., 2001; Poisson et al., 2001; Raux et al., 2000). The LC_8 binding site was subsequently located to a few amino acid residues (Lo et al., 2001) - this motif was found to be conserved in all lyssavirus P proteins (Nadin-Davis et al., 2002). The relationship between binding domain and virus pathogenesis was investigated by mutating the dynein light chain binding site of the rabies virus phosphoprotein. These studies indicated that phosphoprotein contributes to virus pathogenesis by facilitating virus spread because encephalitis with apoptotic neuronal changes was delayed in the experimental mice infected with mutant viruses (Dietzschold et al., 2005; Mebatsion, 2001; Rasalingam et al., 2005).

In addition, the study of the relationship between rabies virus phosphoprotein and virus pathogenesis has progressed recently. The phosphoprotein was reported to enhance viral pathogenicity by preventing induction of interferon in virus-infected cells as an α/β interferon antagonist (Brzozka et al., 2005) and by supporting virus gene expression and formation of infectious particles (Finke et al., 2004).

To better understand the importance of phosphoprotein in virus replication and transcription, P gene-deficient rabies virus was generated and characterized *in vitro* (Shoji et al., 2004). P gene-deficient virus still replicated its genome and produced progeny viruses in cell lines supplemented *in vitro* with phosphoprotein expression, but the virus was apathogenic in adult and suckling mice (Morimoto et al., 2005), and could be further explored as a novel live attenuated vaccine.

Several N-terminally truncated versions of the phosphoprotein can be made in infected cells but their functions are not clearly understood yet (Chenik et al., 1995).

Although few laboratories have been involved in genetically characterizing the rabies virus P gene locus, genetic comparison of RABV Pasteur virus (PV) laboratory strain and a Mokola virus isolate showed that the P gene is a divergent coding region of the virus; Only the transmembrane and intracytoplasmic domains of the G gene exhibit greater variation (Le Mercier et al., 1997). While the reason for a high level of diversity in the central portion of the P gene is unclear, this region is a sensitive target for molecular epidemiological analysis.

Pathogenesis and Pathology of Rabies Virus

Although the precise mechanism by which rabies virus causes a fatal neurological illness is not fully understood, the main features of rabies pathogenesis have been known for some time from animal experimental models.

The victim does not exhibit any symptoms after immediate infection of rabies virus into muscle by a bite exposure of a rabid animal. The incubation period is usually between 6 - 90 days, but, occasionally, it could last for several years (Smith et al., 1991). It is generally believed that the long incubation period is a result of the virus being maintained in the periphery near the bite site, even though the virus may start multiple replications and spread rapidly from the inoculation site to the central nervous system. After the virus has been transported from the entry site into peripheral nerves and then via nerves to the central nervous system, clinical symptoms appear with behavioural alterations due to neurological dysfunction. The acute encephalomyelitis in the central nervous system is manifested in pathology of rabies cases (Jackson and Rossiter, 1997; Mrak and Young, 1994).

Rabies is virtually always lethal, but the recovery of rabid animals and humans has been reported (Jackson and Rossiter, 1997; Jackson, 2005; Willoughby, Jr. et al., 2005). Because each rabies virus strain is adapted to a specific reservoir species, the pathogenesis may vary among different host species, and substantially differ between the species infected by a "spillover" and that found in the reservoir species (Hamir et al., 1996; Wandeler et al., 1994).

Epidemiology of Rabies Virus

Rabies is present in all continents except Antarctica, but the prevalence of rabies varies throughout the world, and its situation and distribution are very different. Although all mammals are susceptible to rabies virus infection, only a few species are natural reservoirs for maintenance of this disease. The disease may be transmitted from the maintenance host to many other species (spill-over event), but this is normally a dead-end for the virus since further transmission to other members of the species is rarely observed. The geographical range of the host species is one factor that determines the range of a particular rabies virus

strain, but natural barriers (e.g. rivers) that may limit contacts between members of the reservoir species, may also limit viral transmission and spread.

In Europe dogs, foxes and wolves were the main source of rabies in the past, while bats and foxes have been major rabies reservoirs since the middle of the 19th century (Barrat and Aubert, 1993).

In most developing countries of Africa, Asia, Latin America and Middle East, dogs still act as the major virus hosts and the primary contributors to human rabies via rabid dog bites. Most of the rabid human cases transmitted from dogs are recorded in India, around 25,000 annually.

In contrast, in North America, the vast majority of rabies cases occur in wild animals such as skunks, bats, raccoons and foxes which serve as reservoirs for discrete viral strains (Wandeler, 1987). The indigenous rabies virus strains in wildlife animals which dominated in some Canadian provinces, such as Manitoba, Saskatchewan and Alberta, were those from foxes (*Alopex lagopus/Vulpes vulpes*) and striped skunks (*Mephitis mephitis*), while in recent years only skunk rabies has predominated in these provinces (Tabel et al., 1974). The arctic fox strain has infected Southern Ontario continuously from the 1950s to the present, but the racoon strain (*Procyon lotor*) of rabies virus entered recently into eastern Ontario and New Brunswick of Canada (Nadin-Davis et al., 2005).

While most studies have discriminated between rabies viruses associated with different host reservoirs (Webster et al., 1985), phylogenetic studies on the arctic fox strain in Canada identified viral variants that were regionally localised (Nadin-Davis et al., 1993; Nadin-Davis et al., 1994; Nadin-Davis et al., 1999).

Rabies Vaccines and Rabies Control

Rabies vaccines have been efficiently and successfully applied to control rabies in humans and animals since the first rabies vaccine developed by Pasteur in the late 19th century.

In terms of rabies virus as a neurotropic virus, the immunologically privileged status of the central nervous system (Barker and Billingham, 1977) and the CD8+ T cell cytotoxic response leading to lysis of infected neurons (Larson et al., 1991; Reddehase et al., 1984; Wiktor et al., 1977; Wiktor et al., 1984), were largely considered during vaccine development. Presently, the killed-virus rabies vaccines containing full-sized intact proteins which generate CD4+ T-cell activation and humoral (B-cell) immune response (Herzog et al., 1992; Lafon and Lafage, 1987; Mifune et al., 1981; Perry and Lodmell, 1991; Smith, 1981) have been predominantly used for humans and domestic animals. In particular, virus-neutralizing antibodies originating from B cells play a critical protective role in virus immunologic reaction (Hooper et al., 1998; Miller et al., 1978; Templeton et al., 1986). In contrast, the use of live-virus vaccines is still limited to the vaccination of wildlife animals (Seder and Hill, 2000).

Rabies virus neutralizing antibodies (VNA) can be detected within 7 to 10 days after the first injection of rabies vaccine and persist more than 2 years according to the function of individual immune memory cells. Since protective VNA is induced only by the glycoprotein (Xiang et al., 1995), subunit and recombinant vaccines expressing the rabies virus glycoprotein are being used for wildlife oral vaccination. A vaccinia-rabies glycoprotein recombinant virus (V-RG) vaccine has been widely distributed for oral vaccination of red foxes (*Vulpes vulpes*), raccoons (*Procyon lotor*) and coyotes (*Canis latrans*) to control wildlife rabies outbreak and prevent rabies transmission from wildlife to domestic animals.

This strategy has been successfully employed in North America and Europe (Roscoe et al., 1998; Vos, 2003).

Recombinant and subunit vaccines have not yet found application for human and domestic animals. The probable reasons are safety issues due to CD8+ T cell cytotoxic responses and efficacy limitations, since only the correct conformation and glycosylation of rabies virus glycoprotein can lead to induction of an appropriate immune response (Kammer and Ertl, 2002; Wiktor et al., 1984).

Rabies in bats poses a major problem with respect to vaccination of the reservoir host. In Latin America, hematophagous bats (*Desmodus rotundus*), one of the main reservoirs of rabies virus, transmit rabies to livestock causing huge economic losses annually. The current control has been attempted through population reduction using anti-coagulants applied to caught vampire bats (Linhart et al., 1972). More recent studies of indirect oral immunization of vampire bats using V-RG vaccine has shown some promise (Almeida et al., 2005). Because vampire bats live in colonies and participate in communal grooming behaviour, the V-RG vaccine is applied in a paste to the back of captured bats; these bats are then returned to the colony where other bats are immunized orally after licking the vaccine during grooming. Protection of livestock from rabid hematophagous bats via pre-exposure treatment is currently being undertaken. However, the control of rabies in insectivorous bats is still problematic.

Rabies and Bat Hosts

Bats are flying mammals that belong to their own special order, the Chiroptera, which have existed since the age of dinosaurs. Of the nearly 1,000 species of bats that are known worldwide, only 40 species inhabit North America. Around 19 species of bats have been found in Canada, 17 species are regular residents and most of them are insectivorous. Some

species are widely distributed across Canada, but little is known about their population structure.

Big brown bats (*Eptesicus fuscus*) are common in many parts of Canada, even in larger cities. Summer roosts are found in hollow trees, caves and buildings including human dwellings. This species forms large maternity colonies of 100-200 individuals. Males are mostly found in clusters. *Eptesicus fuscus* is considered a comparatively sedentary species, moving on average not more than 12 kilometres for hibernation.

Little brown bats (*Myotis lucifugus*) are the most common bats and are widely distributed in Canada. They roost in buildings like attics and natural cavities. The maternity colonies are more commonly found in buildings. This bat species prefers optimal temperature conditions for young growth. They sometimes fall prey to small carnivores, mice, and snakes or other animals occasionally, and *vice versa*. They use night roosts in summer and maternity colonies for hibernation with ten to one thousand individuals together. *Myotis lucifugus* is a gregarious species with a simple social structure. They can migrate several hundreds of kilometres for hibernation.

Within Canada, other *Myotis* species are mainly found in the province of British Columbia. Yuma (*Myotis yumanensis*) and California (*Myotis californicus*) bats like to roost in buildings, but little is known of their biology (Van Zyll De Jong, 1985).

Like many terrestrial mammals, bats are also susceptible to rabies; moreover certain bat species are rabies reservoirs.

Since the first rabid bat was reported in America in 1953 (Scatterday, 1954) and in Canada in 1957 (Brass, 1994), almost all species of bats investigated in North America have been found as rabid (Constantine, 1979). Several species of insectivorous bats are also rabies reservoirs across Canada and in the USA, while in many Latin American countries, Vampire

bats are important rabies reservoirs (Baer, 1975; Nadin-Davis et al., 2001). The high proportion of human cases related to rabid bats has caught significant attention (Lafon, 2005; Morimoto et al., 1996). Data from the Centers for Disease Control and Prevention, in the United States, from 1970 to 1989, have shown that 30% of human rabies cases were caused by bat rabies strains. Even more significantly, bat rabies virus variants have become the predominant cause of human rabies deaths from 1990 to 2000. In Canada, three people have died of rabies after exposure to rabid bats since 1985; they were residents in Alberta, Montreal and Vancouver respectively (Whitney, 2005).

According to the confirmed cases of animal rabies across Canada (data released by Canadian Food Inspection Agency) from January 1, 1998 to December 31, 2003, around 500 rabid bats were identified as rabies virus positive. In the 1990's 40-50 positive cases were diagnosed annually while since 2000 this value has risen to 100-120 per year, primarily due to increased submission numbers from Eastern Canada. Rabid bats are a serious threat to public health in humans and terrestrial animals for several reasons. Firstly, people don't realize bats carry rabies. Secondly, bat bites can be very superficial; consequently, post-exposure prophylaxis probably is often not sought after contact with bats.

The epidemiology of bat rabies is more complicated than that of terrestrial species due to their aerial lifestyle. Viral typing of bat rabies viruses is therefore valuable for understanding the epidemiological trends in bat rabies of Canada. An early study (Webster et al., 1986) divided bat isolates from Canada into four major antigenic groups according to species and provinces: group B-1 covered in *Eptesicus fuscus* from Ontario; group B-2 was found in a variety of bat species ranging from British Columbia eastward into Ontario; group B-3 was associated with *Myotis* species from Ontario and New Brunswick; and group B-4 occurred *Eptesicus fuscus* in the west (Alberta and Saskatchewan). In a later study, at least

13 antigenically and genetically distinct rabies virus variants have been identified (Nadin-Davis et al., 2001). It reported that 5 bat species, *Eptesicus fuscus*, *Myotis lucifugus*, silver-haired bats (*Lasionycteris noctivagans*), hoary bats (*Lasiurus cinereus*) and red bats (*Lasiurus borealis*), are the most commonly diagnosed in Canada. *Eptesicus fuscus* is the species most frequently submitted for rabies testing across the country. Many members of the genus *Myotis* were submitted but only in small numbers including *Myotis lucifugus*, *Myotis californicus*, Keen's Myotis (*Myotis keenii*), Northern Long-eared Myotis (*Myotis septentrionalis*), Western Small-footed Myotis (*Myotis ciliolabrum*), Long-legged Myotis (*Myotis volans*) and *Myotis yumanensis* (Nadin-Davis et al., 2001).

Nadin-Davis et al.(2001), investigating the genetic divergence of rabies viruses from bat species circulating in Canada through characterization of N and G genes of representative rabies virus isolates, identified four principal phylogenetic groups of viruses associated with particular bat species. Additional subgroupings consistent with viral antigenic variations were also noted. The viruses of colonial, non-migratory bats of *Myotis* species, and *Eptesicus fuscus* were organized into 2 subgroups of one large group. The viruses of the solitary, migratory species of *Lasiurus* species, and *Lasionycteris noctivagans* were clustered in another group. Viruses from some *Eptesicus fuscus* bats of British Columbia were organized to a separate group. The phylogenetic analysis confirmed that *Myotis* genus acted as a rabies reservoir. In addition, a strong association was identified between these bat species and the group(s) of viruses associated with them (Nadin-Davis et al., 2001).

Molecular Evolutionary Analysis of Rabies Virus

As an RNA virus, rabies virus has tremendous evolutionary potential to quickly adapt to new hosts. This is a consequence of both high genomic mutation, due to the lack of proof reading capability of the viral RNA polymerase, and a rapid replication rate to yield large

population sizes during infections (Domingo et al., 1996; Holland et al., 1992; Kilbourne, 1991).

The mechanisms of mutations which govern molecular changes at the protein level are generally due to nucleotide substitutions or insertions and deletions at the DNA or RNA level (Ohta, 1992). All substitutions usually consist of either transition or transversion changes. Transitions between nucleotides with a similar molecular structure are more likely to occur than transversions between nucleotides with different molecular structures. However, transversions are more likely to result in amino acid encoding changes than are transitions. Nucleotide substitutions in protein coding genes can be divided into synonymous, that do not result in amino acid changes, and nonsynonymous substitutions, resulting in amino acid changes (Vandamme, 2003). As synonymous (silent) mutations are largely invisible to natural selection, nonsynonymous (amino-acid replacing) mutations will be under much stronger selective pressure. Such genetic changes may become fixed in the population if they provide the organism (in this case a virus) with a selective survival advantage (King and Jukes, 1969; Yang, 2001). The majority of evolution at the molecular level is caused by random genetic drift through mutations that are selectively neutral or nearly neutral. Neutral mutation is an ongoing process which gives rise to genetic polymorphisms (Kimura, 1983b). Positive selection is a Darwinian selection fixing advantageous mutations, which is also termed as "molecular adaptation" and "adaptive molecular evolution" (Kimura, 1983a).

Positive selection is demonstrated at the molecular level if the rate of nonsynonymous substitutions at a site is greater than the rate of synonymous substitutions; this is expressed as the number of nonsynonymous substitutions per nonsynonymous substitution site is much greater than the number of synonymous substitutions per

synonymous substitution site, with a ratio > 1. Positive selection is very important for evolution of new functions and the identification of positive selection infers adaptive evolution. Since the positive selection occurs infrequently at a limited number of amino acids, it is however hard to detect (Stewart et al., 1987; Yang and Nielsen, 1998).

The ability of RNA viruses to quickly adapt and replicate in new hosts has been studied extensively (Domingo and Holland, 1997; Drake, 1993). Benmansour et al. (Benmansour et al., 1992) demonstrated a rapid evolutionary potential of glycoprotein sequences of the rabies virus possible due to heterogeneous population or the quasispecies nature of the virus. A later study on passaging of virus *in vitro* in cell culture, identified certain substitutions in the rabies viral glycoprotein sequence, that changed the nerve tropism of the virus and thereby its virulence. This adaptive process was confirmed by the study in which substantial genetic variation in G gene of viruses passaged through different host species was observed. The large value ($\omega > 1$) determined from the ratio of rate of nonsynonymous (*dN*) over synonymous (*dS*) substitutions per site, indicated positive selection (Kissi et al., 1999). Most molecular evolutionary studies of rabies virus have targeted the nucleoprotein (N) and glycoprotein (G) genes. Although those studies have identified the capability of host adaptation by a laboratory strain of rabies virus, its molecular basis *in vivo* is still unknown. Further studies, which explored host adaptive ability using rabies virus field strains in Europe (Bourhy et al., 1999), North America (Hughes et al., 2005) and globally (Holmes et al., 2002), found very weak if any evidence for positive selection in either viral G or N genes.

Molecular phylogenetics is a tool that uses nucleotide or amino acid sequence data to investigate the evolution or relationships among genes and organisms; it thus tries to reconstruct the evolutionary history of genes and organisms. The result of a phylogenetic

study is normally illustrated by a phylogenetic tree, a diagram of hierarchical branching patterns used for depicting these relationships (Vandamme, 2003).

Molecular phylogenetics has grown in stature for a decade due to several technological improvements. Especially, there has been extensive development of robust tree building methods, and software to apply such methods for phylogenetic tree reconstruction has become readily available. Furthermore, the groundbreaking and powerful method of polymerase chain reaction (PCR) has enormously facilitated the collection of sequence information. The success of phylogenetics in clarifying the evolutionary relationships between humans and other primates, and in elucidating the origins of AIDS (Brown, 1999) are just two examples of the utility of this approach.

Generally, either distance-based or character-based methods are employed to make a phylogenetic tree. The representative character-based methods are maximum parsimony (MP) and maximum likelihood (ML), which use the individual substitutions among the sequences to determine the most likely ancestral relationship. Neighbour Joining (NJ) is the most important method belonging to the distance-based methods which first calculate the overall distance between all pairs of sequences, and generates a tree based on these distances. The distance-based methods are generally much faster than the character-based methods, thus ML is almost unusable with more than a few dozen sequences and MP methods are problematic for more than one hundred sequences. These methods, especially the NJ algorithm, have been widely applied to studies on *Lyssavirus* phylogeny (Nadin-Davis et al., 2002).

Molecular Gene Markers for Genetic Analysis of Animal Populations

Since the 1970s, when the technique of starch gel electrophoresis of allozymic proteins revolutionized population genetics research, the development and application of

molecular tools such as PCR, gel electrophoresis, DNA sequencing, and cloning have played pivotal roles in population genetics. Indeed DNA identification technology has largely replaced the allozyme marker technology. Mitochondrial DNA (mtDNA) (Birky, Jr. et al., 1983) and genomic microsatellite (Bruford and Wayne, 1993; Schlotterer and Pemberton, 1994) gene markers have become the targets of choice for powerful genetic population studies over the last decade.

Microsatellite Gene Markers

Microsatellites or short tandem repeats (STR) are relatively short DNA stretches consisting of tandem repeats of one to five nucleotides (Tautz and Schlotterer, 1994). Microsatellites comprise the Tandemly Repetitive Sequences (TRS) with satellites and minisatellites (Bennett, 2000).

Most microsatellites are dinucleotides, trinucleotides or tetranucleotides located mainly in non-coding regions and distributed randomly throughout the whole genome (Tautz and Schlotterer, 1994). Microsatellites are multi-allelic in a population due to high mutation and bi-allelic (heterozygous) in an individual due to inheritance in a codominant Mendelian manner (Wan et al., 2004).

The human genome contains at least 50,000 microsatellite loci, presenting around one microsatellite locus per 10 kb of genome (Fields et al., 1994). The survey and analysis of microsatellites in various eukaryotic genomes has determined the distribution of microsatellites having different repeat compositions and repeat numbers in different locations of those genomes (Toth et al., 2000). Dinucleotide repeats of AC or GT are most abundant in rodents. In all vertebrates they are mostly observed in intergenic regions and introns and rarely in exons. However, trinucleotide repeats are distributed throughout the genomes. AAC repeats are often found in mammalian introns. Obviously, there are almost

no tetranucleotide repeats in exons, and both AAAG and AAAT repeats are found abundantly in mammals in noncoding regions. Furthermore, the abundance of pentanucleotide repeats is easily observed in introns and intergenic regions of mammalian genomes.

Microsatellite loci are considered polymorphic (more than one allele) owing to their high mutation rates. Human microsatellite repeats exhibit mutation rates of 10^{-3}-10^{-4} per locus per generation (Weber and Wong, 1993). Although the mechanism by which the microsatellite mutates is not well established, the strand-slippage replication mechanism has been accepted as a likely process (Schlotterer and Tautz, 1992; Wierdl et al., 1997). DNA mismatch repair leads to removal or elongation of the repeat during strand-slippage, so that microsatellite mutations are usually observed as either insertion or deletion of a small number of repeat units, and in more than 85% of mutations there is gain or loss of a single repeat unit. Point mutations have been identified as microsatellite mutations recently (Calabrese et al., 2001).

Different microsatellites mutate differently depending on their base composition, repeat number and repeat length, and even on the sequences flanking the microsatellite loci. In general, dinucleotide motifs with a high AT content mutate faster than those with a high GC content. Microsatellite mutations occur more frequently in longer alleles and favour expansion. Tetranucleotides are more stable than dinucleotides and trinucleotides. Microsatelllites having complicated, interrupted or composite repeat structures have a reduced possibility for single step mutational events.

A pedigree study (Brinkmann et al., 1998) of 10,844 parent/child alleles at nine microsatellite loci found that mutations were represented by insertions and deletions of the repeated motif with mostly single-step mutations and seldom two-step mutations. Other

studies focused on mutation rates at different types of microsatellite loci in humans and *Drosophila melanogaster*. Compared to the mutation rates for tetranucleotide loci, dinucleotide motifs have a mutation rate 1.48-2.16 times higher while trinucleotide motifs have a rate 1.22-1.97 times higher (Chakraborty et al., 1997). Based on dinucleotides, GT/CA repeats have the highest mutation rates which are almost 1.1 times as high as that of TC/AG repeats and 1.9 times as high as AT/TA repeats (Bachtrog et al., 2000). Microsatellite allelic diversity is also influenced by microsatellite flanking sequences (Glenn et al., 1996; Grimaldi and Crouau-Roy, 1997). A study on the dinucleotide microsatellite variability in *Drosophila melanogaster* considered microsatellite repeat number as a major factor contributing to the diversity of microsatellite slippage mutation (Bachtrog et al., 2000). Slippage mutations are rare for short repeats, therefore, the longer repeats are the main targets of microsatellite expansion via mutations (Dieringer and Schlotterer, 2003), while contractions exponentially increased in the long motifs. This is the reason why very long alleles (>50 bps) are hardly represented (Amos et al., 1996; Primmer et al., 1996; Twerdi et al., 1999; Xu et al., 2000). Both phylogenetic analysis (Jin et al., 1996; Zhu et al., 2000) and mathematical models (Whittaker et al., 2003) were used to identify the threshold size for microsatellite expansion. Eight nucleotides were confirmed as the threshold size for dynamic expansion via mutation, and almost no mutations were detected in repeat sequences under this threshold size (Rose and Falush, 1998).

Microsatellite loci can be amplified readily using the PCR, and microsatellite alleles can be unambiguously sized and thereby typed by electrophoresis through denaturing polyacrylamide gels (Tautz, 1989). Microsatellite loci are thus very easily scored. Additionally, its unique characters, such as extremely high polymorphism and presumed neutrality make them the most important gene markers for genetic population studies. Such

studies may include genetic comparisons between and within species (Gottelli et al., 1994; Paetkau and Strobeck, 1994; Taylor et al., 1994), effective population size estimation (Allen et al., 1995), population structure determination (Allen et al., 1995; Gottelli et al., 1994) and genetic relationship analysis among different subpopulations (Bowcock et al., 1994; Estoup et al., 1996; Forbes et al., 1995; Lade et al., 1996).

The isolation of microsatellite loci using a hybrid capture technique was originally described in the 1990s (Armour et al., 1994; Kandpal et al., 1994; Kijas et al., 1994); later subsequent studies have followed that technology (Brown et al., 2001; Rotstein et al., 2002), and recently described in detail (Glenn and Schable, 2005).

Cox-1 Gene - One Protein Coding Gene of Mitochondrial DNA Genome

Mitochondrial DNA (mtDNA) markers can be identified by restriction fragment analysis, but this DNA can be more informatically and efficiently analysed by direct sequencing of specific regions generated by PCR (Hoelzel, 1998). Animal mtDNA usually consists of 36 or 37 genes encoding ribosomal RNAs, tRNAs, and subunits of multimeric proteins of the inner mitochondrial membrane. It also has a noncoding sequence, termed the control region (CR), which participates in replication and transcription of mtDNA molecules. The relatively high mutation rate of mtDNA is due to its limited repair ability, with different rates of evolution for different regions. It should be recognized that population genetic studies based on mtDNA differ inherently from those based on genomic DNA due to the maternal inheritance pattern and haploid non-recombining nature of mtDNA (Wan et al., 2004).

The cytochrome oxidase subunit I gene (Cox-1), one of the protein-coding genes in the animal mitochondrial genome, has been identified as a useful target for evaluation of biological diversity, and especially for animal species identification (Hebert et al., 2003a;

Hebert et al., 2003b) for several reasons: robust universal primers lead to recovery of its 5'end from almost all animal representatives (Folmer et al., 1994; Zhang and Hewitt, 1997); the Cox-1 gene yields a wider range of phylogenetic signals than other mitochondrial genes due to slow changes in its amino acid sequence (Hebert et al., 2003a; Lynch and Jarrell, 1993); in addition, as a protein coding gene, a high rate of third base position substitution indicates its great molecular evolution rate (Knowlton and Weight, 2005). Accordingly, studies on Cox-1 gene diversity have been successful in discrimination of closely allied species and in exploring phylogenetic organization within a single species (Cox and Hebert, 2001; Wares and Cunningham, 2001). Taken together, Cox-1 gene sequencing has been considered as a combined method of studying population structure of animal species, such as bats.

Genetic Population Analysis Using Microsatellite Loci

Population genetics is the study of the distribution and change in allele frequencies of genes within and between populations (Consolidated Safety Services, 2005). A gene is a region of a DNA molecule that provides for a specific characteristic of the individual. An allele is one specific form (nucleotide sequence) of a gene. Each and every diploid individual has two alleles of each gene, inheriting one from each parent separately. If two alleles are the same, the individual is then named as homozygote, or homozygous for that gene. Otherwise, the individual is heterozygote, or heterozygous with respect to that gene (Hartl and Clark, 1997a).

The allele frequency is the proportion of all alleles of a particular gene; this value can usually be estimated by sampling and testing its variation within a population or between populations (Nei, 1973; Slatkin, 1995). The allele frequency of that allele is the percentage of

loci that the allele occupies within the population. In population genetics, allele frequencies may differ from one population to another (Pritchard et al., 2000).

Microsatellite gene loci are the hereditary traits transmitted from parent to offspring; different alleles of these loci, generated through stepwise mutation (Valdes et al., 1993), can be recombined in the progeny. Usually in any population with random mating, genotype frequency presented by allele frequency in microsatellites remains constant from generation to generation. Subpopulations of certain species are often caused by substantial topographical features of the landscape that limit mating between individuals from different areas thereby resulting in different allele frequencies in separate subpopulations. Because such allele frequency differs from one subpopulation to another, the observed heterozygosity of microsatellites among a subpopulation is smaller than the expected heterozygosity in the total population (Hartl and Clark, 1997b).

A standard approach for population structure analysis by using microsatellite gene markers (Pritchard et al., 2000) is: 1) sampling DNA microsatellite loci of members from a number of source populations, 2) estimating allele frequencies from these samples in each population at a series of unlinked loci, 3) using the estimated allele frequencies to compute the likelihood that a given genotype originated in each population, and 4) assigning individuals of unknown origin to a population according to these likelihoods. Such an approach has been applied in this study; the allele scores of the targeted microsatellite loci were obtained for multiple samples of different bat species from different geographical locations. The allele frequency of each microsatellite locus was measured within these species, which were then examined for the presence of subpopulations using the software "Structure" (see MATERIALS AND METHODS).

Rational, Hypotheses and Objectives of This Study

In North America, rabid bats have assumed the main responsibility for human deaths from rabies, and rabid bats will continue to be a serious threat to animal and human health for some time, due to frequent occurrence of viral spillover from bats to humans, domestic animals and wildlife. In addition, no present technology is available for control of rabies in insectivorous bats. Furthermore, the epidemiology of bat rabies is poorly understood to date, because of the unique biology, especially, the aerial lifestyle, of bats that complicates study and analysis compared to terrestrial rabies. Also, the existence of bat rabies has been known only since the 1950s'. Therefore, epidemiological analysis, particularly at the molecular level, of rabies virus in bat hosts, merits attention.

This study focused on *Eptesicus fuscus* and *Myotis lucifugus* species which are broadly distributed across Canada in large numbers, and which collectively comprise by far the greatest submitted for rabies testing to Canadian Food Inspection Agency laboratories. These two species are submitted in numbers sufficient for performing host population analysis. All specimens were diagnostic submissions for which detailed records of species and location of origin were well documented. Tissues for both host genomic DNA analysis and rabies virus characterization were available.

As non-migratory and colonial bats, it would be expected that both *Eptesicus fuscus* and *Myotis lucifugus* would exhibit marked sub-population structure. However, no population structure information for those bats is currently available in Canada. Hence a study to explore the application of microsatellite and mitochondrial DNA gene markers to study these Canadian bat species was deemed worthwhile. Evidently, this study would be very valuable for well-understanding rabies in bats in Canada, and for providing a useful reference for the further studies of rabies in other species of bats.

It was hypothesized that certain genetic variants of rabies circulate in a highly species-specific manner in their bat hosts, and a correlation exits between rabies virus variant transmission patterns and their host sub-population structure. To explore the existence of such a correlation the following objectives were established: 1) to group the rabies viruses harboured by certain bat species by genetically characterizing the P gene of rabies viruses from various bat species, 2) to define the population structure of the bat hosts that act as rabies reservoirs, and 3) through mapping of the data generated from viral and host genetic characterization, to explore the possibility that certain viral variants co-localise spatially with discrete sub-populations of bat species.

This study is expected to provide important new information on how host population structure influences the spread of a particular rabies virus variant, thereby providing useful clues and reference on how best to combat such spread in the future through bat population management.

MATERIALS AND METHODS

Specimen Collection

Specimens of bat brain suspensions, brain tissues and lung tissues used for this study were collected through the diagnostic submissions from the Ottawa Laboratory (Fallowfield) and the Lethbridge Laboratory of the Canadian Food Inspection Agency. Most samples from Ottawa (Fallowfield) lab were from Ontario and Quebec provinces, while a few, including *Myotis* specimens were submitted from New Brunswick, Nova Scotia, and Newfoundland. All were collected between the years 2001 to 2004. Samples from the Lethbridge lab came mainly from Saskatchewan, Alberta and British Colombia with a small number (*Myotis*) from Manitoba; years covered were from 1989 to 2004. Rabies viruses were identified by the direct Fluorescent Antibody Test (FAT) (Dean et al., 1996).

Rabies virus positive bat brain tissues were suspended with murine neuroblastoma cell medium to a volume of 1 ml, and used for rabies virus characterization. Rabies positive brain samples as well as brain and lung tissues of rabies negative samples were used for genetic characterization of microsatellite loci and Cox-1 gene. Rabies negative specimens were included for improving the detail of the population structure determination, since the geographic distribution of rabies positive specimens was not broad enough to provide a good regional representation of the bats in Canada. The complete collection consisted of 251 rabies virus positive and 101 rabies virus negative *Eptesicus fuscus* specimens, and 26 rabies positive and 119 rabies negative *Myotis* specimens as summarised in Appendix 1.

The bats of *Eptesicus fuscus and Myotis lucifugus* provided by the Ottawa Laboratory (Fallowfield) were speciated by Dr. Donald McAlpine from Natural Science Department, Museum of New Brunswick, Canada. The speciation of *Myotis* bats supplied by the

Lethbridge Laboratory was confirmed by Dr. David Nagorsen from Mammalia Biological Consulting of British Columbia, Canada.

All the specimens were stored at -20°C until they were processed for DNA and RNA extraction.

Rabies Virus RNA Isolation

Total tissue RNA was extracted using TRIzol LS Reagent (Invitrogen, Burlington, Ontario, Canada) following the supplier's instructions. Essentially, up to 250 µl of brain suspension of each sample was mixed thoroughly by vortexing with 750 µl of TRIzol LS and 5 µg of glycogen (Roche Diagnostics, Laval, Quebec, Canada), as a RNA carrier. The sample mixture was incubated at room temperature for 2 to 3 minutes with vigorous vortexing for 15 seconds each, and then, centrifuged at 12,000 g (13,000 rpm in a standard microfuge) for 15 minutes at 4°C. The aqueous phase was transferred to a fresh 1.5 ml microtube. Total RNA was precipitated by addition of 500 µl of isopropanol and followed by incubation for 10 minutes at room temperature, and pelleted by centrifugation at 12,000 g for 10 minutes at 4°C. Subsequently, the RNA pellet was washed by addition of 1 ml of 75% ethanol with vortexing and then repelleted by centrifugation at 12,000 g for 3 minutes at 4°C. The pellet was dried in a Savant OligoPrep-OP120 concentrator (GMI, Inc. Ramsy, Minnesota, USA) for 15 minutes, and finally dissolved in a certain volume of RNase-free water, according to the pellet size, at room temperature for 15 minutes. The concentration of RNA in each sample was determined by measuring UV absorbance at 260 nm with a spectrophotometer (Genesys 10UV, VWR international, Bridgeport, New Jersey, USA) using a 1/250 dilution of the RNA sample. The RNA samples were stored at -80°C until they were used for rabies virus P gene amplification.

Rabies Virus P gene Amplification by Reverse Transcription PCR (RT-PCR)

All oligo nucleotides applied in this study were synthesized by Integrated DNA Technologies, Inc. (Doralville, Iowa, USA), and all dye labelled oligo nucleotides were synthesized by LI-COR (Lincoln, Nebraska, USA).

1) Annealing of the first primer

Approximately 2 µg of extracted total RNA was incubated with 50 pMoles of RabPFor primer (Nadin-Davis, 1998) described in Table 2, in a total volume of 11 µl brought up with DEPC H_2O. The mix was heated at 65°C for 5 minutes, and then at 37°C for 10-15 minutes.

Table 2. PCR Primers Used in This Study

Primer Name	Nucleotide Sequence 5'-3'	Position
RabPFor	CTACTTCTCCGGGGAAACCAGAAG	1249-1272
RabPRev	GGRAGCCAYAGGTCRTCGTCAT	2575-2596
RabP967	GAGATGGCNGARGAGACTGTWGA	1568-1590
RabP995	CCTTAACTATGTCRTCAAGRTTCA	2208-2230
PSeqFor	GAGATGGCAGARGAGACTGTWGA	1568-1590
CoxFor	TCAACCAACCACAAAGACATTGGCAC	
CoxRev	TAGACTTCTGGGTGGCCAAAGAATCA	
CoxSeqRev	TAGACTTCTGGGTGGCCAAAGAATCA	

The positions identified for the RABV primers refer to the reference PV laboratory strain (GenBank accession number: PV-X03673)

2) Reverse transcription for cDNA synthesis

The annealing reaction was brought to a final volume of 20 µl by addition of 9 µl of a cDNA Master Mix. The final reverse transcription reaction contained 1 x RT buffer

(supplied with the enzyme), 10 mM dithiothreitol (DTT), 1 mM dNTPs, and 200 U of reverse transcriptase (M-MLV) (Invitrogen, Burlington, Ontario, Canada). The reaction was incubated for 1.5-2 hours at 37°C and then terminated by heating at 90-95°C for 5 minutes, chilled on ice and spun down.

3) PCR

The final 50 µl reaction contained 10 µl of cDNA product from step 2) and 40 µl of PCR Master Mix including 1 x PCR buffer (-$MgCl_2$) (included with the enzyme), 1.5 mM $MgCl_2$, 50 pMoles RabPRev primer (Nadin-Davis, 1998) described in Table 2, and 2.5 U Taq DNA Polymerase (Invitrogen, Burlington, Ontario, Canada). Thermal cycling was programmed on a GeneAmp 9700 (Applied Biosystem, Foster City, California, USA) to obtain a PCR product of 1348 base pairs (bp). The cycling file included an initial denaturation at 94.0°C for 2 minutes, followed by 35 cycles at 94.0°C for 30 seconds, 50.0°C for 30 seconds and 72.0°C for 2 minutes, and followed by a 3 minute extension at 72.0°C and then a hold at 4.0°C. After thermal cycling, an aliquot of each reaction was analysed by standard electrophoresis through a 1% agarose gel. DNA bands were visualized under UV light after ethidium bromide staining. If amplified PCR products were not visualized at gel documentation, the nested PCR using internal primers was applied to generate a smaller 664 bp amplicon for those samples.

4) Nested PCR

Two µl of the first round PCR product was mixed with 48 µl nested PCR Master Mix comprising 1 x PCR buffer (-$MgCl_2$), 25 pMoles RabP967, 25 pMoles primer RabP995 (Nadin-Davis, 1998) described in Table 2, 200 µM dNTP, 1.5 mM $MgCl_2$ and 2.5 U Taq DNA polymerase. The cycling profile consisted of an initial denaturation of 95.0°C for 2 minutes, and then 30 cycles of 95.0°C for 45 seconds, 50.0°C for 30 seconds and 72.0°C for

1 minute, a final extension at 72.0°C for 2 minutes, and then a hold at 4.0°C. The PCR product was analysed by 1% agarose gel electrophoresis as described above.

Purification of PCR Products of Rabies Virus P Gene

A Promega Wizard PCR Preps DNA purification system was used for purifying PCR products of P gene. Each PCR product was transferred to a 1.5 ml microtube and mixed with 100 µl of direct DNA purification buffer with a brief vortex. One ml of Wizard® PCR Preps Resin (Promega, Madison, Wisconsin, USA) was added and the mixture was vortexed briefly 3 times. The whole mixture was transferred to a minicolumn attached to a syringe barrel at the upper end and onto a vacuum manifold at the other. After drawing the resin-bound DNA into the minicolumn, 2 ml of 80% isopropanol were applied to wash the minicolumn and draw through by applying vacuum. The DNA in the minicolumn was eluted using 50 µl of 1 x TLE buffer prewarmed to 70°C and collected by spinning the minicolumn for 20 seconds in a microcentrifuge. The purified PCR products were stored at -20°C.

Partial Rabies Virus P gene Sequencing

The thermo sequenase primer cycle sequencing kit (Amersham Biosciences, Little Chalfont Buckinghamshire, England) was used for the cycle sequencing of P gene PCR products. A P gene internal primer PSeqFor (Nadin-Davis, 1998) labelled with an IR 700 dye (LI-COR, Lincoln, Nebraska, USA) was employed to prime the sequencing reaction. The cycling profile included a denaturation step at 95.0°C for 3 minutes, 33 cycles of 95.0°C for 35 seconds, 50.0°C for 35 seconds, and 72.0°C for 45 seconds, and followed by a final extension at 72°C for 2 minutes, and a hold at 4°C. Three µl of IR stop solution (LI-COR, Lincoln, Nebraska, USA) were added into each tube and samples were denatured at 95°C for 3 minutes just before the sample loading. Usually, 0.8-0.9 µl of each sample was loaded to a

3.7% denaturing polyacrylamide gel, 66 cm long and 0.20 mm wide. Sequencing gels were run for 14 hours at 3000 constant volts on a LI-COR 4200L automated sequencer.

Base calling was performed automatically using the LI-COR program e-Seq, version 2. Sequences were aligned and edited manually using program AlignIR, version 2 (LI-COR, Lincoln, Nebraska, USA).

Phylogenetic Analysis of Rabies Virus P Gene Sequences

Using a sequence length trimmed to 597 bp, 206 rabies virus partial P gene sequences from *Eptesicus fuscus* specimens and 25 sequences from *Myotis* species specimens were employed for phylogenetic analysis. The precise sequence window was defined according to its position in the reference RABV PV laboratory strain (Tordo et al., 1986a). A specimen of the Ontario arctic fox strain of rabies virus (Fox_ON) was applied as an out group for this analysis.

These DNA sequences were imported as a FASTA format file into CLUSTAL X program, version 1.8 (Thompson et al., 1994) to generate an alignment, that could be imported into the PHYLIP 3.6 package of programs (Felsenstein, 2005) used for phylogenetic analysis.

The SEQBOOT program was used for generating bootstrapped replicates (100 or 1000) of the original data set. The DNADIST program, set up with the F84 model (Felsenstein and Churchill, 1996; Kishino and Hasegawa, 1989) and analysed with multiple or single data sets, was applied to compute a distance matrix for all nucleotide sequences. The NEIGHBOR program constructed a NJ tree using the distance matrix generated from bootstrapped data. The CONSENSE program was applied to generate a consensus tree from all trees generated from the NJ analysis, and another output file that provided the bootstrap values for each cluster. The RETREE program was used to unroot the consensus tree. The

FITCH program was used together with a distance matrix of the original single data set for reapplying distance information to branches of the consensus tree.

The DNAPARS program was applied to make a MP tree using a bootstrapped dataset. The CONSENSE program was again used to generate a consensus tree from the outtree file and bootstrap values were recovered from the outfile.

For all trees, only bootstrap values above 70 percent were considered significant.

Treeview (Page, 1996) software was used to manipulate all the output tree files to provide graphical representation.

Comparison of Selected Sequences with Reference Sequences of Rabies Virus P Gene

Certain P gene sequences (Appendix 1) selected according to their distribution in representative clades of the NJ tree, were compared to sequences of reference bat rabies virus samples which were described previously (Nadin-Davis et al., 2001) and subsequently characterized at the P gene locus (Nadin-Davis et al., 2002).

Two to eight samples from each clade and subclade were included in this analysis. A NJ tree was generated using 37 selected sequences and 19 reference sequences. The detailed information on the selected and reference samples was summarized in Appendix 1 and Table 3, respectively.

Analysis of Predicted Partial P Protein Sequences

The partial P gene sequences from rabies virus positive bats of *Eptesicus fuscus* and *Myotis* species were selected according to the year of isolation and geographical location of origin; see list in Appendix 1. Forty one isolates of *Eptesicus fuscus*, selected from clades I-VI, and 16 isolates of *Myotis* species from clade VII, were representative of the genetic diversity of all rabies viruses in this study.

Table 3. Reference Sample List Used for Comparative Analysis of Rabies Viral P Gene

Isolate Reference No.	Bat Species Submitted	Province/State or Country of Origin	Year of Submission
EF22	*Eptesicus fuscus*	BC, CA	1988
EF23	*Eptesicus fuscus*	BC, CA	1993
EF25	*Eptesicus fuscus*	BC, CA	1990
EF31	*Eptesicus fuscus*	SASK, CA	1989
EF32	*Eptesicus fuscus*	ONT, CA	1993
EF33	*Eptesicus fuscus*	ONT, CA	1993
EF55	*Eptesicus fuscus*	NB, CA	1997
EF71	*Eptesicus fuscus*	CT, USA	1998
EF72	*Eptesicus fuscus*	CT, USA	1998
LAN13	*Lasionycteris noctivagans*	ONT, CA	1980
LB5	*Lasiurus borealis*	TX, USA	unknown
LB6	*Lasiurus borealis*	ONT, CA	1991
LC1	*Lasiurus cinereus*	ALTA, CA	1992
ME1	*Myotis evotis*	BC, CA	1992
ML4	*Myotis lucifugus*	BC, CA	1992
ML6	*Myotis lucifugus*	NS, CA	1994
ML8	*Myotis lucifugus*	ONT, CA	1997
MYO1	*Myotis*	ONT, CA	1996

The translation of P gene sequences was conducted by DNASIS software (Hitachi Software Engineering Co., Ltd, Yokohama, Kanagawa Prefecture, Japan). Alignment of amino acid sequences was performed with CLUSTALX version 1.8 (Thompson et al., 1994).

Rabies Virus Evolutionary Analysis

1) Dataset generation

Fifty seven partial P gene sequences described above were organized into two sub-data sets represented by the two bat hosts, *Eptesicus fuscus* and *Myotis* species.

2) Nucleotide sequence alignment and phylogenetic tree construction

Separate sequence alignments were generated for the 2 datasets, represented by the two bat hosts *Eptesicus fuscus* and *Myotis* species, using the CLUSTAL X program.

The DNAML program was used to construct a phylogenetic tree by the DNA ML algorithm, characterized by allowing different rates of evolution at different sites, of the PHYLIP 3.6 package (Felsenstein, 2005). The ML trees for each dataset were used as the input tree files of the CODEML program (Goldman and Yang, 1994).

3) Tests for positive selection

The CODEML program of the PAML package, a program of maximum likelihood analysis of protein-coding DNA sequences using various codon substitution models, was employed for selection analysis. Six models designated as M0, M1, M2, M3, M7 and M8, which vary constraints on the values of nonsynonymous substitutions per nonsynonymous site (*dN*) and synonymous substitutions per synonymous site (*dS*) and their ratio (ω) were applied to explore whether positive selection was operating (Woelk et al., 2001; Woelk and Holmes, 2001) on both datasets. The different specified models provide various opportunities for the d*N*/d*S* ratio (ω) to vary among different sites (Nielsen and Yang, 1998; Yang et al., 2000). Positive selection can be inferred by comparison of implemented models through likelihood ratio tests. Bayesian methods were employed to identify the specific codons which have $\omega > 1$ if there was evidence of positive selection. The detailed methods for model selection were described elsewhere (Hughes et al., 2005; Woelk et al., 2002). The ω value from model 0 (between the bounds of 0 and 1) was accepted as the mean value if there was no evidence suggested for positive selection (Hughes et al., 2005).

4) Estimation of molecular evolution rates

The program BEAST (Drummond and Rambaut, 2003) can be used to estimate the rate of molecular evolution (substitutions per site per year: μ). The BEAST program analyzes

molecular sequences by using a Bayesian MCMC (Markov chain Monte Carlo) model without requiring tree topology (Drummond et al., 2002). It needs two complementary programs to assist with the complete analysis. The program BEAUTi is used to create the input file for the BEAST program by importing the data and setting the evolutionary models to be tested. The program TRACER is used to analyse the output from the BEAST program and plot the results.

NEXUS format files were generated by CLUSTAL X and were imported into the BEAUTi program; each sequence was dated with the year of isolation. The analysis was performed with a substitution model set as HKY85 (Hasegawa et al., 1985) without site heterogeneity model, prior coalescent model set as constant population size, and the length of the MCMC set as 100,000 or 1,000,000 to achieve a reasonably effective sample size (ESS≥100). Three replicate BEAST runs were operated for each estimation of both datasets to check the the convergence of the output values.

Bat microsatellite Loci Library Establishment

1) Principle of the method for Microsatellite loci Isolation

The method employed here has been summarized by the Savannah River Ecology Laboratory (Glenn and Schable, 2005).

Genomic DNA is cut into small fragments (400-900 bp) using a restriction endonuclease RsaI which recognizes the motif GT^AC of dsDNA. Oligonucleotide linkers, SuperSNX24 forward and reverse are ligated to the cut DNA; these linkers provide the primer binding sites for PCR to amplify the linker-DNA fragments, as subsequently verified by running PCR products on an agarose gel. The DNA is then denatured to produce single stranded fragments for hybridization to a microsatellite probe biotinylated at its 3' end. The biotin moiety of the probe can irreversibly bind to streptavidin coated magnetic beads,

thereby providing a mechanism to enrich via hybridization of microsatellite loci having repeats similar to those in the probe. DNA microsatellite loci are recovered by application of a small magnet which pulls the beads out of solution. The enriched DNA is recovered and cloned into a plasmid that is transformed into a bacterial host. After positive colonies are stored, the inserts are amplified by PCR and then size selected on an agarose gel. The DNA insert of selected clones is sequenced to confirm presence of a microsatellite for primer design and for microsatellite locus amplification. Figure 3 illustrates the entire process for microsatellite locus isolation.

2) Genomic DNA extraction using CTAB method

The protocol was modified from an anonymous author.

a) Hexadecyl Trimethyl Ammonium Bromide (CTAB) solution preparation

The CTAB solution contained 19.84 mg/ml of Hexadecyl Trimethyl Ammonium Bromide (Sigma, St. Louis, Missouri, USA), 0.1 M Tris, 1.4 M NaCl, 0.02 M EDTA, and 0.20% (v/v) β-mercaptoethanol (Fisher Scientific, Fair Lawn, New Jersey, USA).

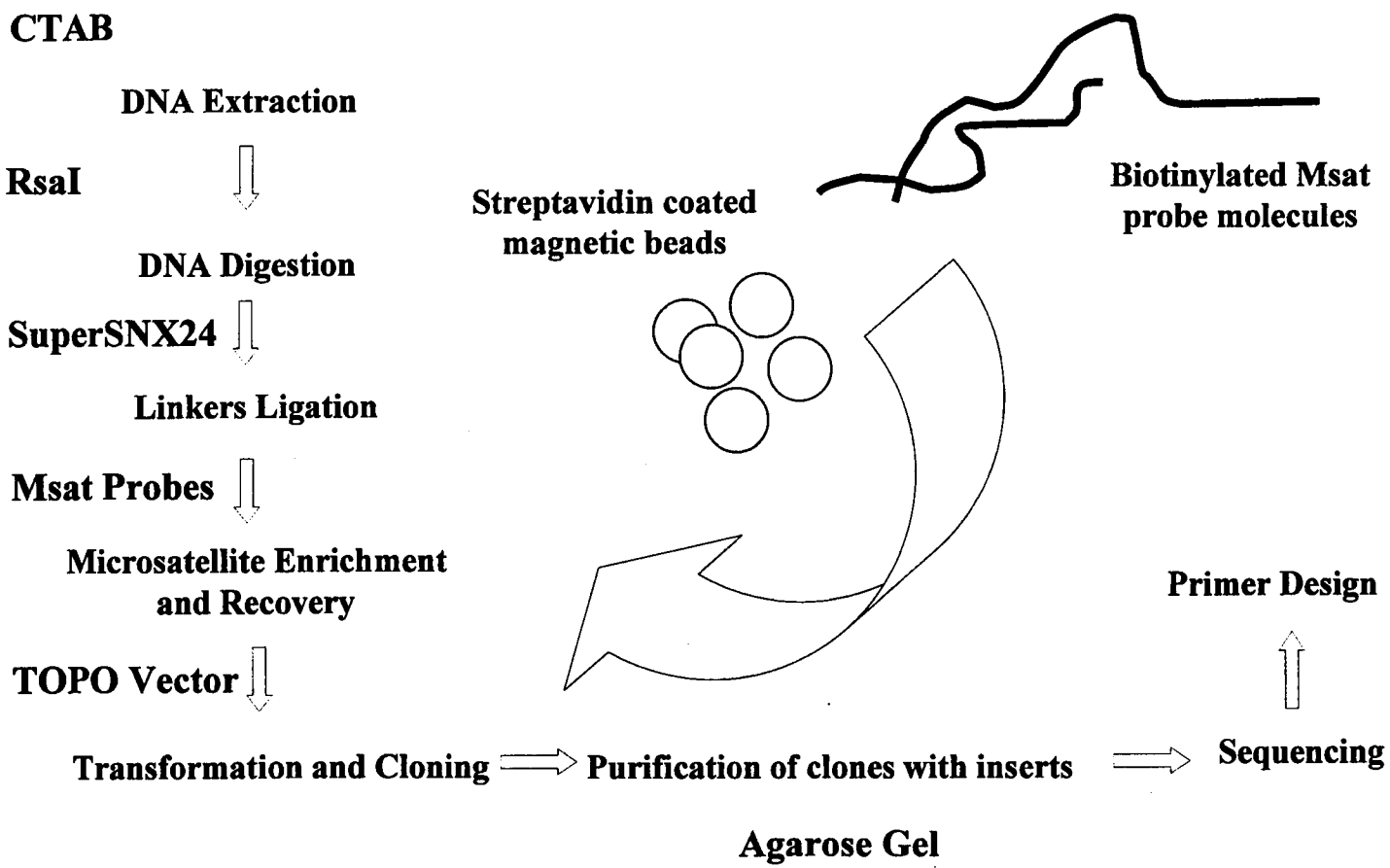
This solution should be freshly prepared for DNA extraction use.

b) DNA extraction

For each 500 µl brain suspension (or approximately 50 mg brain tissue or lung tissue) in a 1.5 ml microtube, 50 µl of CTAB solution was added and the tissue ground with a micro pestle. Another 550 µl of CTAB solution were used for rinsing each pestle for each sample. The microtube was capped tightly and incubated at 65°C water bath for 40-60 minutes, and then centrifuged for 7 minutes at 13 K g. The upper phase of each sample was transferred into a new 1.5 ml microtube. Five hundred and fifty µl of chloroform were added to each microtube, which was finger vortexed and centrifuged for 30 minutes at 13 K g. The upper phase was transferred into a new 1.5 ml microtube containing 750 µl of cold isopropanol

Figure 3. The Process of Microsatellite Loci Isolation

Msat Library Construction



(chilled to -20°C before use). The samples were placed at -20°C for 30 minutes or overnight, and then centrifuged for 30 minutes at 13 K g to pellet the DNA. The supernatant of each sample was carefully decanted and discarded. The DNA pellet was washed with 200 µl of cold 70% ethanol (chilled to -20°C before use), dried in a Savant OligoPrep-OP120 concentrator (GMI, Inc. Ramsy, Minnesota, USA) around 30 minutes, and then redissolved in a certain volume of 1 x TLE buffer, according to the pellet size, at room temperature overnight. The concentration of each DNA sample was measured by examining UV absorbance at 260 nm with a spectrophotometer (Genesys 10UV, VWR international, Bridgeport, New Jersey, USA) using an aliquot of the DNA sample diluted 250 times. The extracted DNA samples were stored at -20°C until use.

3) Restriction endonuclease digestion – to cut DNA into approximately 500 base pair blunt end fragments using the enzyme RsaI which cleaves nucleotides between GT and AC.

Preparation of a master mix of RsaI solution:

10 x Ligase Buffer (New England Biolabs, Beverly, MA, USA)	2.50 µl
100 x Bovine Serum Albumin (BSA) (New England Biolabs, Beverly, MA, USA)	0.25 µl
5 M NaCl	0.25 µl
Rsa I (New England Biolabs, Beverly, MA, USA)	1.00 µl
Xmn I (New England Biolabs, Beverly, MA, USA)	1.00 µl
Genomic DNA (200 ng/µl)	20.0 µl

The mixture was incubated in a water bath at 37°C temperature for 30 - 60 minutes. The incubation time was adjusted, after checking the fragment size of the digested DNA (3 µl) on a 1% agorose gel, so as to generate DNA fragments of the required size.

4) Ligation of SuperSNX linkers to DNA fragments – to provide the primer binding site for subsequent PCR steps

a) Preparation of double strand SuperSNX linkers

SuperSNX24 Forward: 5'-GTTTAAGGCCTAGCTAGCAGAATC-3'

SuperSNX24+4P Reverse: 5'-pGATTCTGCTAGCTAGGCCTTAAACAAAA-3'

Equal volumes of equal molar amounts of SuperSNX24 and SuperSNX24+4p primers were mixed and 5 M NaCl was added to make final concentration of SuperSNX linkers as 100 mM. The mixture was heated to 95°C and cooled down to room temperature.

b) Preparation of a master mix of linker-ligation solution

dsSuperSNX linkers (prepared from step a))	7.0 µl
10 x Ligase Buffer (New England Biolabs, Beverly, MA, USA)	1.0 µl
DNA ligase (New England Biolabs, Beverly, MA, USA, 400 U/µl)	2.0 µl
Total	10.0 µl

The master mix was added into the entire cut DNA from step 3). Then the mixture was incubated at room temperature for more than 2 hours. The ligation procedure should be checked by PCR.

c) Preparation of PCR master mix

10 x PCR buffer (-$MgCl_2$)	2.5 µl
BSA (250 µg/ml)	2.5 µl
SuperSNX-24 (10 µM)	1.3 µl
dNTP's (25 mM each)	1.5 µl
$MgCl_2$ (25 mM)	2.0 µl
dH_2O	13.0 µl
Taq DNA Polymerase (5 U/µl)	0.2 µl
Linker ligated DNA fragments	2.0 µl

The PCR cycling was an initial denaturation at 95.0°C for 2 minutes; then, 20 cycles of 95.0°C for 20 seconds, 60.0°C for 20 seconds, a final extension at 72.0°C for 1.5 minutes, followed by a hold at 15.0°C. The results of the linker-ligation process were evaluated by electrophoresis of the PCR product on a 1.5% agarose gel. A successful ligation should

generate a smear of fragments, centered at about 500 bp without any specific DNA bands being disproportionately represented. The PCR product thus generated was used for microsatellite enrichment.

5) Dynabead enrichment of microsatellites

Dynabeads were washed twice in 1 x TE buffer and 1 x Hybridization solution individually, and then resuspended in 150 µl of 1 x Hybridization solution for later use. 3' biotin labelled oligonucleotides of $(ATC)_8$ and $(GT)_9$ were prepared as microsatellite probes. In a 0.2 ml PCR tube, the following mix was prepared:

2 x Hybridization Solution	25.0 µl
Biotinylated microsatellite probe (1 µM each)	10.0 µl
Linker ligated DNA PCR product	10.0 µl
dH_2O	5.0 µl
Total	50.0 µl

The hybridization of these oligonucleotides and DNA fragments was performed in a thermal cycler by using a touch down program. The program denatured the DNA and probe mixture at 95°C, then ramped quickly to 70°C and stepped down 0.2°C every 5 seconds for 99 cycles, and finally quickly ramped down to 15°C.

The above mixture was added to dynabeads solution and incubated at room temperature for more than 30 minutes on a rotator. The beads were captured using a Dynal MPC-S Magnetic Particle Concentrator (Dynal A.S., Oslo, Norway) and washed twice sequentially with 400 µl 2 x SSC, 0.1% SDS, and 400 µl of 1 x SSC, 0.1% SDS at 50°C. After washing, the captured beads were suspended in 200 µl of 1 x TLE, vortexed and incubated at 95°C for 5 minutes. The supernatant was removed quickly to a new tube and precipitated by addition of 22 µl of 3 M NaOAc and 444 µl of 95% ethanol. The tube was placed on ice for more than 15 minutes and centrifuged at 15 K g for 10 minutes. The

invisible DNA pellet was washed by 0.5 ml of 70% ethanol, air dried and resuspended in 25 µl of 1 x TLE buffer at room temperature overnight.

6) PCR recovery of enriched DNA

A master mix for each PCR was prepared as follows:

10 x PCR buffer (-$MgCl_2$)	2.5 µl
BSA (250 µg/ml)	2.5 µl
dNTPs (2.5 mM)	1.5 µl
SuperSNX-24 (10 µM)	1.3 µl
$MgCl_2$ (25 mM)	2.0 µl
dH_2O	13.0 µl
Taq DNA polymerase (5 U/µl)	0.2 µl
Microsatellite DNA fragments	2.0 µl

The PCR was processed on a thermal cycler with a PCR profile thus: an initial denaturation at 95°C for 2 minutes; followed by 25 cycles of 95°C for 20 seconds, 60°C for 20 seconds, and 72°C for 1.5 minutes; a final extension at 72°C for 30 minutes and then a hold at 15°C. The PCR product was evaluated by electrophoresis on a 1.0% agarose gel to check for the recovery of DNA. Ideally, a smear of fragments centered at about 500 bp should be recovered.

7) Preparation of LB agar plates with ampicillin

One litre of LB medium containing 10 g tryptone, 5 g yeast extract and 10 g NaCl was mixed, adjusted to a pH of 7.5, and 15 g bacto-agar was added before autoclaving for 20 minutes at 121°C. The sterile medium was cooled to 50°C. One ml of ampicillin (Scientific Imaging Systems, EASTMAN KODAK COMPANY, Rochester, New York, USA) stock (100 µg/ml) was added and mixed with the LB medium. The well-mixed medium was poured into sterile Petri dishes and allowed to cool and solidify. LB agar plates were stored at 4°C until use.

Around one hour prior to transformation, the required number of LB plates with ampicillin were prewarmed at 37°C. Forty µl of 40 mg/ml X-Gal (Diagnostic Chemicals Ltd., Charlottetown, Prince Edward Island, Canada) were spread on each LB plate and incubated at 37°C until use.

8) Ligation and transformation of enriched DNA into plasmids

TA cloning of the microsatellite-enriched DNA into a TOPO vector using TOP 10 *chemically competent E. coli* cells (Invitrogen, Burlington, Ontario, Canada) from Invitrogen's TOPO TA cloning kits (Invitrogen, Burlington, Ontario, Canada) were employed for ligation and transformation of microsatellite fragments, following manufacturer's instructions. Briefly, the TOPO cloning reaction, consisting of 2 µl PCR product, 0.5 µl of salt solution, 0.5 µl of TOPO vector, was mixed gently and incubated for 5-15 minutes at room temperature (22-23°C), and then placed on ice.

One vial of TOP 10 competent cells was thawed on ice and 2 µl of TOPO cloning reaction were added to it. The above mixture was incubated on ice for 30 minutes, then heated for 30 seconds at 42°C in a water bath, and transferred back onto ice immediately. Finally, 250 µl of room temperature SOC medium was added, and the tube shaken horizontally at 37°C for 1 hour before plating out the transformed bacteria. Two volumes, 25 µl and 50 µl of transformation mixture were plated onto the prepared LB plates with ampicillin. The plates were incubated overnight at 37°C to allow bacterial colony growth.

9) Storing positive colonies with PCR

a) Selection of positive colonies (white)

White colonies were transferred by sterile toothpick to culture tubes containing 300 µl LB medium supplemented with ampicillin (100 µg/ml). Cultures were grown at 37°C overnight.

b) The preparation of PCR master mix for amplifying the DNA microsatellite inserts (25 µl reaction)

250 µg/ml BSA	2.50 µl
10 x PCR buffer (-$MgCl_2$)	2.50 µl
10 µM M13 forward primer (Invitrogen, Burlington, Ontario, Canada)	0.625 µl
10 µM M13 reverse primer (Invitrogen, Burlington, Ontario, Canada)	0.625 µl
25 mM $MgCl_2$	1.50 µl
2.5 mM dNTPs (2.5 mM each)	1.50 µl
dH_2O	14.65 µl
Taq DNA polymerase	0.10 µl
DNA template from bacteria colony grown up in LB broth	1.00 µl

The PCR was processed with the following thermal cycle program: an initial denaturation at 95.0°C for 3 minutes, then 35 cycles of 95.0°C for 20 seconds, 50.0°C for 20 seconds, and 72.0°C for 1 minute and 30 seconds, and a final hold at 15.0°C. The DNA inserts were evaluated by electrophoresis of 2 µl of the M13/bacterial PCR product on a 1% agarose gel at 80 volts for 30 to 40 minutes.

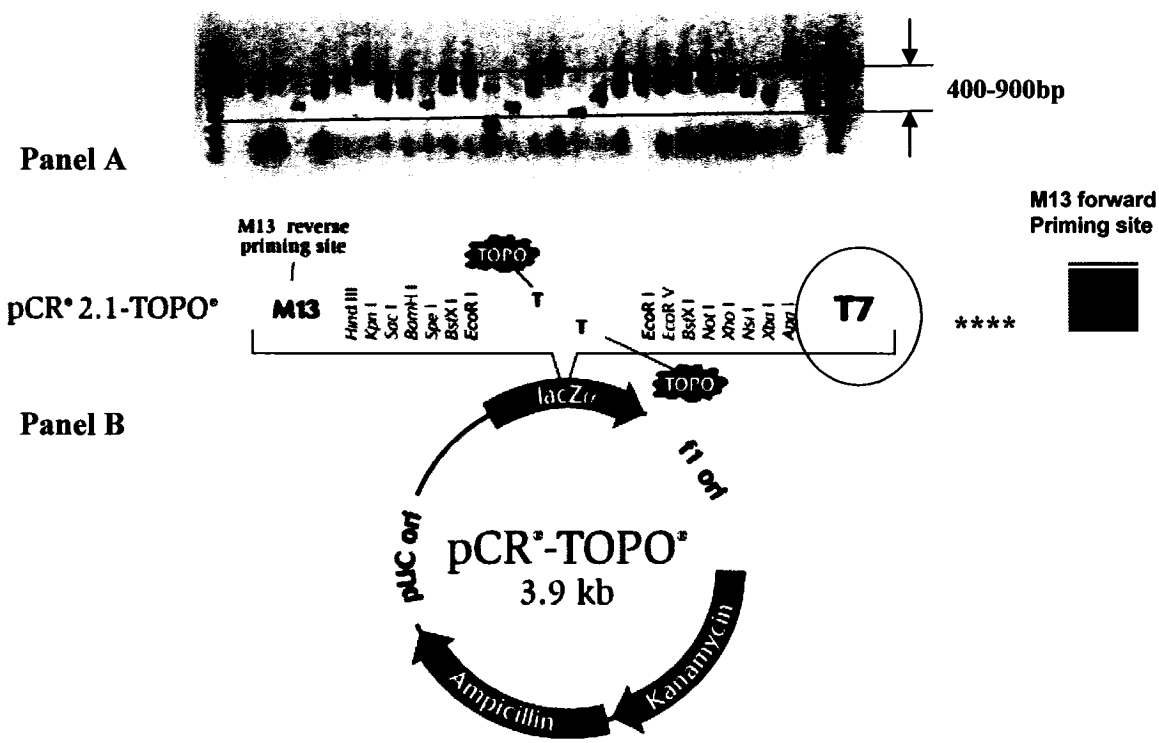
c) Identification of DNA inserts containing microsatellites

DNA inserts of 400-900 bp were chosen for DNA sequencing. Prior to sequencing, the PCR products were purified using a Promega Wizard PCR Preps DNA purification system as described in section "Purification of Rabies Virus P Gene PCR Products". A 700 IR dye labelled T7 promoter primer (LI-COR, Lincoln, Nebraska, USA) was employed in a cycle sequencing reaction with an annealing temperature of 48°C in the thermal cycling program (Figure 4). The sequencing reactions were performed as described previously using the LI-COR 4200L automated sequencer and the e-Seq V2 software (LI-COR, Lincoln, Nebraska, USA).

d) Construction of Microsatellite Loci Libraries

The *Eptesicus fuscus* and *Myotis lucifugus* two microsatellite loci libraries were

Figure 4. DNA Inserts with Microsatellite Loci and TOPO Cloning Map
Panel A. Agarose gel electrophoresis of PCRs generated from 28 colonies recovered from a microsatellite DNA library. A 100 bp DNA ladder (Invitrogen) is shown in the first and last lanes. The 2 red horizontal lines show the limits of the fragment of the range suitable for further characterization.
Panel B. Schematic of the PCR-TOPO vector (Invitrogen) used for cloning. The locations of the M13 for/rev primer sites used for PCR of the inserts are indicated as is the location of the T7 promoter primer binding site used for DNA sequencing.


Panel A
400-900bp
M13 forward
Priming site
M13 reverse
priming site
pCR® 2.1-TOPO®
M13
TOPO
T7
****
Panel B
TOPO
pCR®-TOPO®
3.9 kb
Ampicillin
Kanamycin

constructed by using $(GT)_9$ and $(ATC)_8$ microsatellite probes. For each bat genus, 298 positive clones were picked, cultured in LB broth at 37°C overnight, then amplified by PCR, and analysed on a 1% agarose gel. Four hundred clones having DNA inserts, with sizes between 400 and 900 bases, were recovered for *Eptesicus fuscus* (186) and *Myotis lucifugus* (214) respectively. All selected DNA inserts from the two species were sequenced to identify the microsatellite loci and determine the flanking sequences present on both sides of the microsatellites.

Primer 3 software (Rozen and Skaletsky, 2000) available online was applied to design primers flanking suitable microsatellite loci; primers were designed to minimize self-complementarities and primer-dimer formation. Primer 3 software was also employed for calculation of PCR product size of selected DNA inserts containing useful microsatellite loci.

Microsatellite Loci Evaluation

Each of ten *Eptesicus fuscus* and ten *Myotis lucifugus* DNA samples were amplified individually for 9 microsatellite loci (*Eptesicus fuscus*) and 7 microsatellite loci (*Myotis lucifugus*) respectively. The following PCR amplification program was employed on a thermal cycler (GeneAmp 9700, Applied Biosystem, Foster City, California, USA): an initial denaturation at 95°C for 4 minutes, then 33 cycles of 95°C for 30 seconds, 60°C for 35 seconds, and 72°C for 3 minutes, a final extension at 72°C for 7 minutes and a hold at 4°C. Each 25 µl of PCR contained 1 x PCR buffer (-$MgCl_2$), 200 µM dNTPs, 1.5 mM $MgCl_2$, 40 pMoles each of the forward and reverse microsatellite primers, 1 U Taq DNA polymerase and 100 ng genomic DNA were included. The polymorphism of each microsatellite locus was evaluated by electrophoresis of PCR product on a 2% agarose gel.

Multiplex Amplification of Microsatellite Loci

A process for microsatellite amplification described by LI-COR, in which one of each microsatellite primer pair (forward primer) is tagged by addition of the M13 reverse primer sequence at its 5' end, was employed. The reaction includes a separate "M13" 700 IR dye-labelled primer, to incorporate dye into the product (Kovar et al., 2005). The following protocol for amplifying multiple microstatellites using multiplex PCR was employed: each 10 µl reaction included 1 x PCR buffer (-$MgCl_2$), 200 µM dNTPs, 0.5 pMole of each microsatellite primer, 1 pMole M13 IR 700-dye labelled reverse primer (LI-COR, Lincoln, Nebraska, USA), 1 U Taq DNA polymerase, and 1 µl (20 ng) of genomic DNA. The volume of dH_2O was adjusted according to the number of microsatellite primers used in each reaction. The reaction was processed in a GeneAmp 9700 (Applied Biosystem, Foster City, California, USA) thermal cycler. The cycling profile had an initial heat denaturation of 95.0°C for 2 minutes, followed by 30 cycles of 95.0°C for 30 seconds, 55.0°C for 40 seconds, 65.0°C for 1 minute, and concluded by a final extension at 65.0°C for 7 minutes followed by a hold at 4.0°C. Each 10 µl of PCR product were mixed with 4 µl of blue stop solution (LI-COR, Lincoln, Nebraska, USA), and denatured by heating at 95°C for 3 minutes before polyacrylamide gel electrophoresis on a 42 cm long, 0.25 mm wide, 6% polyacrylamide denaturing gel on a LI-COR 4200L sequencing system. Each gel was run at 1400 volts for 2 hours; a 50-350 DNA size standard with labelled dye IR 700 (LI-COR, Lincoln, Nebraska, USA) was electrophoresed in several wells across the gel (Figure 5, 6).

An automated genotyping software, $SAGA^{GT}$ (LI-COR, Lincoln, Nebraska, USA), was used for automatic allele size scoring of microsatellite loci after electrophoresis. The software used project models to manage each step of the process. The molecular weight standards manager was used to assign the molecular weight sets to accurately define the base

Figure 5. Polyacrymide Gel Image of Microsatellites for *Eptesicus fuscus*
Lane 1, 12, 23, 34, 45: Molecular weight standards (50-350 bp).
Lane 2-11: three microsatellite loci presented per lane.
Lane 12-48: two microsatellite loci presented per lane.

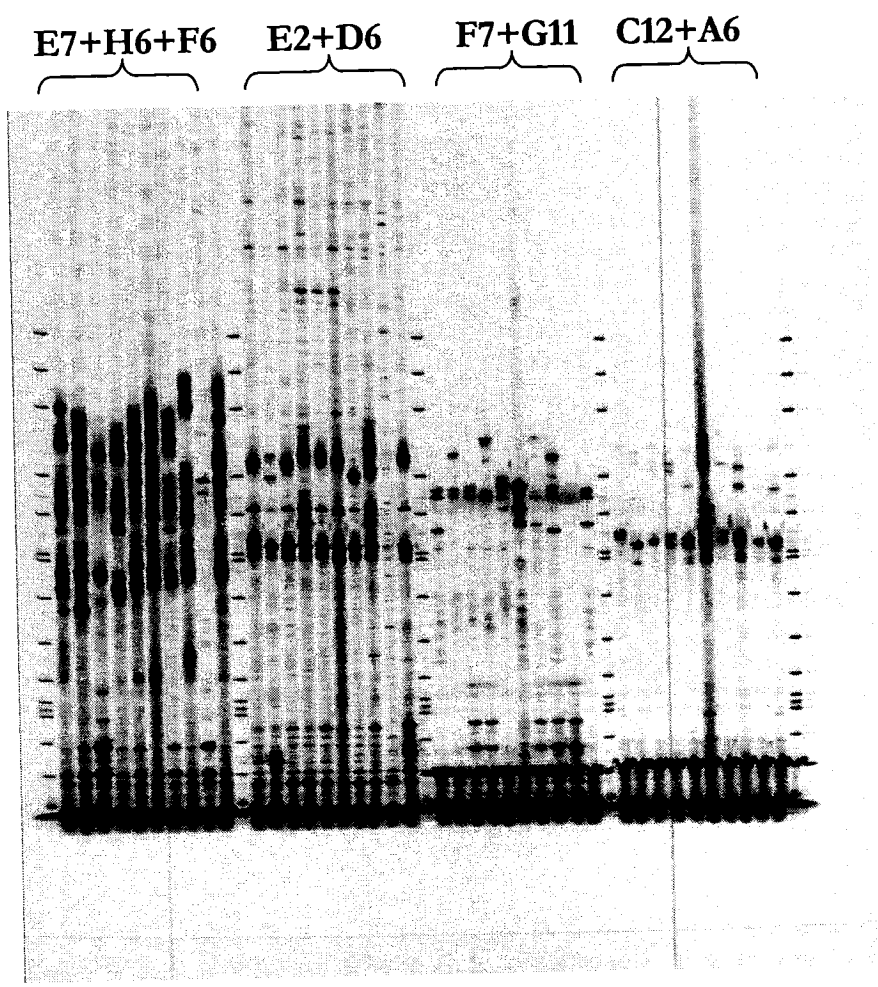
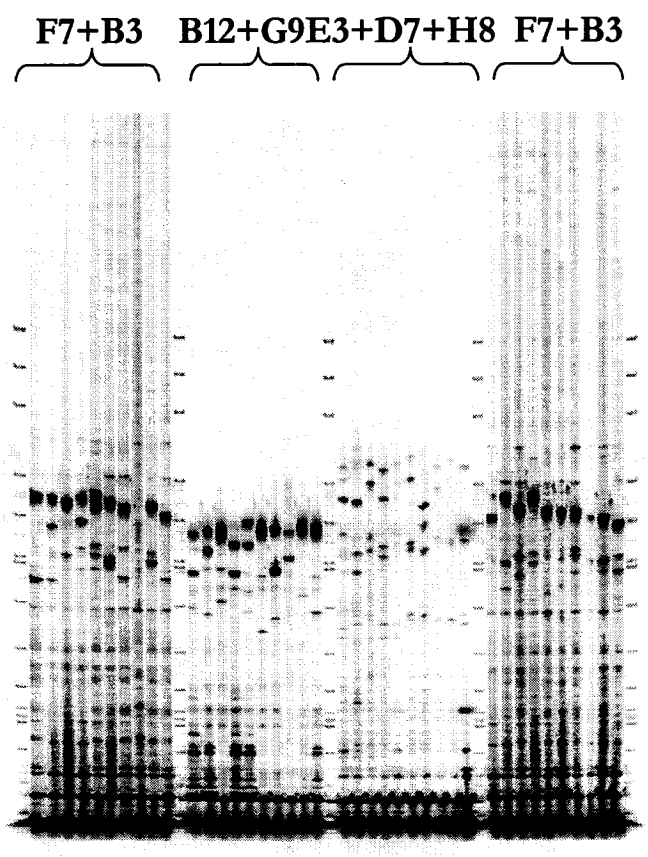

E7+H6+F6
E2+D6
F7+G11
C12+A6

Figure 6. Polyacrymide Gel Image of Microsatellite for *Myotis lucifugus*
Lane 1, 12, 23, 34, 45: Molecular weight standards (50-350 bp).
Lane 24-33: Three microsatellite loci presented per lane.
Lane 2-11, lane 13-22 and lane 35-44: Two microsatellite loci presented per lane.


F7+B3
B12+G9E3+D7+H8
F7+B3

pair size of each sample. The locus manager was used to arrange all microsatellite loci in the database so that the size range would be determined upon analysis. The individual DNA data were added to and handled by the source manager. Gel electrophoresis information was manipulated by the gel manager. Subsequently, the gel image files were automatically transferred from the DNA analyzer to the application server. Sample microsatellite loci sizes were then calibrated and their location and size range were identified through the locus information entered during project set up (LI-COR, 2004).

For some samples, multiplex PCR didn't work efficiently due to the overlapping in allele size of certain loci; these samples were therefore scored by amplifying each microsatellite locus separately.

Use of the "Structure" Software to Infer Population Structure from Microsatellite Data

The software "Structure" (Falush et al., 2003; Pritchard et al., 2000) applies a Bayesian model-based clustering method, based upon the probability of sample population frequencies and population allele frequencies, to infer numbers of source population or population structure and to assign individuals to populations by using multilocus genotype data, such as microsatellites. This program is supposed to cluster individuals which have a similar genotype to a distinct group by assuming each group (population) is characterized by a set of allele frequencies, no matter if a predefined population exists or not.

The software "Structure" calculates the probability of an observed genotype of the individual sampled in a certain population in which the allele frequencies are different from other populations. It follows the assumptions of Hardy-Weinberg and complete linkage equilibrium. Under these assumptions each allele at each locus in each genotype is an independent draw from the appropriate frequency distribution, and this completely specifies the probability distribution Pr (X|Z, P), in which X denotes the observed genotypes of the

individual sampled, Z denotes the individual population of origin, and P denotes the allele frequencies in that population.

The software "Structure" assumes a model in which there are K populations (K maybe unknown) where K can be defined by the user in advance. K is characterized by a set of allele frequencies at each locus. Individuals may belong to multiple clusters in which their ancestors have close genetic relationship. "Structure" identifies the numbers of clusters into which the sample data (X) can fit with posterior probability Pr (X|K). This software was thought to be the most suitable for analyzing microsatellite data sets for inferring bat population structure in Canada, due to unavailability of predefined bat population. This software was employed to analyze the microsatellite loci datasets generated from *Eptesicus fuscus* and *Myotis lucifugus*. Two models, admixture (each individual has inherited some fraction of his/her genome from ancestors in population K) or non-admixture (each individual comes from one of the K populations), were employed in separate analyses, combined with correlated allele frequency and 100,000 burn-in period with 10,000 iterations.

The GenAlEx6 software (Peakall and Smouse, 2005) is a population genetics research tool that runs with Microsoft Excel. It applies frequency and distance models to create varieties of population genetic methods, which include population assignment, analysis of molecular variance (AMOVA), Nei genetic distance, and tests for Hardy-Weinberg Equilibrium, etc.

The software RSTCALC 2.2 (Goodman, 1997) can be used to analyse population structure, genetic differentiation and gene flow using microsatellites. Fst (Wright's F-statistics) (Wright, 1946; Wright, 1951; Wright, 1965) is the inbreeding coefficient within subpopulation, relative to the total. Fst provides a measure of the genetic differentiation among populations. That is, the proportion of the total genetic diversity (heterozygosity) that

is distributed among the populations. Fst can range from 0 (no genetic differentiation) to 1 (fixation of alternative alleles). An Fst value (0-0.05) indicates little genetic differentiation among population, Fst (0.05-0.15) represents moderate differentiation and Fst (0.15-0.25) represents great differentiation. An Fst value (>0.25) interprets very great differentiation. Rst (Slatkin, 1995), analogous to Fst is usually applied to measure genetic differentiation based on a stepwise mutation model (SMM) used specifically for microsatellite datasets.

The 9 microsatellite loci were genotyped for all 295 *Eptesicus fuscus* DNA samples individually, while 7 microsatellite loci were genotyped for all individuals of 126 *Myotis lucifugus* DNA samples. A cluster analysis was implemented with the "Structure" program using a setting $2 \leq K \leq 5$.

The total number of alleles, observed and expected heterozygosity was calculated using GenAIEx6 software.

The Rst measuring genetic differentiation based on stepwise mutation model (SMM) was calculated by the RST CALC 2.2 software program.

Cox-1 Gene Amplification of *Myotis lucifugus* Genomic DNA

Two PCR primers, CoxFor and CoxRev (Table 2) (Ward et al., 2005) were used to amplify a product of around 650 bp containing partial gene portion of Cox-1 gene sequence. The 25 µl reaction consisted of 40 pMoles of each forward and reverse primers, 200 µM dNTPs, 1 x PCR buffer (-$MgCl_2$), 1.5 mM $MgCl_2$, 1 U Taq DNA polymerase, and 200 ng of *Myotis lucifugus* genomic DNA template. Thermal cycling for PCR reaction was performed in a GeneAmp 9700 (Applied Biosystem, Foster City, California, USA) with the cycling program: an initial denaturation at 94.0°C for 4 minutes, followed by 30 cycles of 94.0°C for 15 seconds, 50.0°C for 35 seconds, 72.0°C for 1 minute 30 seconds, a final extension at 72.0°C for 5 minutes and concluded by a hold at 4.0°C.

Purification of PCR Products of Cox-1 Gene

Promega Wizard PCR Preps DNA purification system was used for purifying the Cox-1 gene PCR products as described previously (see section "Purification of Rabies Virus P Gene PCR Products").

Cox-1 Gene Sequencing

The thermo sequenase primer cycle sequencing kit (Amersham Biosciences, Little Chalfont Buckinghamshire, England) was used for the sequencing of Cox-1 gene PCR products. An IR 700 labelled primer of CoxSeqRev (Table 2), directed to internal amplicon sequence was employed with the cycling file: an initial denaturation at 95.0°C for 3 minutes, followed by 33 cycles of 95.0°C for 35 seconds, 50.0°C for 35 seconds, and 72°C for 45 seconds, then an final extension at 72°C for 1 minute 30 seconds and a hold at 4°C.

Sequencing reactions were processed by the Li-COR 4200L automated sequencer as described previously.

Cox-1 Gene Phylogenetic Analysis

Cox-1 gene sequences of 552 nucleotides in length were aligned using CLUSTAL X. NJ algorithm of the PHYLIP 3.6 package was used to predict the genetic relationship among the 106 *Myotis lucifugus* DNA samples using human Cox-1 gene sequence as an out group. These analyses were perfomed as described in the MATERIALS AND METHODS section "Phylogenetic Analysis of Rabies Virus P Gene Sequences".

Mapping of RABV Variants and Host Populations: *Eptesicus fuscus*

ArcView 8 software (http://gislounge.com/ll/arcview8.shtml), widely used for visualizing, managing, creating, and analyzing geographic data, was employed to create maps of the data generated during this study. The Universal Transverse Mercator (UTM) grids for providing data location were converted into the coordinates of latitude and

longitude; thereby the geographic data pattern was identified by plotting the data collection on the map. Access 2000 is the database which ArcView 8 software uses to manipulate the dataset.

Sample subsets, as based on viral P gene sequences and *Eptesicus fuscus* microsatellite loci, were input and analyzed in the software ArcView 8, separately.

RESULTS

RABV P Gene Sequence Analysis

A total of 231 bat rabies viral isolates of which only 25 specimens were collected from *Myotis* species of bats, were characterized by sequencing of a 597 nucleotide region of the P gene. This sequence window corresponded to bases 112 to 717 of the RABV PV laboratory strain P gene open reading frame (ORF) (Tordo et al., 1986a). The alignment of all RABV 231 sequences did not introduce any gaps except for four *Eptesicus fuscus* isolates of BB93L02011, BB93L02244, BB95L01057, BB00L01762 and one *Myotis evotis* isolate of EV96L01358, for which a two base insertion and a two base deletion were noted at residues 214 to 215 and 219 to 220 of the PV reference strain sequence, respectively. The alignment including the PV strain introduced four base, two base and single base deletions and three base, two base and 2 single base insertions at PV nucleotide residues 187 to 190, 230 to 231, 425, and 196 to 198, 222 to 223, 202, 419, respectively. The inclusion of all insertions and deletions, resulting in 9 gaps, generated a 606 nucleotide alignment. The intragenotypic nucleotide similarity within a 597 base sequence length for this 231 P gene dataset is 69.0%. Referring to the alignment at the PV reference sequence, the most divergent portion of the sequence window is at base positions between 161 and 214 with an overall base similarity of 50.0%, another divergent sequence is at base positions 418 to 519 with an intragenotypic base similarity of 53.0%.

Phylogenetic Analysis of Rabies Virus P Gene Sequences

For easier identification of geographic location, the area covering provinces of British Columbia, Alberta, Saskatchewan, and Manitoba was termed as West, or western area. The area belonging to Ontario, Quebec and New Brunswick, Nova Scotia, Newfoundland was referred to as East, or eastern area.

The Neighbour-Joining (NJ) algorithm was employed for phylogenetic analysis of these 231 partial P gene sequences. The resulting tree (Figure 7 and Appendix 3) depicts eight groups, divided according to rabies virus host species and host geographic localization. The samples of clades I-VI and clade VIII comprised primarily *Eptesicus fuscus* specimens as well as some *Myotis* isolates. The seventh clade consisted exclusively of *Myotis* associated specimens.

The first clade (group I) consisted of 98 isolates recovered in years 2001, 2003 and 2004, of which 93 *Eptesicus fuscus* isolates originated from the province of Ontario, 2 *Eptesicus fuscus* isolates from Quebec province, and 3 isolates recovered from *Myotis lucifugus* bats in Ontario. Clade two (group II) comprised of 11 *Eptesicus fuscus* isolates collected from Quebec and 8 *Eptesicus fuscus* isolates from Ontario, recovered in the same years as group I. Six and 13 viral isolates comprised clades III (group III) and IV (group IV) respectively; they were all *Eptesicus fuscus* isolates from the province of British Columbia, except one isolate in clade III that was recovered from a *Myotis yumanensis* specimen YU95L01017 in British Columbia. Specimens in clade III were recovered in 1995, 1999, 2000, 2003 and 2004, while clade IV specimens were recovered in 1995, 1996, 1998, 2000, and 2002 to 2004. The fifth clade (group V) included 22 samples isolated from *Eptesicus fuscus* bats of western provinces; 21 were from British Columbia and 1 was from Saskatchewan. Clade V also included two *Myotis* samples from British Columbia, *Myotis yumanensis* YU00L01855 and *Myotis volans* VO03L00692. The samples of clade V were recovered in the years 1989, 1993 to 1996, 1998 to 2000, and 2002 to 2004. The sixth clade (group VI) was composed of viral isolates of *Eptesicus fuscus* distributed broadly among western and eastern areas; 5 originated from the weste coast, 12 were from Alberta province, 17 were collected in the province of Saskatchewan, 15 were recovered from Ontario, and two

Figure 7. Phylogenetic Neighbor Joining tree structure of 231 rabies viral isolates from *Eptesicus fuscus* and *Myotis* bat species. The detailed tree information is depicted in Appendix 3.


77.5
35.2
46.9
99.9
54.3
51.8
94.8
100
54.4
100
100
75.9
49.9
70.0
100
43.2
98.2
50.4
I
VI
III
IV
II
V
VIII
VII
100

Myotis specimens grouped in this clade were from two species, *Myotis septentrionalis* SE02L00687 and *Myotis yumanensis* YU93L02414, recovered in British Columbia. The samples were recovered in this clade over a long period, from 1989 to 2004. Within this clade, the western samples clustered together, while the eastern specimens were separated to two small outlying groups.

Clade seven (group VII) was a very distinct grouping comprising of 16 specimens isolated from *Myotis* species, including 3 *Myotis lucifugus* from British Columbia, 1 *Myotis lucifugus* from Ontario, 1 *Myotis lucifugus* from Quebec, and another one from New Brunswick, recovered in years of 1996, 1999, 2001 and 2003. Additional viral specimens in this clade were recovered from other species of *Myotis* bats, including 5 from *Myotis evotis*, 3 from *Myotis septentrionalis*, and 1 from *Myotis keenii*, all recovered from British Columbia between the years 1994 to 2004.

Clade VIII (group VIII), composed 4 isolates circulating in *Eptesicus fuscus* recovered in years of 1993, 1995 and 2000 - two from British Columbia and two from Alberta respectively, and one from *Myotis evotis* of British Columbia recovered in 1996.

The bootstrap value for clade I at 77.5% is considered significant, while clades II, III IV and VIII were all strongly supported with bootstrap values of 100%. The bootstrap value for clade V was 75.9%, but clade VI had a low bootstrap value of 51.8%. The bootstrap value of the clade comprising of clade I and clade VI was 99.9%, while the clade comprising of clade I, III and VI had quite a low bootstrap value (54.3%). Clade VII, with a very high bootstrap value of 98.2%, was well supported, with limited support for any further subdivision.

The genetic distance within each clade I, III, IV, VI was less than 0.0084, whilst the genetic distance within clade II, clade VIII, clade VII and clade V was less than 0.0169,

0.0640, 0.0641 and 0.0682 respectively. Considering the entire tree, clades I and VI had the closest genetic distance (less than 0.0169). Next were clades III and VI (less than 0.0520), clades III and IV (less than 0.0552), clades II and IV (less than 0.0976), clades II and VII (less than 0.1164), clades V and VII (less than 0.1187), and clades V and clade VIII (less than 0.1464) sequentially. Clade VIII had the farthest genetic distance compared to all other clades.

A Maximum Parsimony (MP) tree (Appendix 4) and its tree structure (Figure 8), analysed with a single data set was also generated using 231 P gene sequences. Unfortunately, use of bootstrapped data with this method overwhelmed computer capacity. Compared to the NJ tree, the MP tree had a slightly different structure, while clades I to V, VII and VIII were predicted similar to the NJ analysis. Clade VI split into two separate groups (Clade VIa and Clade VIb) outlying clade I in the MP tree. This subdivision was random with respect to the original samples from western and eastern regions of the country.

Comparison of Selected Samples and Reference Samples of Viral P Gene

Thirty seven representative samples (Appendix 1) selected from each of the 8 clades identified by the NJ tree were compared with 19 reference bat rabies virus isolates characterized previously at N, G and P gene sequences (Nadin-Davis et al., 2001; Nadin-Davis et al., 2002). This comparison was performed to examine whether the relatively large sampling of *Eptesicus fuscus* and *Myotis* bat rabies used in this study had uncovered variants not previously identified and to correlate the phylogenetic clades identified here to those described before (Nadin-Davis et al., 2001).

The reference and selected specimens representing the eight genetic groups of bat rabies virus identified were compared by generation of a NJ tree (Figure 9) in this study. The reference samples of EF33, EF55, EF71 and EF72 were identified in clade II. EF23 and

Figure 8. Phylogenetic Maximum Parsmony tree structure of 231 rabies viral isolates from *Eptesicus fuscus* and *Myotis* bat species (see Appendix 1) and the detailed tree information is presented in Appendix 4.

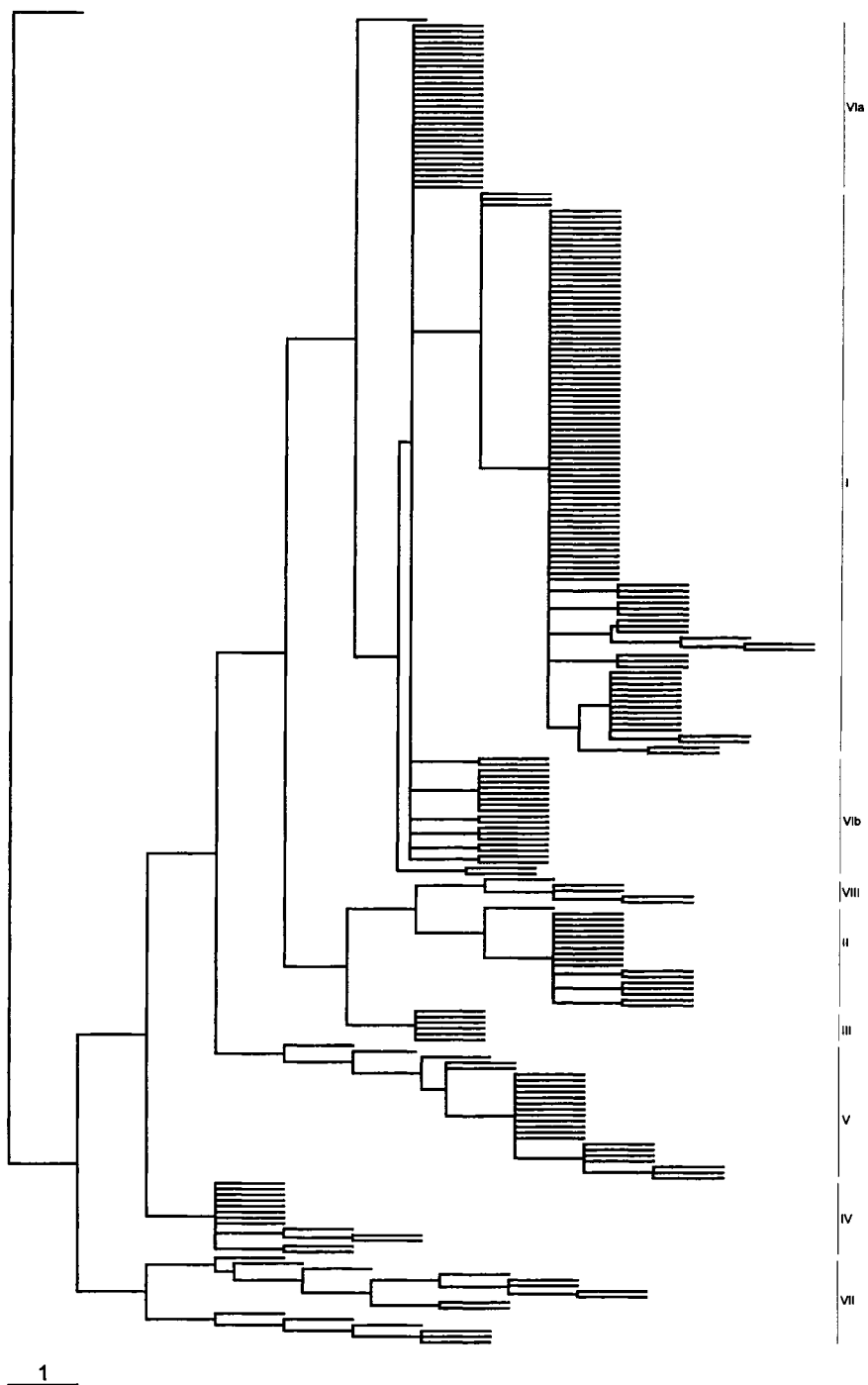
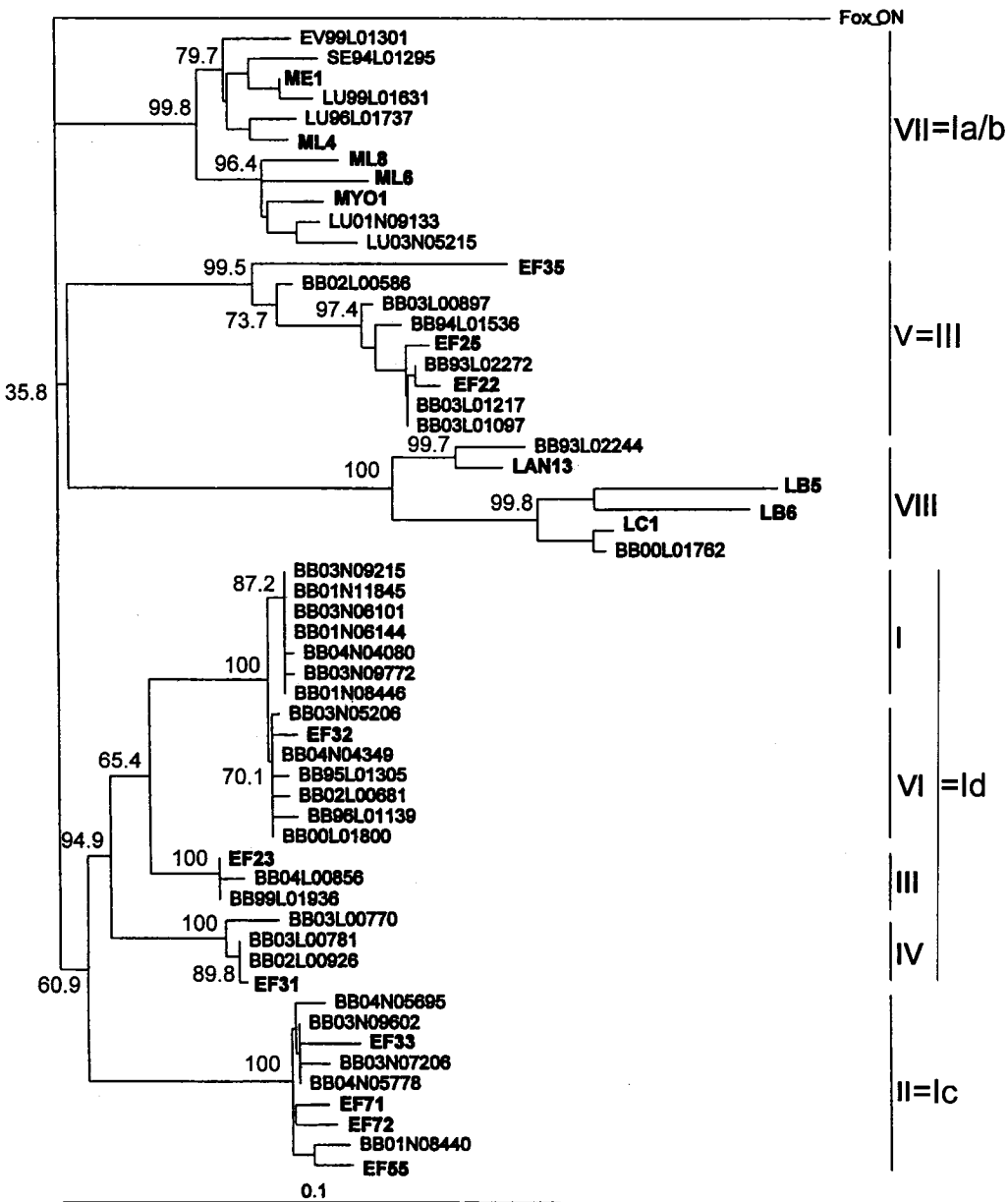

VIa
I
VIb
VIII
II
III
V
IV
VII
1

Figure 9. Phylogenetic tree of 37 selected rabies viral isolates from bats of *Eptesicus fuscus* and *Myotis* species (see Appendix 1) and 19 reference bat viruses characterized previously (see Table 3), analysed by a NJ algorithm applied to a 597 bp P gene sequence window. Numbers to the left of a branch indicated the number of times out of 1000 bootstrap replicates that the branch was supported. Numbers on the right-hand side of the figure indicated the main phylogenetic clades according to the grouping determined from the NJ tree (Appendix 3) followed by the corresponding group designations as defined previously by Nadin-Davis *et al.*(2001). Ontario fox rabies virus P gene sequence (Fox_ON) was the outgroup. Distance data were reapplied to this consensus tree using the FITCH program of PHYLIP v3.63; a genetic distance scale is shown below the tree

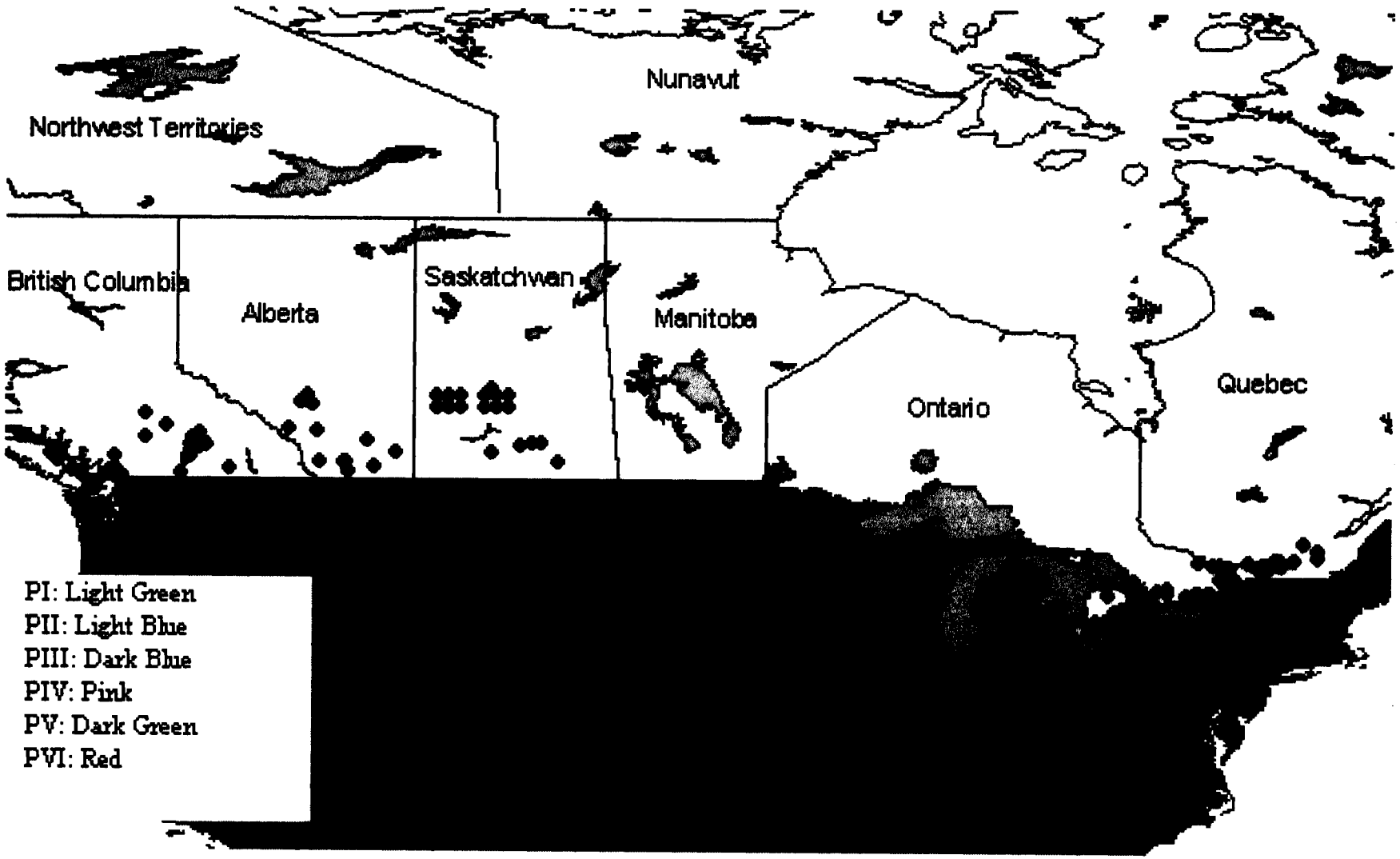

Fox_ON
EV99L01301
SE94L01295
ME1
LU99L01631
LU96L01737
ML4
ML8
ML6
MYO1
LU01N09133
LU03N05215
EF35
BB02L00586
BB03L00897
BB94L01536
EF25
BB93L02272
EF22
BB03L01217
BB03L01097
BB93L02244
LAN13
LB5
LB6
LC1
BB00L01762
BB03N09215
BB01N11845
BB03N06101
BB01N06144
BB04N04080
BB03N09772
BB01N08446
BB03N05206
EF32
BB04N04349
BB95L01305
BB02L00681
BB96L01139
BB00L01800
EF23
BB04L00856
BB99L01936
BB03L00770
BB03L00781
BB02L00926
EF31
BB04N05695
BB03N09602
EF33
BB03N07206
BB04N05778
EF71
EF72
BB01N08440
EF55
0.1
99.8
79.7
96.4
99.5
73.7
97.4
35.8
99.7
100
99.8
87.2
100
65.4
70.1
94.9
100
100
89.8
60.9
100
VII=Ia/b
V=III
VIII
I
VI
=Id
III
IV
II=Ic

EF31 were collected to clade III and clade IV respectively. EF22, EF25, EF35 were clustered in clade V. EF32 was organized to clade VI. ME1, ML4, ML6, ML8 and MYO1 were grouped in clade VII. LAN13, LB5, LB6 and LC1 were grouped into clade VIII representing *Lasiurus* and *Lasionycteris* viral variants introduced to four *Eptesicus fuscus* and one *Myotis evotis* by spill-over transmission. Thus clade VIII was identified as not being maintained by either of the two species under investigation in this study and isolates of this clade were removed from further analysis.

Such phylogenetic analysis confirmed that no new viral variants, associated with *Eptesicus fuscus* and *Myotis* species in Canada, were identified in this study, although no reference P gene sequences corresponded to the clade I viral variant. The specimens used for N gene characterization probably covered this type of viral variant (personal communication with Dr. Nadin-Davis). Moreover, it is evident in these phylogenetic trees that clades I and VI were very closely related.

When the viral specimens (from *Eptesicus fuscus* only) were plotted on a map according to their predicted phylogenetic groupings (I-VI) (Figure 10), the distribution of these specimens displayed very distinct geographic trends. The clade I specimens were distributed throughout the province of Ontario, including two specimens from Quebec located on the border of Ontario and Quebec provinces. Clade II specimens were distributed across Quebec and Ontario. Samples of clades III and IV were recovered from the province of British Columbia only. The samples of clade V were recovered primarily from the British Columbia, except for specimen of BB89L02317 from Saskatchewan. The specimens of clade VI were widely distributed across the country except in the province of Quebec.

Figure 10. Map of Location of *Eptesicus fuscus* Isolates of Rabies Virus
Six types of rabies viral variants circulating in *Eptesicus fuscus* bat species were identified using P gene phylogeny. More than one sample may be represented by each square symbol.


Nunavut
Northwest Territories
British Columbia
Alberta
Saskatchewan
Manitoba
Ontario
Quebec
PI: Light Green
PII: Light Blue
PIII: Dark Blue
PIV: Pink
PV: Dark Green
PVI: Red

Partial Phosphoprotein Sequence Prediction and Analysis

The primary sequences of 57 partial rabies virus P protein deduced from the partial P gene nucleotide sequences encoded a 199 amino acid sequence of P protein from residue 38 to 236 in length compared to the PV reference strain, no gaps being introduced (see Figure 11). Intragenotypic identity of these proteins was 67.84%. There were two highly variable domains VD1 between positions 61 and 80, and VD2 between positions of 134 and 180 with intragenotypic identity of 30.00% and 51.06% respectively. The partial protein sequences of isolates clustering within the same clade were very similar. The intragenotypic identity of clade I and clade IV was 99.50%; clade II was 98.99%; clade III and clade VI was 98.49%; clade V was 94.97%, and clade VII had the lowest percentage, 85.93%.

The high proportion of both acidic and serine residues could be identified in these partial P protein sequences. A total of twenty-one of conserved aspartic and glutamic acid residues, and 18 conserved serine and threonine residues were identified throughout 199 amino acid sequences.

The partial P protein sequences only covered three of four internal Met codons 53, 69, and 83 which the rabies challenge virus standard (CVS) strain uses to direct the synthesis of four N-terminally truncated P proteins by controlling their translational initiation (Chenik et al., 1995). Met_{53} was conserved in 57 selected samples at all clades. Met_{69} was much conserved in Clades I, III, IV and V. Met_{69} in three samples of *Eptesicus fuscus* BB04N05695, BB03N04543 and BB01N08905 of Clade II were all mutated to Isoleucine (I), while two specimens (*Myotis septentrionalis* SE04L01384 and *Myotis lucifugus* LU96L01737) in clade VII had a mutation from Methionine (M) to Threonine (T) at position 69. Met_{83} was conserved at each and every clade except for clade V. Met_{83} in nine out of ten specimens at clade V was mutated to Isoleucine (I).

Figure 11. Prediction and Comparison of Selected Phosphoprotein Sequences
Fifty seven selected rabies virus partial phosphoprotein sequences from amino acid residues 38 to 236 were compared and predicted (see Appendix 1 for details on selected isolates). The difference from the PV reference strain was indicated. "." indicated identity at that position; "*" represented conserved acidic residues of Glutamic Acid (Glu or E) and Aspartic Acid (Asp or D); "#" indicated the positions of conserved ser/thr residues; "$" marked the positions of 3 of 4 methionines for internal translation initiation in the CVS strain; "&" emphasized the binding domain of phosphoprotein and dynein light chain

```
                  *  * *    **$          #     $    *     **  $ * *
PV                HLQGEPIEVD NLPEDMGRLH LDDGKSPNPG EMAKVGEGKY REDFQMDEGE
-------------------------------------------------------------------------
BB89L01903  .......... S.....K... .E.E.PSGL. ....S..S.C Q.........
BB95L00952  .......... S.....K... .E.E.PSGL. ....S..S.C Q.........
BB94L00580  .......... S.....K... .E.E.PSGL. ....S..S.C Q.........
BB99L01070  .......... S.....K... .E.E.PSGL. ....S..S.C Q.........
BB92L01816  .......... S.....K... .E.E.PSGL. ....S..S.C Q.........
BB97L01771  .......... S.....K... .E.E.PSGL. ....S..S.C Q.........
BB96L01181  .......... S.....K... .E.E.PSGL. ....S..S.C Q.........  VI
BB00L01869  .......... S.....K... .E.E.PSGL. ....S..S.C Q.........
BB93L02139  .......... S.....K... .E.E.PSGL. ....S..S.C Q.........
BB02L00681  .......... S.....K... .E.E.PSGL. ....S..S.C Q.........
BB98L01773  .......... S.....K... .E.E.PSGL. ....S..S.S Q.........
BB01N08639  .......... S.....K... .E.E.PSGL. ....S..S.C Q.........
BB04N04417  .......... S.....K... .E.E.PSGL. ....S..S.C Q.........
BB03N05206  .......... S.....K... .E.E.PSGL. ....S..S.C Q.........
-------------------------------------------------------------------------
BB03N09215  .......... S.....K... .E.E.PSGL. ....S..S.C Q.........
BB01N10259  .......... S.....K... .E.E.PSGL. ....S..S.C Q.........  I
BB04N05590  .......... S.....K... .E.E.PSGL. ....S..S.C H.........
-------------------------------------------------------------------------
BB00L00835  .......... S.....R... ...A.PSGL. ....A..S.S H.........
BB04L00856  .......... S.....R... ...A.PSGL. ....A..S.S H.........
BB03L01081  .......... S.....R..Q ...A.PSGL. ....A..S.S H.........  III
BB95L01007  .......... S.....R... ...A.PSGL. ....A..S.S H.........
BB99L01936  .......... S.....R... ...A.PSGL. ....A..S.S H.........
-------------------------------------------------------------------------
BB96L01752  .......... S.....R... ...S.PSGL. ....A..S.C Q.........
BB98L01172  .......... S.....R... ...S.PSGL. ....A..S.C Q.........
BB02L00827  .......... S.....R... ...S.PSGL. ....A..S.C Q.........  IV
BB95L01382  .......... S.....R... ...S.PSGL. ....A..S.C Q.........
BB00L01756  .......... S.....R... ...S.PSGL. ....A..S.C Q.........
BB03L00770  .......... S.....R... ...S.PSGL. ....A..S.C Q.........
-------------------------------------------------------------------------
BB04N05695  .......... S.....R... ...E.PSGL. VI..A..S.C Q.........
BB03N04543  .......... S.....R... ...E.PSGL. VI..A..S.C Q.........  II
BB01N08905  .......... S.....R... ...E.PSGL. VI..A..S.C Q.........
-------------------------------------------------------------------------
BB96L01557  ....D..... ......R... ...E.PSG.D G......S.C QD...IG.A.
BB89L02317  ....D..... ......R... ...E.PSG.D G......S.C QD...IG.A.
BB99L01771  ....D..... ......R... ...E.PSG.D G......S.C QD...IG.A.
BB98L01766  ....D..... ......R... ...E.PSG.D G......S.C QD...IG.A.
BB04L00745  ....D..... ......R... ...E.PSG.D G......S.C QD...IG.A.
BB95L00920  ....D..... ......R... ...E.PSG.D G......S.C QD...IG.A.  V
BB99L01395  ....D..... ......R... ...E.PSG.D G......S.C QD...IG.A.
BB03L00506  ....D..... ......R... ...E.PSG.D G......S.C QD...IG.A.
BB02L00586  ....D..... ......R... ...E.PSGL. G...A..S.C QD....G...
BB94L01536  ....D..... ......R..Q .N.E.PSGPD G......S.C QD...IG.A.
-------------------------------------------------------------------------
EV02L01055  .......... S.....R... ..NE.PSDL. ....A..S.C Q.........
EV03L01088  .......... S.....R... ..NE.PSDL. ....A..S.C Q.........
LU99L01631  .......... S.....R... ..NE.PSDL. ....A..S.C Q.........
LU99L00631  .......... S.....R... ..NE.PSDL. ....A..S.C Q.........
SE00L01396  .......... S.....R... ..NE.PSDL. ....A..S.C Q.........
SE94L01295  .......... S.....R... ..NE.PSGL. ....A..S.C Q.........
EV03L00798  .......... S.....R... ..NE.PSGL. ....A..S.C Q.........
KE03L00671  .......... S.....R... ...ERTSGL. ....A..N.C Q.........  VII
SE04L01384  .......... S.....R... ...A.PSG.. .T..G..S.S Q.........
LU03N05215  .......... S.....R... ...A.PSG.. ....G..SRC Q.........
LU03N08135  .......... S.....R... ...A.PSG.. ....G..S.C Q.........
LU01N09133  .......... S.....R... ...A.PSG.. ....G..S.C Q.........
EV04L01219  .......... S.....R... ...A.PSG.. ....A..S.C Q.........
CI02L00604  .......... S.....R... ..EA.PSGL. ....A..S.C Q.........
EV99L01301  .......... S.....R... ...E.PSGL. ....A..S.C Q.........
LU96L01737  .......... S..D..R... ...E.PSGL. .T..A..S.C Q.........
-------------------------------------------------------------------------
```

```
                88
                 *          *            # *       # # **  #
PV               DPSLLFQSYL DNVGVQIVRQ IRSGERFLKI WSQTVEEIIS YVAVNFPNPP
-------------------------------------------------------------------------
BB89L01903       ..A.......  .....HM...  M.........  ..H.......  ..M....SL.
BB95L00952       ..A.......  .....HM...  M.........  ..H.......  ..M....SL.
BB94L00580       ..A.......  .....HM...  M.........  ..H.......  ..M....SL.
BB99L01070       ..A.......  .....HM...  M.........  ..H.......  ..M....SL.
BB92L01816       ..A.......  .....HM...  M.........  ..H.......  ..M....SL.
BB97L01771       ..A.......  .....HM...  M.........  ..H.......  ..M....SL.
BB96L01181       ..A.......  .....HM...  M.........  ..H.......  ..M....SL.
BB00L01869       ..A.......  .....HM...  M.........  ..H.......  ..M....SL.
BB93L02139       ..A.......  .....HM...  M.........  ..H.......  ..M....SL.
BB02L00681       ..A.......  .....HM...  M.........  ..H.......  ..M....SL.
BB98L01773       ..A.......  .....HM...  M.........  ..H.......  ..M....SL.
BB01N08639       ..A.......  ......M...  M.........  ..H.......  ..M....SL.
BB04N04417       ..A.......  ......M...  M.........  ..H.......  ..M....SL.
BB03N05206       ..A.......  ......M...  M.........  ..H.......  ..M....SL.
-------------------------------------------------------------------------
BB03N09215       ..A.......  ......M...  M.........  ..H.......  ..M....SL.
BB01N10259       ..A.......  ......M...  M.........  ..H.......  ..M....SL.
BB04N05590       ..A.......  ......M...  M.........  ..H.......  ..M....SL.
-------------------------------------------------------------------------
BB00L00835       ..A.......  ......M...  M.........  ..H.......  ..M....SL.
BB04L00856       ..A.......  ......M...  M.........  ..H.......  ..M....SL.
BB03L01081       ..A.......  ......M...  M.........  ..H.......  ..M....SL.
BB95L01007       ..A.......  ......M...  M.........  ..H.......  ..M....SL.
BB99L01936       ..A.......  ......M...  M.........  ..H.......  ..M....SL.
-------------------------------------------------------------------------
BB96L01752       ..A.......  ......M...  M.........  ..H.......  ..M....SL.
BB98L01172       ..A.......  ......M...  M.........  ..H.......  ..M....SL.
BB02L00827       ..A.......  ......M...  M.........  ..H.......  ..M....SL.
BB95L01382       ..A.......  ......M...  M.........  ..H.......  ..M....SL.
BB00L01756       ..A.......  ......M...  M.........  ..H.......  ..M....SL.
BB03L00770       ..A.......  ......M...  M.........  ..H.......  ..M....SL.
-------------------------------------------------------------------------
BB04N05695       ..A.......  ..I...M...  M.........  ..........  ..S....SL.
BB03N04543       ..A.......  ..I...M...  M.........  ..........  ..S....SL.
BB01N08905       ..A.......  ..I...M...  M.........  ..........  ..T....SL.
-------------------------------------------------------------------------
BB96L01557       ..A.......  ....I.M...  M.........  ..........  ..MI...SL.
BB89L02317       ..A.......  ....I.M...  M.....L...  ..........  ..MI...SL.
BB99L01771       ..A.......  ....I.M...  M.........  ..........  ..M....SL.
BB98L01766       ..A.......  ....I.M...  M.........  ..........  ..M....SL.
BB04L00745       ..A.......  ....I.M...  M.........  ..........  ..M....SL.
BB95L00920       ..A.......  ....I.M...  M.........  ..........  ..M....SL.
BB99L01395       ..A.......  ....I.M...  M.........  ..........  ..M....SL.
BB03L00506       ..A.......  ....I.M...  M.........  ..........  ..M....SL.
BB02L00586       ..A.......  ....I.M...  M.........  ..........  ..M....SL.
BB94L01536       ..A.......  ....I.M...  M.........  ..........  ..M....SL.
-------------------------------------------------------------------------
EV02L01055       ..IF......  ......V...  M.........  ..........  ..T....DLS
EV03L01088       ..IF......  ......V...  M.........  ..........  ..T....DLS
LU99L01631       ..IF......  ......V...  M.........  ..........  ..T....DLS
LU99L00631       ..IF......  ......V...  M.........  ..........  ..T....DLS
SE00L01396       ..VF......  ......V...  M.........  ..........  ..T....DLS
SE94L01295       ..VF......  ..........  M.........  ..........  ..T....DLS
EV03L00798       ..VF......  ..........  M.........  ..........  ..T....DLS
KE03L00671       ..VF......  ..........  M.........  ..........  ..T....DLS
SE04L01384       ..LF......  ..........  M.........  ..........  ..T....DLS
LU03N05215       ..LF......  ..........  M.........  ..........  ..T....DLS
LU03N08135       ..VF......  ..........  M.........  ..........  ..T....DLS
LU01N09133       ..VF......  ..........  M.........  ..........  ..T....DLS
EV04L01219       ..VF......  ..........  M.........  ..........  ..T....DLS
CI02L00604       ..VF......  ..........  M.........  ..........  ..S....DLS
EV99L01301       ..VF......  ..........  M.........  ..........  ..T....DLS
LU96L01737       ..VF...L..  ......M...  M.........  ..........  ..T....GLL
-------------------------------------------------------------------------
```

```
          138 &&&&&&&&&&
              #**  #  #   *       #  #  *    #       #  #     * #
PV            GKSSEDKSTQ TTGRELKKET TPTPSQRESQ SSKARMAAQT ASGPPALEWS
--------------------------------------------------------------------|
BB89L01903    R......A.. .VN.....G. VSVS...... L...K.V... ..........|
BB95L00952    R......A.. .VN.....G. VSVS...... L...K.V... ..........|
BB94L00580    R......A.. .VN.....G. VSVS...... L...K.V... ..........|
BB99L01070    R......A.. .VN.....G. VSVS...... L...K.V... ..........|
BB92L01816    R......A.. .VN.....G. VSVS...... L...K.V... ..........|
BB97L01771    R......A.. .VN.....G. VSVS...... L...K.V... ..........|
BB96L01181    R......A.. .VN.....G. VSVS...... L...K.V... ..........|
BB00L01869    R......A.. .VN.....G. VSVS...... L...K.V... ..........|
BB93L02139    R......A.. .VN.....G. VSVS...... L...K.V... ..........|
BB02L00681    R......A.. .VD.....G. VSVS...... L...K.V... ..........|
BB98L01773    R......A.. .VN.....G. VSVS...... L...K.V... ..........|
BB01N08639    R......A.. .VN.....G. VSVS...... L...K.V... ..........|
BB04N04417    R......A.. .VN.....G. VSVS...... L...K.V... ..........|
BB03N05206    R......A.. .VN.....G. VSVS...... L...K.V... ..........|
--------------------------------------------------------------------|
BB03N09215    R......A.. .VN.....G. VSVS...... L...KIV... ..........|
BB01N10259    R......A.. .VN.....G. VSVS...... L...KIV... ..........|
BB04N05590    R......A.. .VN.....G. VSVS...... L...KIV... ..........|
--------------------------------------------------------------------|
BB00L00835    R......A.. .VN.....G. VSVS...... ....KIM... ..........|
BB04L00856    R......A.. .VN.....G. VSVS...... ....KIM... ..........|
BB03L01081    RM.....A.. .VN.....G. VSVS...... ....KIM... ..........|
BB95L01007    R......A.. .VN.....G. VSVS...... ....KIM... ..........|
BB99L01936    R......A.. .VN.....G. VSVS...... ....KIM... ..........|
--------------------------------------------------------------------|
BB96L01752    R......A.. .VN..... VSVS...... ....KI.... ..........|
BB98L01172    R......A.. .VN..... VSVS...... ....KI.... ..........|
BB02L00827    R......A.. .VN..... VSVS...... ....KI.... ..........|
BB95L01382    R......A.. .VN..... VSVS...... ....KI.... ..........|
BB00L01756    R......A.. .VN..... VSVS...... ....KI.... ..........|
BB03L00770    R......A.. .VS..... VSVS...... ....KI.... ..........|
--------------------------------------------------------------------|
BB04N05695    R.A....A.. .ANQ....K. MSVS.....K ....K..... ..........|
BB03N04543    R.A....A.. .ANQ....K. MSVS.....K ....K..... ..........|
BB01N08905    R.A....A.. .ANQ...NK. MSVS.....K ....K..... ..........|
--------------------------------------------------------------------|
BB96L01557    R.V....A.. .AS..... VSAS...... ....K..... ....S.....|
BB89L02317    R.V....A.. .AS..... VSAS...... ....K..... ....S.....|
BB99L01771    R.V....A.. .AS..... VSAS...... ....K..... ....S.....|
BB98L01766    R.V....A.. .AS..... VSAS...... ....K..... ....S.....|
BB04L00745    R.V....A.. .AS..... VSAS...... ....K..... ....S.....|
BB95L00920    R.V....A.. .AS..... VSAS...... ....K..... ....S.....|
BB99L01395    R.V....A.. .AS..... VSAS...... ....K..... ....S.....|
BB03L00506    R.V....A.. .AN..... VSAS...... ....K..... ....S.....|
BB02L00586    R.A....A.. .AS..... VSAS...... ....K..... ....S.....|
BB94L01536    R.V....A.. .AN..... VSAS...... ....K..... ....S.....|
--------------------------------------------------------------------|
EV02L01055    .......A.. .AN...R... VSA..... ...TK..... ..........|
EV03L01088    .......A.. .AN..... VSA..... ...TK..... ..........|
LU99L01631    .......A.. .AN..... VSA..... ...TK..... ..........|
LU99L00631    .......A.. .AN..... VSA..... ...TK..... ..........|
SE00L01396    .......A.. .AN..... VSA..... ...TK..... ..........|
SE94L01295    .......A.. .AD..... ASAS..... ...TK..... ..........|
EV03L00798    .......A.. .AN..... VSAS..... ...TK..... ..........|
KE03L00671    .......A.. .AN..F...I VSAS..... ...TK..... ..........|
SE04L01384    .......A.. .AN..... VSA..... ...TK..... ..........|
LU03N05215    .......A.. .AN..... ASA..... ...TK..... ..........|
LU03N08135    .......A.. .AN..... VSA..H.... ...TK..... ..........|
LU01N09133    .......A.. .AN..... VSA..... ...TK..... ..........|
EV04L01219    .......A.. .AN..... VSAS..... ...TKT.... ..........|
CI02L00604    .......A.. .AN..... VSAS..... ...TK..... .......D..|
EV99L01301    .......A.. .AN.....DA VSAS..... ...TK..... ..........|
LU96L01737    .......A.. .AN..... VSAS....N. ...TK..... ..........|
--------------------------------------------------------------------|
```

```
          188                                                                 236
              ***  #  * *      * # #      #  ##        *      **
PV          ATNEEDDLSV EAEIAHQIAE SFSKKYKFPS RSSGILLYNF EQLKMNLDD
-----------------------------------------------------------------------------|
BB89L01903  .......... .......... .......... .....F.... .........|
BB95L00952  .......... .......... .......... .....F.... ........E|
BB94L00580  .......... .......... .......... .....F.... .........|
BB99L01070  .......... .......... .......... .....F.... .........|
BB92L01816  .......... .......... .......... .....F.... .........|
BB97L01771  .......... .......... .......... .....F.... .........|
BB96L01181  .......... .......... .......... .....F.... .........|
BB00L01869  .......... .......... .......... .....F.... .........|
BB93L02139  .......... .......... .......... .....F.... .........|
BB02L00681  .......... .......... .......... .....F.... .........|
BB98L01773  .......... .......... .......... .....F.... .........|
-----------------------------------------------------------------------------|
BB01N08639  .......... .......... .......... .....F.... .........|
BB04N04417  .......... .......... .......... .....F.... .........|
BB03N05206  .......... .......... .......... .....F.... .........|
BB03N09215  .......... .......... .......... .....F.... .........|
BB01N10259  .......... .......... .......... .....F.... .........|
BB04N05590  .......... .......... .......... .....F.... .........|
-----------------------------------------------------------------------------|
BB00L00835  .......... .......... .......... .....F.... .........|
BB04L00856  .......... .......... .......... .....F.... ......V..|
BB03L03108  .......... .......... .......... .....F.... .........|
BB95L01007  .......... .......... .......... .....F.... .........|
BB99L01936  .......... .......... .......... .....F.... .........|
-----------------------------------------------------------------------------|
BB96L01752  .......... .......... .......... .....F.... .........|
BB98L01172  .......... .......... .......... .....F.... .........|
BB02L00827  .......... .......... .......... .....F.... .........|
BB95L01382  .......... .......... .......... .....F.... .........|
BB00L01756  .......... .......... .......... .....F.... .........|
BB03L00770  .......... .......... .......... .....F.... .........|
-----------------------------------------------------------------------------|
BB04N05695  .......... .......... .......... .....F.... .........|
BB03N04543  .......... .......... .......... .....F.... .........|
BB01N08905  .......... .......... .......... .....F.... .........|
-----------------------------------------------------------------------------|
BB96L01557  .A.....P.. .......... .......... .....F.... .........|
BB89L02317  .A.....P.. .......... .......... .....F.... .........|
BB99L01771  .A.....P.. .......... .......... .....F.... .........|
BB98L01766  .A.....P.. .......... .......... .....F.... .........|
BB04L00745  .A.....P.. .......... .......... .....F.... .........|
BB95L00920  .A.....P.. .......... .......... .....F.... .........|
BB99L01395  .A.....P.. .......... .......... .....F.... .........|
BB03L00506  .A.....P.. .......... .......... .....F.... .........|
BB02L00586  .A.....P.. .......... .......... .....F.... .........|
BB94L01536  .A.....P.. .......... .......... .....F.... .........|
-----------------------------------------------------------------------------|
EV02L01055  .A........ .......... .......... .....F.... .........|
EV03L01088  .A........ .......... .......... .....F.... .........|
LU99L01631  .A........ .......... .......... .....F.... .........|
LU99L00631  .A........ .......... .......... .....F.... .........|
SE00L01396  .A........ .......... .......... .....F.... .........|
SE94L01295  .A........ .......... .......... .....F.... .........|
EV03L00798  .A........ .......... .......... .....F.... .........|
KE03L00671  .A....N... .......... .......... .....F.... .........|
SE04L01384  .A........ .......... .......... .....F.... .........|
LU03N05215  .......... .......... .......... .....F.... .........|
LU03N08135  .......... .......... .......... .....F.... .........|
LU01N09133  .......... .......... .......... .....F.... .........|
EV04L01219  .A........ .......... .......... .....F.... .........|
CI02L00604  .......... .......... .......... .....F.... .........|
EV99L01301  .......... .......... .......... .....F.... .........|
LU96L01737  .A........ .......... .......... .....F.... .........|
-----------------------------------------------------------------------------|
```

The recently reported binding site for the host dynein light chain is located at P protein residues 139 to 151 (N'-KSSEDKSTQTTGR-C') of the PV train (Poisson et al., 2001). This region of the P protein was included in the predicted sequences of these bat virus P genes. In all cases, the conserved LC_8 binding sequence, EDKATQT, was present in agreement with the conserved LC_8 binding motif [K/R]XTQT was recently described (Lo et al., 2001). In many positions the presence of amino acid replacements specific to a particular clade is apparent (e.g. D of residues 42 and 79 in clade V); in some or many cases certain substitutions are shared between clades.

Rabies Virus Evolutionary Analysis

1) Positive selection analysis

Two datasets of selected *Eptesicus fuscus* and *Myotis* species sequences were generated and analyzed (see MATERIALS AND METHODS) using various codon substitution models in the CODEML program in the PAML package. As indicated, the adaptive molecular evolution of rabies virus can be explored by comparing synonymous and nonsynonymous substitution rates in protein-coding DNA sequences. Based on the dN/dS ratio (ω) shown in Table 4, there was no evidence for positive selection identified in either dataset.

The CODEML program was also used to infer the transition/transversion (Ti/Tv) ratio (κ), a value expressing the relative probabilities of different types of nucleotide changes for correction of genetic distance measures, of the two datasets, as displayed in Table 4.

2) Estimation of the rate of molecular evolution

Two datasets of selected *Eptesicus fuscus* and *Myotis* species sequences were tested using Bayesian MCMC models analyzed by BEAST program. The estimated substitution data are presented at Table 4.

Table 4. Datasets and Results of Rabies Virus Evolutionary Analysis

Dataset	Host species	Years	N	ω	μ			κ
					SiteModel			
					1	2	3	
EF	*Eptesicus fuscus*	16	41	0.1874	2.545E-5	3.408E-5	1.700E-4	4.77323
MY	*Myotis* species	14	16	0.1870	4.096E-4	4.107E-4	2.146E-3	5.27596

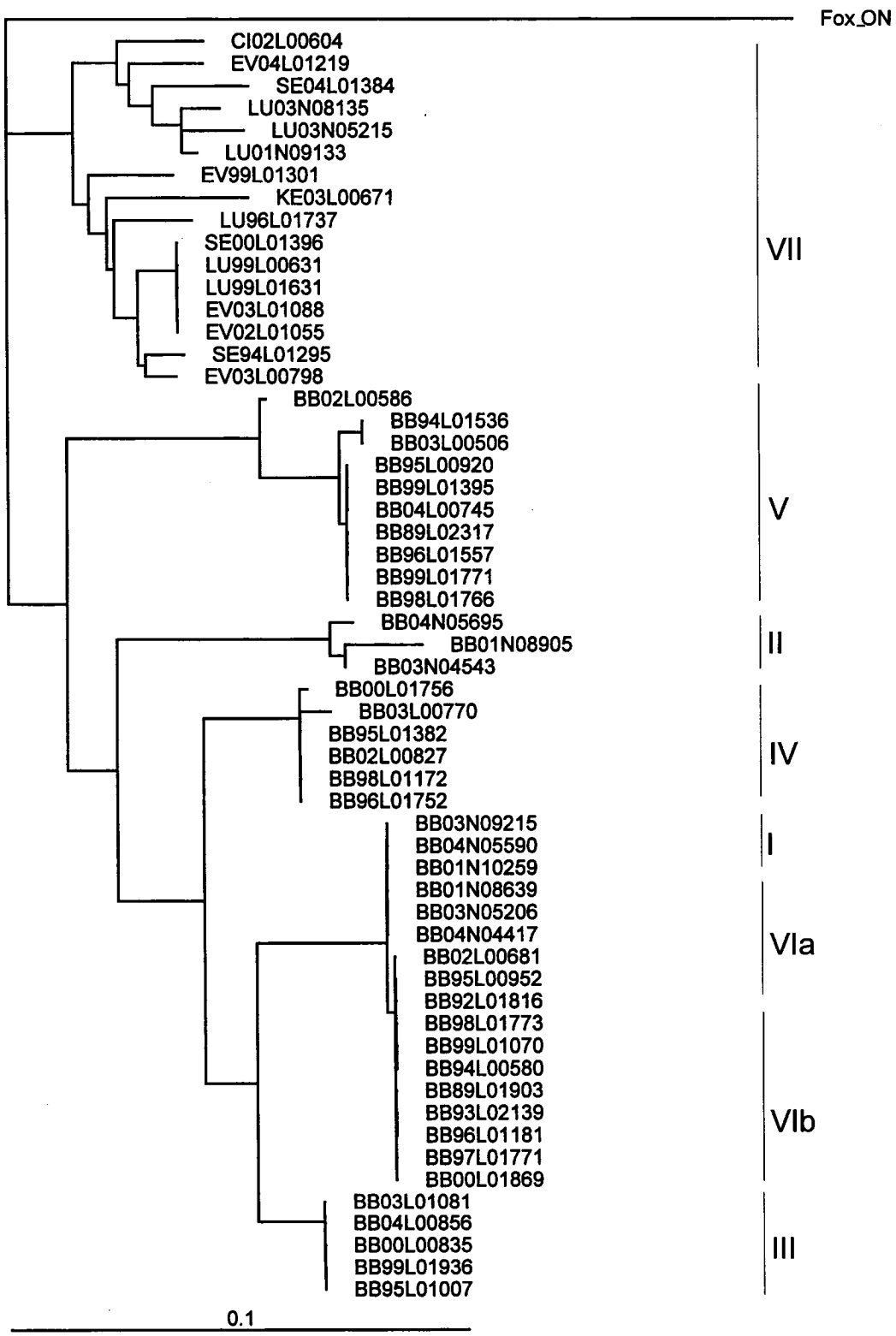
Years: Total number of years over which the sequences were isolated
N: Number of sequences within each dataset
ω: Ratio of non-synonymous substitutions per non-synonymous site (*dN*) to synonymous substitutions per synonymous site (*dS*)
μ: Substitutions per site per year
κ : Ratio of transition and transversion

The ML tree generated (Figure 12) for implementation of rabies virus evolutionary analysis identified seven clades in agreement with the clade organization of the NJ tree (Appendix 3).

Microsatellite Locus Library Information

Two hundred and ninety eight positive clones were cultured and amplified from each microsatellite library; 186 *Eptesicus fuscus* clones and 214 *Myotis lucifugus* clones with DNA inserts of appropriate size were sequenced. The final outcome of the library screening was the isolation of 9 microsatellite loci for *Eptesicus fuscus* and 7 microsatellite loci for *Myotis lucifugus*, all of which were of value for extensive screening due to their polymorphism in initial trials. The 9 microsatellite loci of *Eptesicus fuscus* were all perfect

Figure 12. Phylogenetic ML tree of 57 selected rabies viral isolates from bats of *Eptesicus fuscus* (41) and *Myotis* species (16) used for evolutionary analysis (see Appendix 1). Numbers on the right-hand side of the figure indicated the main phylogenetic groupings as identified by the NJ tree (Appendix 3). Ontario fox rabies virus P gene sequence (Fox_ON) was the outgroup.


Fox_ON
CI02L00604
EV04L01219
SE04L01384
LU03N08135
LU03N05215
LU01N09133
EV99L01301
KE03L00671
LU96L01737
SE00L01396
LU99L00631
LU99L01631
EV03L01088
EV02L01055
SE94L01295
EV03L00798
BB02L00586
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BB96L01557
BB99L01771
BB98L01766
BB04N05695
BB01N08905
BB03N04543
BB00L01756
BB03L00770
BB95L01382
BB02L00827
BB98L01172
BB96L01752
BB03N09215
BB04N05590
BB01N10259
BB01N08639
BB03N05206
BB04N04417
BB02L00681
BB95L00952
BB92L01816
BB98L01773
BB99L01070
BB94L00580
BB89L01903
BB93L02139
BB96L01181
BB97L01771
BB00L01869
BB03L01081
BB04L00856
BB00L00835
BB99L01936
BB95L01007
VII
V
II
IV
I
VIa
VIb
III
0.1

dinucleotide repeats except for locus BNS26E2, which had the motif $(TG)_4(AG)(TG)_{11}$. One trinucleotide locus was included in the 7 microsatellite loci recovered from *Myotis lucifugus*, while the other 6 were all perfect dinucleotide repeats except for one, LS44D7 locus of $(CG)_2(TG)_{17}$.

The selected microsatellite loci designation, their primers, and their amplicon sizes are summarized in Tables 5 and 6, which also indicate their organization into different sets for the purpose of scoring by multiplex PCR. However, upon application of multiplex PCR to a large sample number, it was found that 2 loci BS45F7 and BS45G11 of the third set of

Table 5. Nine Pairs of Primers of Microsatellite Loci for *Eptesicus fuscus*

Clone ID	**MSAT Motifs**	**Amplicon (bp)**	**Dye**	**Set**	**Primer (5' -> 3')****
BS45E7	$(GT)_{18}$	162	700	1	TGGGCAAAAGTTTCTTCAGG
					CAGTTGGTCCGACAGTTGTG
BS45H6	$(TG)_{19}$	208	700	1	AGGGCCAGACAGCTGATAAA
					TGTCTGTGAGGCAGAGCAAC
BS26F6	$(TG)_{13}$	239	700	1	GGGAAGTGGGATAGTCATGG
					AACCGGTTTTGGCTATGGTA
BNS26E2	$(TG)_4(AG)(TG)_{11}$	190	700	2	TGCCCTTATTTTGGATGTTG
					AGATGGAAATTGCCAACACG
BNS26D6	$(TG)_{18}$	234	700	2	CATTATGCAATGCCCCTCTC
					AACAGAGACTGGCAGAGTGGA
BS45F7	$(TG)_{18}$	218	700	3	CCAGCAGAGGAATGGAAGAC
					GCTGAATGTTGGACAAGTGC
BS45G11*	$(GT)_{18}$	247	700	3	TTCTTTTGGGCTTGTCATCA
					AAATCAGCCTCACTGTTGAGC
BNS26C12	$(GT)_{12}$	186	700	4	TACAGGGACAGGCAAAGACC
					CTTGGGTGCTCAGGACAGAT
BS45A6*	$(GT)_{17}$	227	700	4	GCTTGGAGATGGGGTGATTA
					CTTTGACATCTGGGGCTTTG

"*" Indicates double volumes of primers were used for microsatellite locus amplification
"**" Indicates the M13 reverse primer sequence was added to the 5' end of the forward primers in each pair of microsatellite primers for the large-scale screening

Table 6. Seven Pairs of Primers of Microsatellite Loci for *Myotis lucifugus*

Clone ID	MSAT Motifs	Amplicon (bp)	Dye	Set	Primers (5' -> 3')**
LS24E3	$(TCA)_{12}$	154	700	1	ACAGTCTCCTGTGGCATCCT
					CAGAGGAGGAATTGCAGACC
LS44D7	$(CG)_2(TG)_{17}$	192	700	1	AGTCAGCTGGCCGACAAC
					GCAAGTGCTCGGTAAACCAT
LS44H8*	$(GT)_{13}$	234	700	1	CAACACCCCGCAAAAATAAT
					TCTGCATTCTCACCAGCAGT
LNS24F7	$(TG)_{20}$	183	700	2	TCTCTGTGGGCTGTTTTCCT
					GCTCCTTTTGGCTTTTTGAG
LS24B3*	$(GT)_{21}$	225	700	2	CCCTGAAGATCCACAAAGTG
					GTGAAAGTGATGGGGAGAGG
LS24B12	$(TG)_{14}$	157	700	3	GCCTTCTTCCTCCACCATTC
					TGGAGGCTATTGATTCTGGA
LS44G9	$(TG)_{18}$	192	700	3	TTCAGGAATGACTCCCACTTG
					ACCCTGAGAGCAAAACCAGA

"*" Indicates double volumes of primers were used for microsatellite locus amplification
"**" Indicates that the M13 reverse primer sequence was added to the 5' end of the forward primers in each pair of microsatellite primers for the large-scale screening

Eptesicus fuscus microsatellites were overlapping in the size ranges of their amplified PCR fragments. The amplification of the loci of this microsatellite set was therefore processed individually for most DNA samples of *Eptesicus fuscus*.

Comparative Measures of Microsatellite Genetic Variation by Software GenAlEx6.0

1) *Eptesicus fuscus*

Genotyping of 295 *Eptesicus fuscus* specimens at 9 microsatellite loci enabled scoring of 338 distinct alleles. A high polymorphism (100% polymorphic) was observed at all 9 microsatellite loci. It indicated the usefulness of these microsatellites for population studies. For all 295 *Eptesicus fuscus* DNA samples, five microsatellites (BS45E7, BS45H6, BS26F6, BS45F7, and BS45G11) were successfully amplified. Only one out of 295 samples

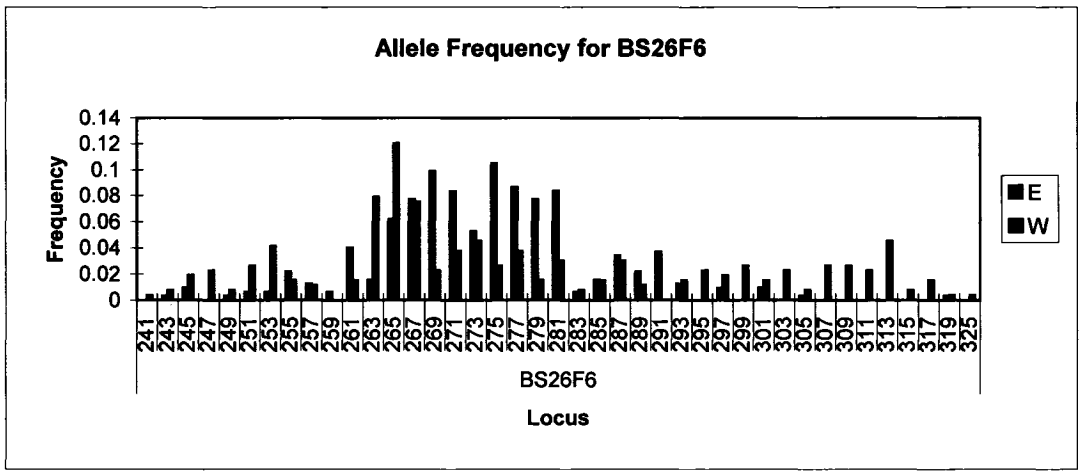
failed to be amplified at microsatellite loci BNS26D6, BNS26C12 and BS45A6. Two out of 295 samples failed to be amplified at locus BNS26E2. The overall success rate of amplification over all microsatellite loci for 295 samples was 98.64%. The numbers of alleles for each locus in population I (East) and population II (West), predicted by the "Structure" analysis (See next section "Bat Population Structure Determination Using Microsatellites"), ranged from 13 to 29 (mean = 21.89 $\pm$ 5.84) and 15 to 39 (mean = 21.67 $\pm$ 7.83) respectively. The expected heterozygosity in each locus in two populations was substantially higher than the observed heterozygosity, while their individual mean values across all loci were 0.875 $\pm$ 0.078, 0.528 $\pm$ 0.078 and 0.861 $\pm$ 0.079, 0.483 $\pm$ 0.175 respectively.

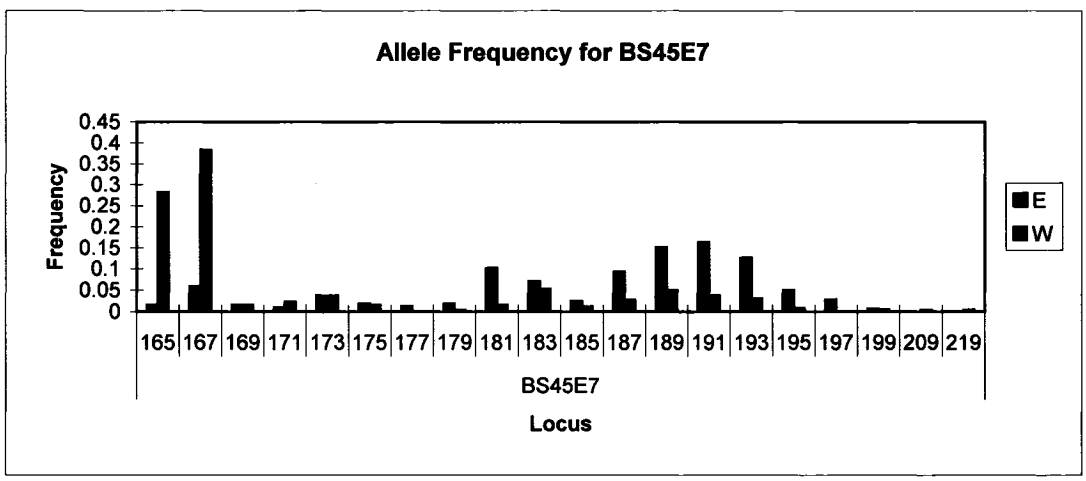
For each locus a graph of locus allele frequency in each population as a function of allele size is presented in Figure 13. For each of the 2 populations, the effective number of alleles (Na), the observed heterozygosity (Ho) and the expected heterozygosity (He) are displayed in Table 7.

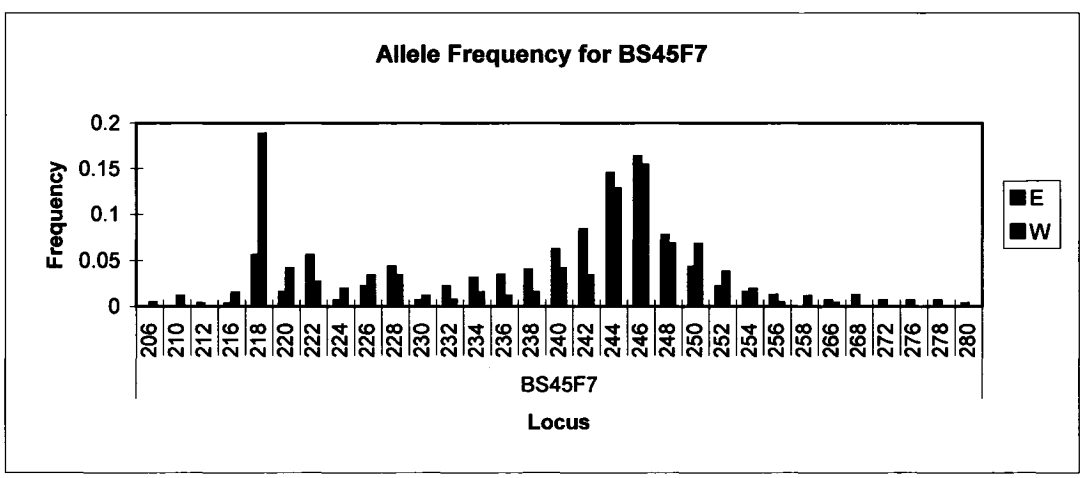
2) *Myotis lucifugus*

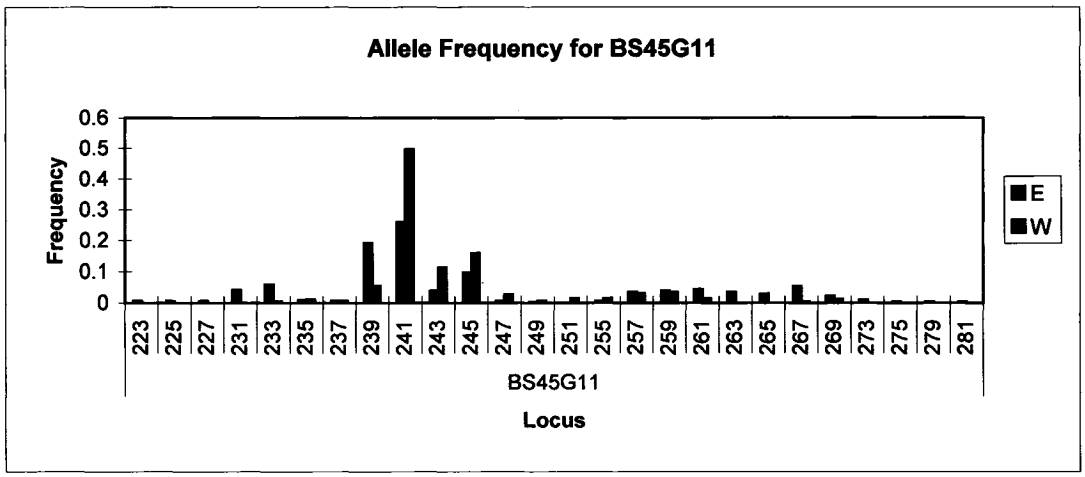
Genotyping of 126 *Myotis lucifugus* DNA specimens at 7 microsatellite loci enabled scoring of 144 distinct alleles. A high polymorphism (100% polymorphic) was observed at all 7 microsatellite loci indicating their applicability for population studies. The number of microsatellite alleles amplified per locus ranged from 16 to 25 (mean = 20.57 $\pm$ 2.70). Of all 126 DNA samples, 3 microsatellite loci, LS44H8, LNS24F7, and LS44G9 were amplified successfully in all cases. The failure rate of locus amplification for LS24E3, LS24B3, and LS24B12 was 2.38%, 3.17%, and 1.59%, respectively. The overall mean rate of successful amplification of all microsatellite loci was 93.65%. The observed heterozygosity (0.535 $\pm$ 0.025) of all 7 alleles was lower than the expected heterozygosity (0.906 $\pm$ 0.025). Locus

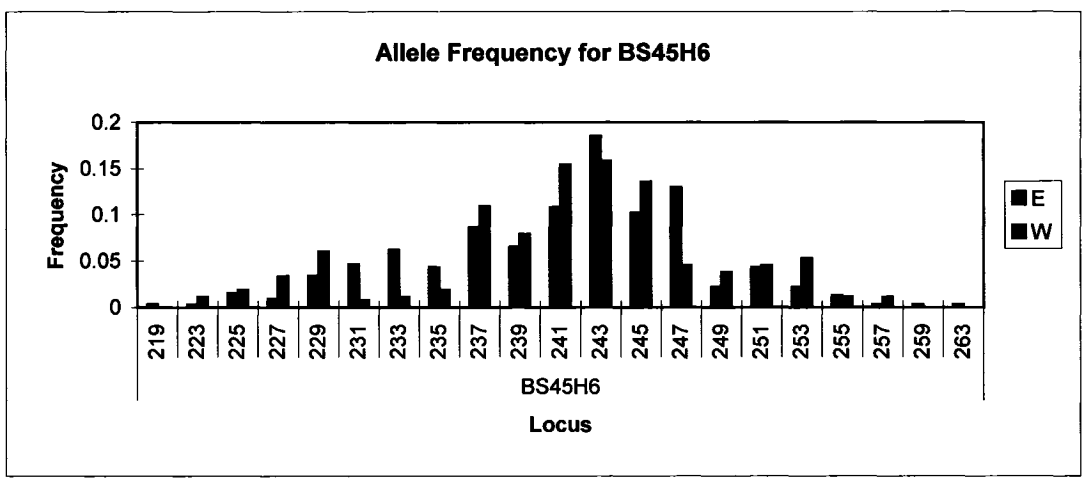
Figure 13. Allele Frequencies Identified by 2 Populations and 9 Microsatellite Loci of *Eptesicus fuscus*

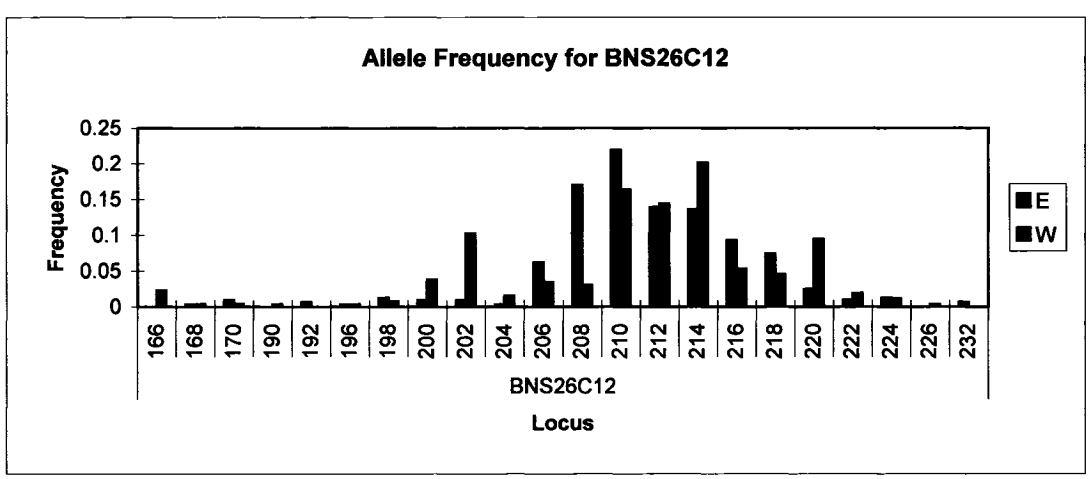

Allele Frequency for BS26F6
Frequency
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
241 243 245 247 249 251 253 255 257 259 261 263 265 267 269 271 273 275 277 279 281 283 285 287 289 291 293 295 297 299 301 303 305 307 309 311 313 315 317 319 325
BS26F6
Locus
E
W

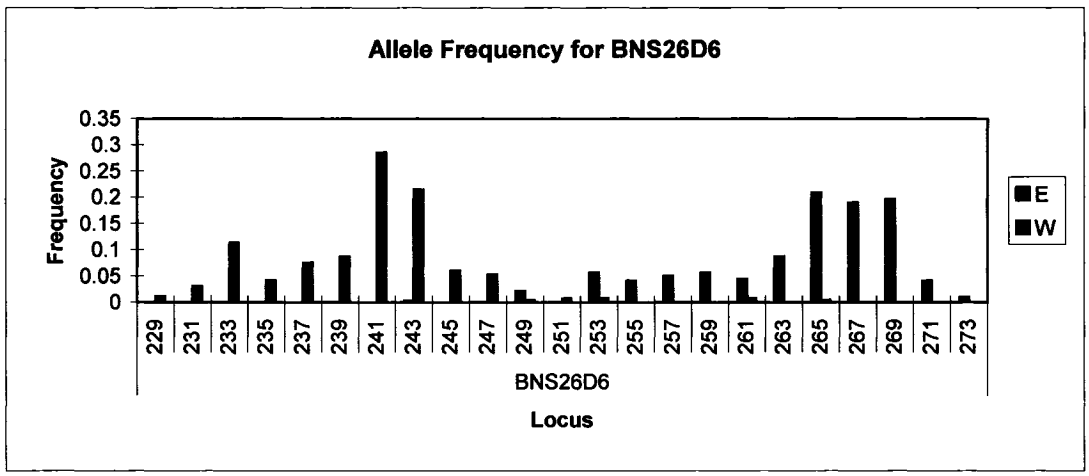

Allele Frequency for BS45E7
Frequency
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
165 167 169 171 173 175 177 179 181 183 185 187 189 191 193 195 197 199 209 219
BS45E7
Locus
E
W


Allele Frequency for BS45F7
Frequency
0.2
0.15
0.1
0.05
0
206 210 212 216 218 220 222 224 226 228 230 232 234 236 238 240 242 244 246 248 250 252 254 256 258 266 268 272 276 278 280
BS45F7
Locus
E
W


Allele Frequency for BS45G11
Frequency
0.6
0.5
0.4
0.3
0.2
0.1
0
223 225 227 231 233 235 237 239 241 243 245 247 249 251 255 257 259 261 263 265 267 269 273 275 279 281
BS45G11
Locus
E
W


Allele Frequency for BS45H6
Frequency
0.2
0.15
0.1
0.05
0
219 223 225 227 229 231 233 235 237 239 241 243 245 247 249 251 253 255 257 259 263
BS45H6
Locus
E
W


Allele Frequency for BNS26C12
Frequency
0.25
0.2
0.15
0.1
0.05
0
166 168 170 190 192 196 198 200 202 204 206 208 210 212 214 216 218 220 222 224 226 232
BNS26C12
Locus
E
W


Allele Frequency for BNS26D6
Frequency
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0.3
0.25
0.2
0.15
0.1
0.05
0
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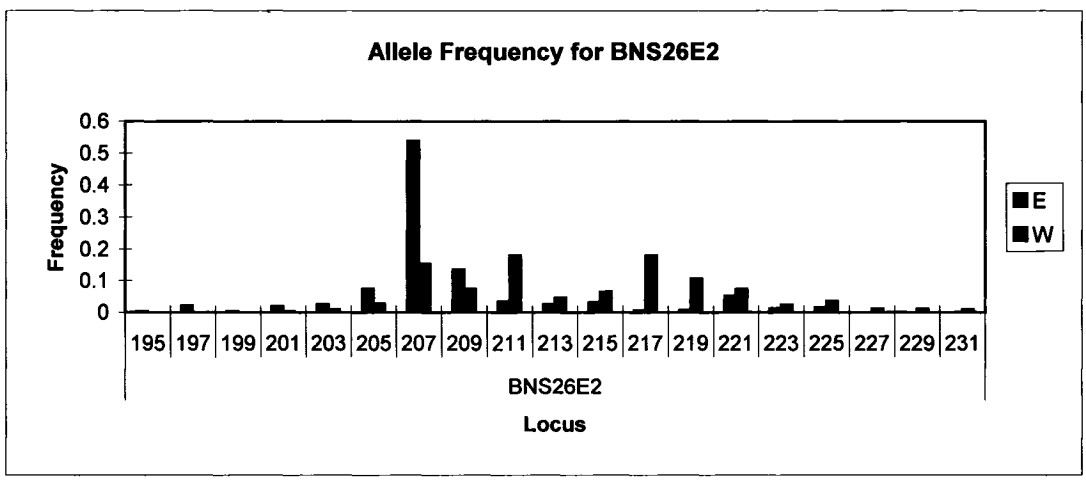
BNS26D6
Locus
E
W


Allele Frequency for BNS26E2
Frequency
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0.5
0.4
0.3
0.2
0.1
0
195 197 199 201 203 205 207 209 211 213 215 217 219 221 223 225 227 229 231
BNS26E2
Locus
E
W

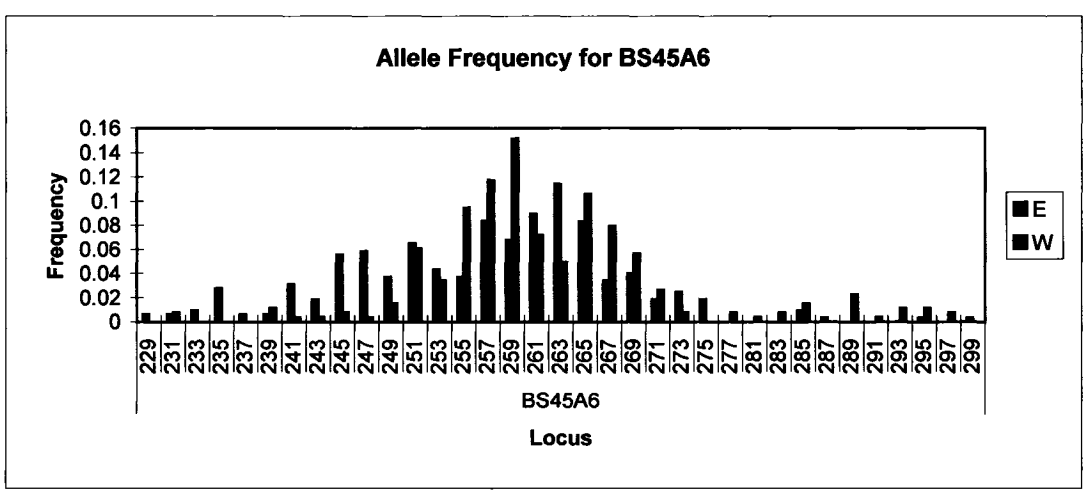

Allele Frequency for BS45A6
Frequency
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
229 231 233 235 237 239 241 243 245 247 249 251 253 255 257 259 261 263 265 267 269 271 273 275 277 281 283 285 287 289 291 293 295 297 299
BS45A6
Locus
E
W

Table 7. Genetic Diversity of Microsatellite Loci of *Eptesicus fuscus*

	East (PI)				West (PII)			
	N	Na	Ho	He	N	Na	Ho	He
BS26F6	162	29	0.559	0.933	133	39	0.519	0.954
BS45E7	162	18	0.543	0.900	133	18	0.316	0.762
BS45F6	162	28	0.660	0.919	133	25	0.602	0.904
BS45G11	162	25	0.420	0.871	133	16	0.233	0.710
BS45H6	162	21	0.636	0.903	133	18	0.632	0.901
BNS26C12	162	19	0.648	0.866	132	20	0.780	0.883
BNS26D6	161	13	0.267	0.861	133	15	0.368	0.837
BNS26E2	162	16	0.481	0.680	131	16	0.527	0.881
BS45A6	162	28	0.500	0.939	132	28	0.371	0.919

N: Number of Samples
Na: Number of Alleles
Ho: Observed Heterozygosity
He: Expected Heterozygosity

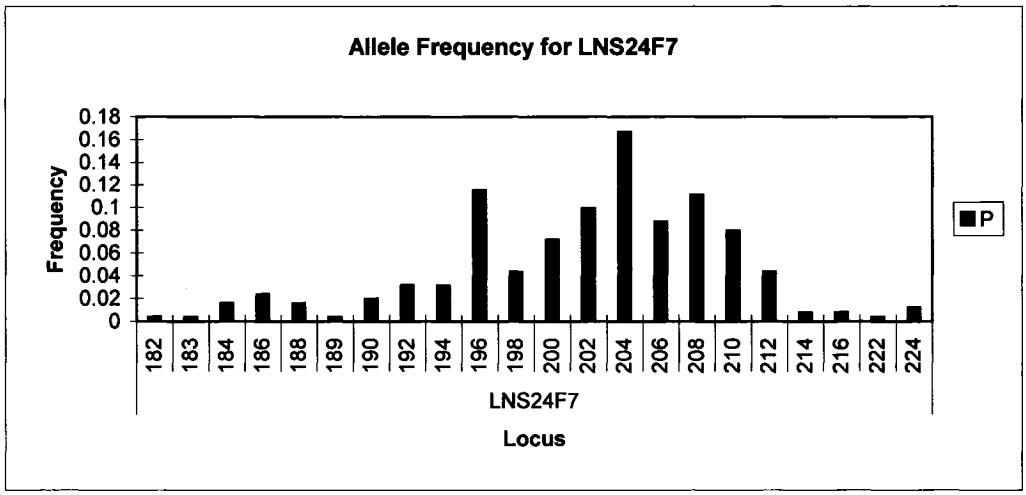
LS24B3 presented the biggest difference between observed and expected heterozygosity (-0.641).

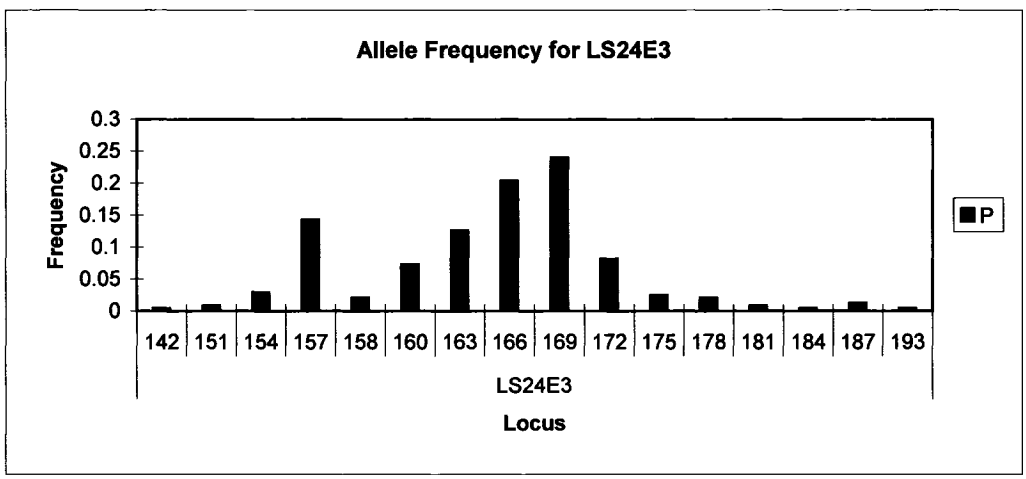
For each locus, a graph of locus allele frequency as a function of allele size is shown in Figure 14. The effective number of alleles (Na), the observed heterozygosity (Ho), and the expected heterozygosity (He) for the complete dataset are shown in Table 8.

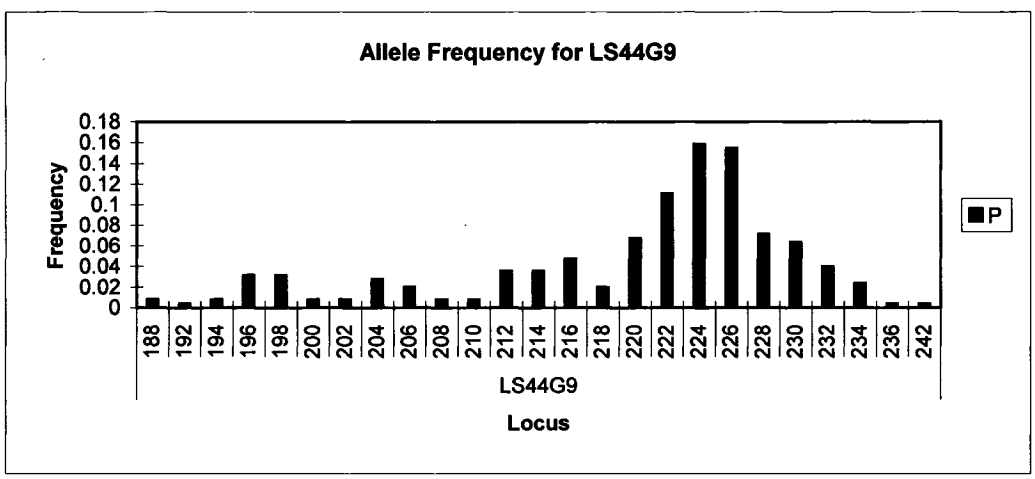
Bat Population Structure Determination Using Microsatellites

The group of 295 *Eptesicus fuscus* DNA samples (162 of them from Ontario and Quebec and 133 of them from British Colombia, Albert and Saskatchewan) were partitioned into two clusters of East and West by the "Structure" analysis of all 9 genotyped

Figure 14. Allele Frequencies Identified by 1 Population and 7 Microsatellite Loci of *Myotis lucifugus*


Allele Frequency for LNS24F7
Frequency
0.18
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
182 183 184 186 188 189 190 192 194 196 198 200 202 204 206 208 210 212 214 216 222 224
LNS24F7
Locus
P


Allele Frequency for LS24E3
Frequency
0.3
0.25
0.2
0.15
0.1
0.05
0
142 151 154 157 158 160 163 166 169 172 175 178 181 184 187 193
LS24E3
Locus
P


Allele Frequency for LS44G9
Frequency
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0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
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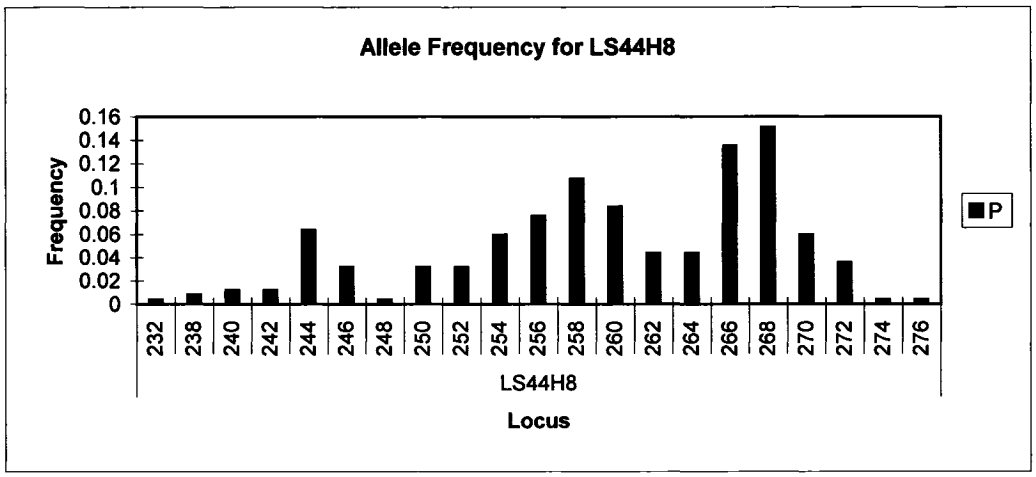
LS44G9
Locus
P


Allele Frequency for LS44H8
Frequency
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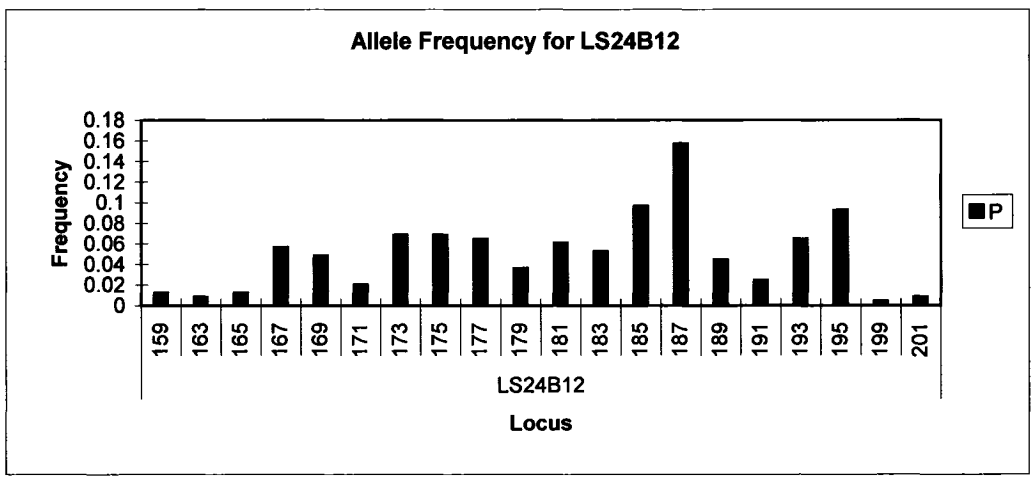
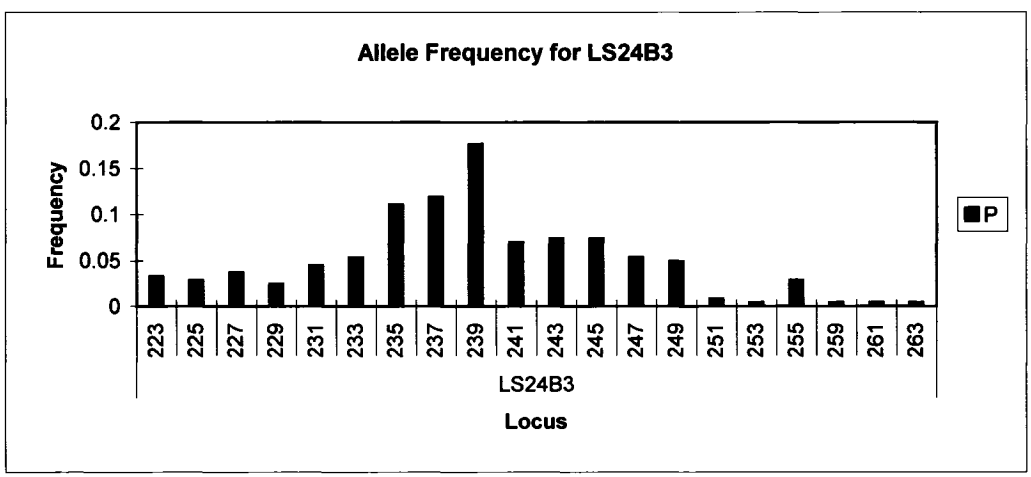
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
232 238 240 242 244 246 248 250 252 254 256 258 260 262 264 266 268 270 272 274 276
LS44H8
Locus
P


Allele Frequency for LS24B12
Frequency
0.18
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
159 163 165 167 169 171 173 175 177 179 181 183 185 187 189 191 193 195 199 201
LS24B12
Locus
P


Allele Frequency for LS24B3
Frequency
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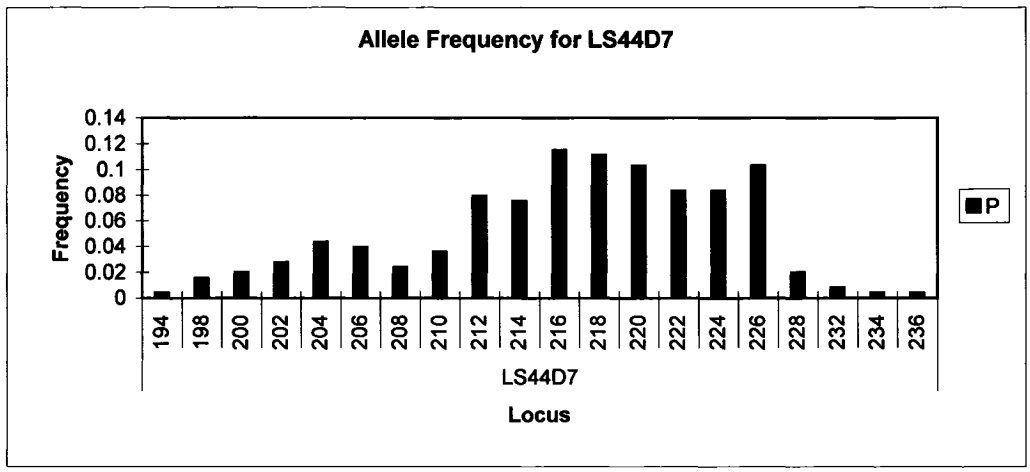
0.15
0.1
0.05
0
223 225 227 229 231 233 235 237 239 241 243 245 247 249 251 253 255 259 261 263
LS24B3
Locus
P


Allele Frequency for LS44D7
Frequency
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0.12
0.1
0.08
0.06
0.04
0.02
0
194
198
200
202
204
206
208
210
212
214
216
218
220
222
224
226
228
232
234
236
LS44D7
Locus
P

Table 8. Genetic Diversity of Microsatellite Loci of *Myotis lucifugus*

	N	Na	Ho	He
LNS24F7N	126	22	0.556	0.910
LS24E3	125	16	0.496	0.851
LS44G9	126	25	0.690	0.914
LS44H8	126	21	0.579	0.915
LS24B12	126	20	0.500	0.922
LS24B3	126	20	0.270	0.911
LS44D7	126	20	0.651	0.920

N: Number of Samples
Na: Number of Alleles
Ho: Observed Heterozygosity
He: Expected Heterozygosity

microsatellite loci. Only one sample from British Columbia was clustered into the eastern group and two samples from Ontario were clustered into the western group; however these samples had very close partial membership in both clusters. The percentage of samples for which their membership coefficients were larger than 0.85 in their inferred clusters averaged 91.52% for all 295 samples. The extremely high correspondence between genetically inferred group and sampling geographic location was detected at K = 2.

The structure program was rerun to explore clustering of 162 eastern and 133 western samples grouped separately based on their microsatellite scoring. For the 162 samples of the eastern dataset assuming K = 2, the first group contained 79 samples, of which 61 samples collected from Ontario and 18 samples collected from Quebec, with a high membership coefficient (0.85) of 74.69%. The second group, containing 83 samples, of which 64 samples circulated in Ontario and 19 samples collected from Quebec, had a high membership

coefficient (0.85) of 69.88%. For 133 samples of the western dataset, the third group consisted of 76 specimens, comprising 12 specimens from Saskatchewan, 14 specimens from Alberta and 50 specimens from British Columbia, with high membership coefficient (0.85) of 68.14%. The fourth group consisted of 57 specimens with a high membership coefficient (0.85) of 71.93%, and comprising 14 specimens from Saskatchewan, 11 specimens from Alberta and 32 specimens from British Columbia. Detailed information of this population structure is summarized in Table 9. When these four groups were plotted on a map of Canada (Figure 15), eastern and western groupings were clearly evident by their geographic

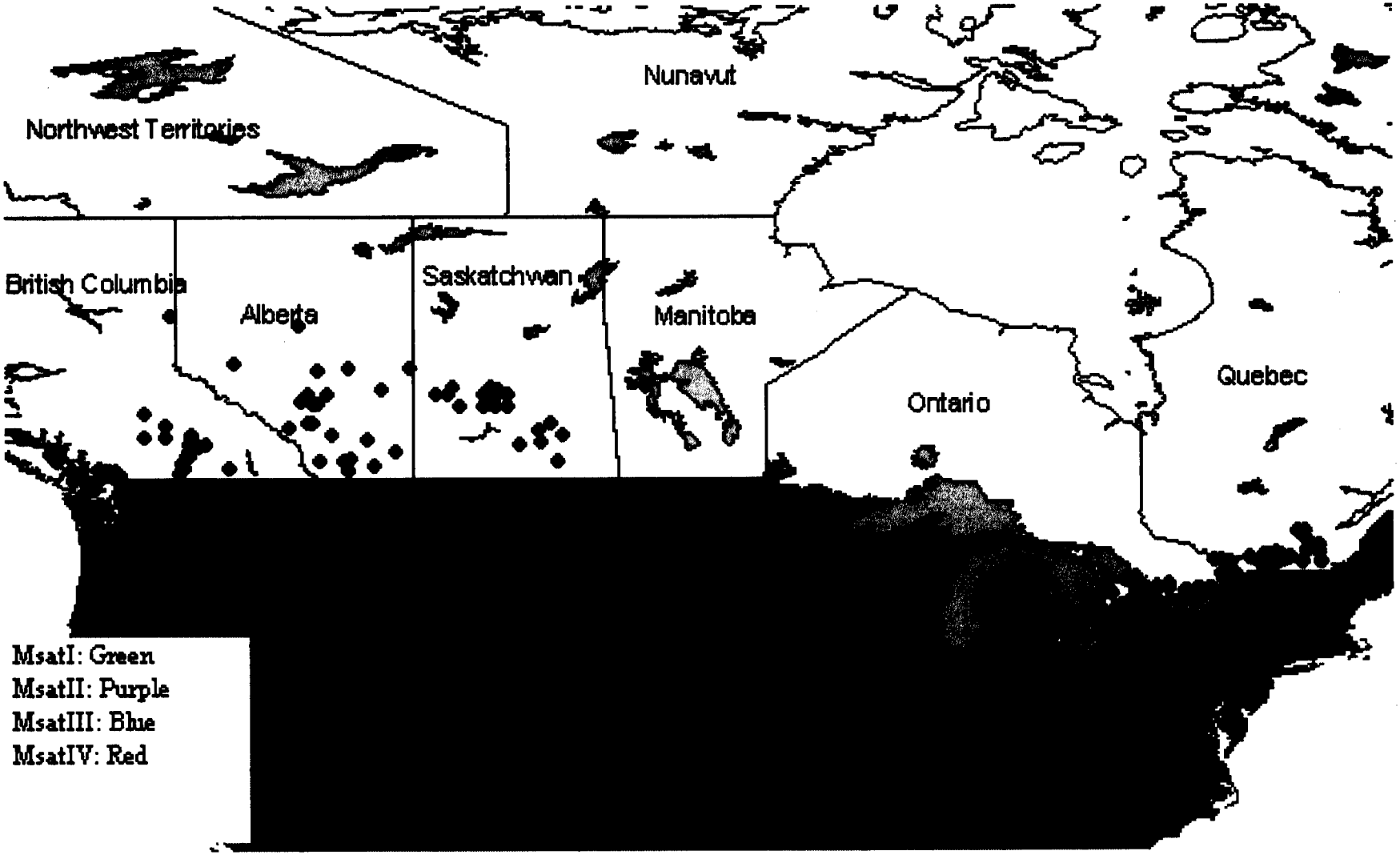
Table 9.The *Eptesicus fuscus* Specimen Distribution under Software Structure Analysis (K = 4)

	Eastern Group (162)			**Western Group (133)**	
	Group I	Group II		Group III	Group IV
ON	61	64	**BC**	50	32
QC	18	19	**AB**	14	11
Total	79	83	**SK**	12	14
(0.85)	74.69%	69.88%	**Total**	76	57
			(0.85)	68.14%	71.93%

distribution, however, further subdivision into four groups didn't exhibit any clear regional localization. The microsatellite data developed in this study most strongly suggest that two genetic populations of *Eptesicus fuscus* exist in Canada.

The dataset of 126 *Myotis lucifugus* DNA samples, genotyped at 7 microsatellite loci was analysed with Structure software. When K = 2, a total of 65 specimens (27 from British Columbia, 9 from Alberta, 2 from Saskatchewan, 1 from Manitoba, 8 from Ontario, 15 from Quebec, 2 from New Brunswick and 1 from Newfoundland) were clustered into group I,

Figure 15. Map Showing Distribution of *Eptesicus fuscus* Specimens Grouped according to Microsatellite Genotyping
Two groups: Population I or East (MsatI + MsatII) and Population II or West (MsatIII + MsatIV) were identified. More than one sample may be represented by each square symbol


Nunavut
Northwest Territories
British Columbia
Alberta
Saskatchwan
Manitoba
Ontario
Quebec
MsatI: Green
MsatII: Purple
MsatIII: Blue
MsatIV: Red

whilst 61 specimens (6 from British Columbia, 3 from Alberta, and 1 from Manitoba, 18 from Ontario, 23 from Quebec, 7 from New Brunswick, 1 from Newfoundland and 2 from Nova Scotia) comprised the second group. The high membership coefficient (0.85) for all 126 specimens was 83.33%. At K = 3, three clusters were distributed as follows. One group consisted of 39 specimens of *Myotis lucifugus* DNA samples (21 from British Columbia, 9 from Alberta, 2 collected in Saskatchewan, 1 from Manitoba, 4 from Ontario, 2 from Quebec). Another group comprised 39 *Myotis lucifugus* samples - 7 from British Columbia, 2 from Alberta, 1 from Manitoba, 5 from Ontario, 19 from Quebec, and 5 from New Brunswick. The third group contained 48 specimens of bats *Myotis lucifugus* DNA samples - 5 from British Columbia, 1 from Alberta, 17 from Ontario, 17 from Quebec, 4 from New Brunswick, 2 from Newfoundland, and 2 from Nova Scotia. The percentage of high membership coefficient (0.85) is 52.38%. A summary of this information is presented in Table 10.

Table 10. *Myotis lucifugus* Specimen Distribution under Software Structure Analysis

	K=2		**K=3**		
	GI	**GII**	**GI**	**GII**	**GIII**
BC	27	6	21	7	5
AB	9	3	9	2	1
SK	2	0	2	0	0
MAN	1	1	1	1	0
ON	8	18	4	5	17
QC	15	23	2	19	17
NB	2	7	0	5	4
NL	1	1	0	0	2
NS	0	2	0	0	2
Total	65	61	39	39	48
(0.85)	83.33%			52.39%	

Overall, the conclusion from Structure software analysis was that the microsatellite dataset of *Myotis lucifugus* didn't display any clustering trends correlated with the geographical localization of these samples.

Rst (ρ) Measure Using RSTCALC 2.2

Rst (ρ) values were estimated between two populations - population I (East) and population II (West) of bats *Eptesicus fuscus* set up with 1000 bootstrap value and 1000 permutation test. ρ value (averaging variance components) was 0.3170. ρ value (averaging over loci) was 0.2187. Both ρ values supported the very great and great genetic differentiation between the two populations, respectively.

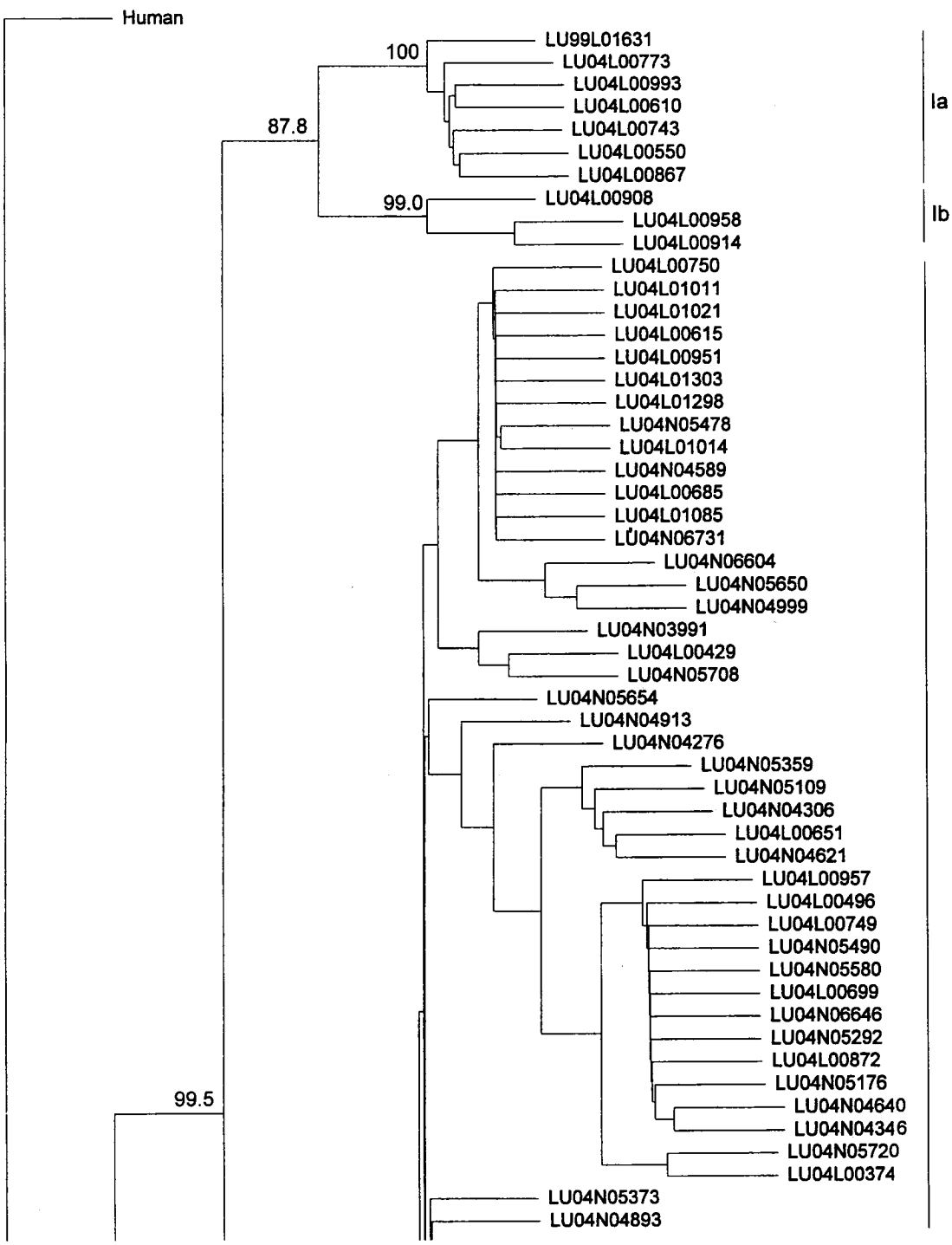
Population Structure Determination of *Myotis lucifugus* Using Cox-1 Gene

Since the microsatellite analysis had failed to identify any population structure in *Myotis lucifugus* bats, an alterative method of genotyping was explored in which mitochondrial Cox-1 gene sequences were employed in a phylogenetic analysis. The combination of microsatellite DNA and mitochondrial DNA genotyping methodology has been suggested for determination of bat population structure due to the complication of bat biology (Burland and Wilmer, 2001; Ruedi and Castella, 2003).

One hundred and six *Myotis lucifugus* Cox-1 gene sequences were characterized over a 552 bp region. An alignment of all sequences showed that no insertions or deletions were necessary to align these sequences and most substitutions were transitions with very few transversion evident. Using all 106 Cox-1 gene sequences and a human Cox-1 gene sequence as an outgroup, a NJ tree was constructed from the sequence alignment (Figure 16).

The NJ tree suggested four primary clusters; bootstrap values for the first three clades were 87.8% (I), 97.3% (II) and 99.5% (III) respectively. Groups I (divided into two subgroups with bootstrap values of 100% and 99.0%) and II comprised *Myotis lucifugus*

Figure 16. Phylogenetic tree of 106 mtDNA specimens from *Myotis lucifugus* bats (see Appendix 1) analysed by a NJ algorithm using a 552 bp partial sequence of the Cox-1 gene. Numbers to the left of a branch indicated the number of times out of 1000 bootstrap replicates that the branch was supported. Numbers on the right-hand side of the figure indicated the main phylogenetic groupings. A human Cox-1 gene sequence was the outgroup.


Human
100
87.8
99.0
99.5
LU99L01631
LU04L00773
LU04L00993
LU04L00610
LU04L00743
LU04L00550
LU04L00867
LU04L00908
LU04L00958
LU04L00914
LU04L00750
LU04L01011
LU04L01021
LU04L00615
LU04L00951
LU04L01303
LU04L01298
LU04N05478
LU04L01014
LU04N04589
LU04L00685
LU04L01085
LU04N06731
LU04N06604
LU04N05650
LU04N04999
LU04N03991
LU04L00429
LU04N05708
LU04N05654
LU04N04913
LU04N04276
LU04N05359
LU04N05109
LU04N04306
LU04L00651
LU04N04621
LU04L00957
LU04L00496
LU04L00749
LU04N05490
LU04N05580
LU04L00699
LU04N06646
LU04N05292
LU04L00872
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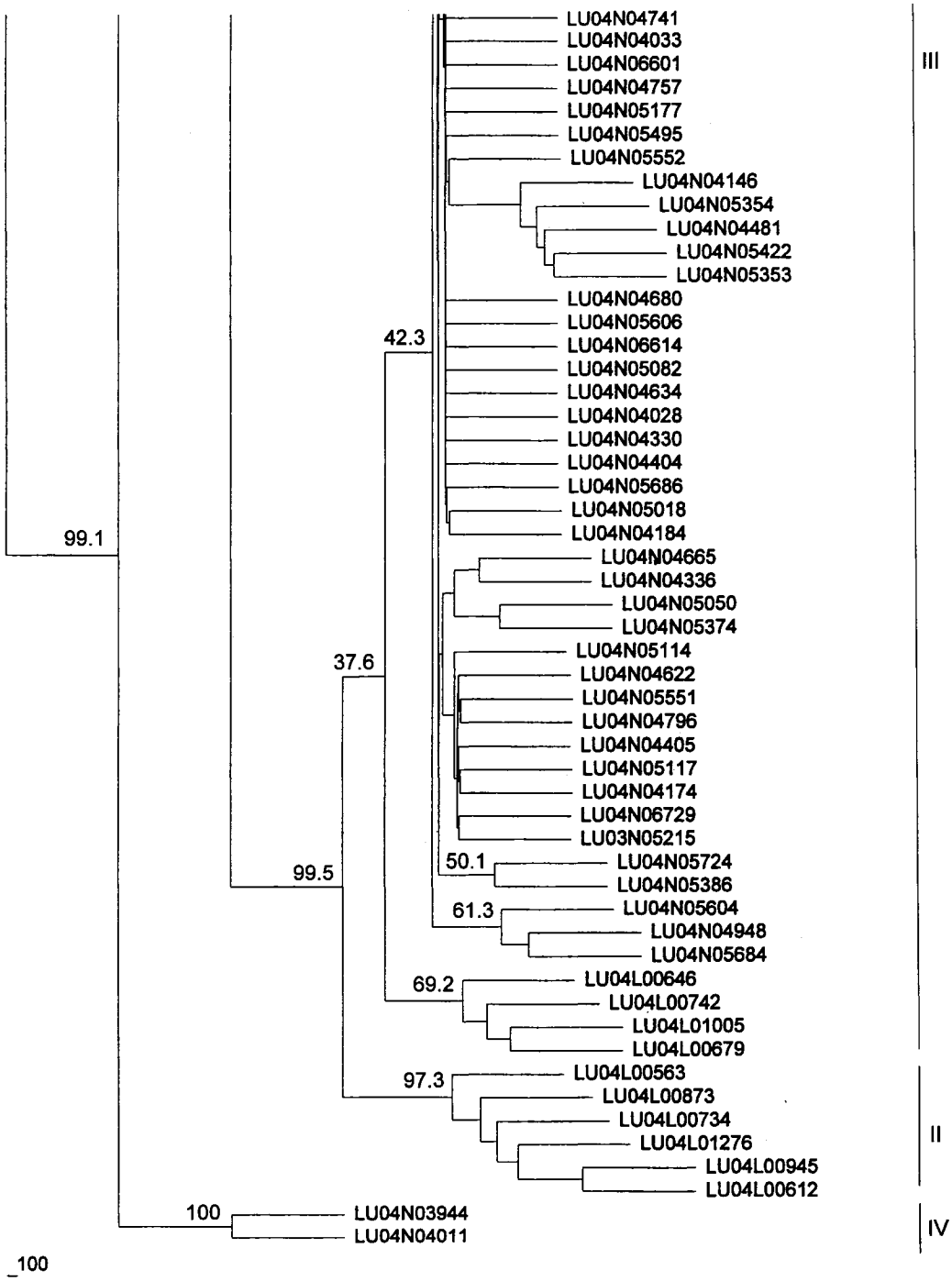
LU04L00374
LU04N05373
LU04N04893
Ia
Ib


LU04N04741
LU04N04033
LU04N06601
LU04N04757
LU04N05177
LU04N05495
LU04N05552
LU04N04146
LU04N05354
LU04N04481
LU04N05422
LU04N05353
LU04N04680
LU04N05606
LU04N06614
LU04N05082
LU04N04634
LU04N04028
LU04N04330
LU04N04404
LU04N05686
LU04N05018
LU04N04184
LU04N04665
LU04N04336
LU04N05050
LU04N05374
LU04N05114
LU04N04622
LU04N05551
LU04N04796
LU04N04405
LU04N05117
LU04N04174
LU04N06729
LU03N05215
LU04N05724
LU04N05386
LU04N05604
LU04N04948
LU04N05684
LU04L00646
LU04L00742
LU04L01005
LU04L00679
LU04L00563
LU04L00873
LU04L00734
LU04L01276
LU04L00945
LU04L00612
LU04N03944
LU04N04011
42.3
99.1
37.6
99.5
50.1
61.3
69.2
97.3
100
100
III
II
IV

samples exclusively from British Columbia. One large cluster, group III, comprised samples from across Canada including the provinces of British Columbia, Alberta, Saskatchewan, Manitoba, Ontario, Quebec, New Brunswick, Newfoundland, and Nova Scotia. Clades I to III formed a highly supported major grouping with a bootstrap value of 99.5%. Two samples (LU04N03944 and LU04N04011) formed a fifth clade outside of this group that clustered quite separately from all other *Myotis lucifugus* samples in this tree; it is suspected that these two specimens may be misspeciated.

In summary, Cox-1 gene sequence analysis identified two discrete *Myotis lucifugus* populations in the province of British Columbia, but failed to identify any population tructure in the remaining sample set that included specimens right across Canada. When the results of microsatellite and mitochondrial DNA genotyping were compared, there was lilttle correspondence in the population structure predicted by these two methods. Thus this study failed to clearly define significant *Myotis lucifugus* population structure across Canada.

DISCUSSION

Bats, mammals with an aerial life style, constituting the order Chiroptera, are susceptible to rabies, and some bat species have been identified as the maintenance reservoirs of rabies. In Canada at least thirteen distinct rabies virus variants circulating in bats have been characterized. The phylogenetic study of rabies virus N gene, G gene and P gene inferred that the identification of rabies viral variants from bats was generally based on the species and geographical localisation of bat hosts (Nadin-Davis et al., 2001; Nadin-Davis et al., 2002). Of the insectivorous bat species that act as rabies virus reservoirs, *Eptesicus fuscus* is most frequently submitted for rabies diagnosis and *Myotis lucifugus* is the species most commonly reported for rabies infection in the *Myotis* genus.

This study explored the possibility that certain rabies viral variants co-localize with discrete sub-populations of *Eptesicus fuscus* and *Myotis lucifugus*. It was hypothesized that landscape features that affect population structure of rabies hosts will also impact on transmission patterns of rabies viruses such that discrete rabies virus variants associate with particular host sub-groups. While this is certainly true of terrestrial and carnivores (Nadin-Davis et al., 1993), the aerial lifestyle of the chiroptera could complicate this picture. However, the bat species targeted in this study are generally regarded as non-migratory and sedentary, which might suggest that the above hypothesis would hold true. These analyses were achieved by genetically characterizing both the host and rabies virus; hence, RT-PCR, DNA sequencing and phylogenetic studies were employed to characterize the rabies viral variants circulating in *Eptesicus fuscus* and *Myotis lucifugus* (*Myotis* species) using specimens recovered throughout the country. The microsatellite genotyping technology and direct sequencing of mitochondrial Cox-1 gene were applied for determination of population (sub-population) structure of the bat hosts.

Rabies Virus Divergence

The central region of rabies virus P gene is a divergent *Lyssavirus* target, as suggested by previous studies of the rabies virus P gene ORF (Nadin-Davis et al., 1997; Nadin-Davis et al., 2002) and Mokola virus P gene ORF (Le Mercier et al., 1997), which indicated that the most poorly conserved portion of P gene was between 183 bp to 591 bp. This study characterized the central portion of P gene using a 597 nucleotide sequence window corresponding to bases 112 to 711 of the RABV PV laboratory strain. The most divergent region (from 161 bp to 210 bp) and a second diverse region (from 419 bp to 518 bp) were recognized in agreement with previous studies (Nadin-Davis et al., 2002).

The reason for the high divergence of this segment of the rabies virus P gene is not known, though the portion of the protein encoded by this segment of the gene may function as a hinge between the two more conserved ends of the protein responsible for interacting with nucleoprotein and RNA polymerase. Some important characteristics of the P protein sequences include a high proportion of both conserved acidic and serine residues, and limited conservation of four internal Met residues that can direct synthesis of truncated P proteins. In addition, the binding domain for dynein light chain, EDKATQT, was found to be well conserved in this region.

Phylogenetic Analysis of Partial Rabies Virus P Gene Sequences

Both the NJ algorithm and MP method were applied to generate different phylogenetic trees from the RABV P gene dataset generated in this study. The MP tree was analysed without bootstrapping due to the large collection of P gene sequences available, so as to compare results with the NJ tree, for which bootstrap values, used for assessment of the robustness of the consensus tree are available.

The NJ tree identified eight rabies viral clades, clades I to VI and VIII containing isolates associated primarily with *Eptesicus fuscus*, and clade VII comprising exclusively *Myotis* species, with a high bootstrap confidence value of 98.2%. The distribution of the first six clades of viral variants recovered from *Eptesicus fuscus* displayed strong geographical localization: clade I consisting of viral isolates from Ontario; clade II containing viral isolates from Quebec and Ontario; clade III and IV comprising viral variants specifically from British Columbia; clade V containing viral specimens from the western area, covering British Columbia and Saskatchewan; clade VI including viral samples broadly from across the country, except Quebec. Clade VIII was subsequently found to represent viruses typical of *Lasionycteris* and *Lasiurus* variants, introduced into four *Eptesicus fuscus* and one *Myotis evotis* by spill-over transmission.

The major groupings were reproduced using the different phylogenetic approach of MP; the MP tree generated using a single dataset predicted just a slight difference in tree structure in which Clade VI was split into two small groups outlying clade I. This division of clade VI samples did not reflect their geographical origins. The concordant results of P gene phylogeny generated from two different methods support the proposed division of rabies viral variants circulating in *Eptesicus fuscus* and *Myotis* species in Canada in this study.

A previous study (Nadin-Davis et al., 2001) that characterized rabies viral N and G genes, segregated non-migratory bats *Eptesicus fuscus* and *Myotis* species from other bats in Canada as one clade, of which *Myotis* species from central and eastern Canada and those from British Columbia were clustered into subclade a and subclade b, respectively. *Eptesicus fuscus* isolates from central or eastern regions were grouped to subclade c, and *Eptesicus fuscus* specimens from across the country were found in subclade d. Within subclade d, certain strong geographical trends were identified including cluster d_4 from British Columbia

and cluster d_3 from British Columbia and Saskatchewan. Thus consistent results were obtained by phylogenetic study of rabies viruses associated with non-migratory bats in Canada, regardless of the viral locus (N, G or P gene) targeted. A substantial correlation exists between rabies viral variants, maintenance species and geographical distribution of the bat hosts.

Clades I, III, IV, VI in this study are equivalent to group Id (Nadin-Davis et al., 2001), clade II (this study) is equivalent to group Ic (Nadin-Davis et al., 2001), and clade VII in this study is equivalent to group Ia and Ib (Nadin-Davis et al., 2001), while clade V (this study) is equivalent to group III (Nadin-Davis et al., 2001).

The viral "spillover" from *Eptesicus fuscus* to *Myotis* species certainly occurred, and the nine samples for which xuch an event had occurred involved 4 species: *Myotis lucifugus*, *Myotis yumanensis*, *Myotis septentrionalis* and *Myotis volans*. Two specimens of *Myotis lucifugus* LU03N01864 and LU03N09437 from Ontario were grouped in clade I. *Myotis yumanensis* YU95L01017 from British Columbia was clustered in clade III. Two *Myotis* samples (*Myotis yumanensis*: YU00L01855 and *Myotis volans*: VO03L00692) from British Columbia were organized into clade V. The other two *Myotis* specimens of *Myotis yumanensis* (YU93L02414) and *Myotis septentrionalis* (SE02L00687) from British Columbia were found in clade VI. These findings are in agreement with previous results (Nadin-Davis et al., 2001; Smith, 1988) which indicated the complication of antigenic typing of *Myotis* isolates. As indicated above, spillover from *Lasionycteris/Lasiurus* reservoirs were also identified in specimens of *Eptesicus fuscus* and *Myotis evotis* which represented clade VIII.

Some correlation between certain amino acid replacements and particular phylogenetic groups of viruses was observed. This kind of replacement occurred most

frequently in viruses of group V, which exhibited several specific mutations. The amino acid sequences of the closely related viruses of groups I and VI were similar but differed from that of other viral groups, at certain amino acid sequence positions. At position 88, group III viruses exhibited a mutation from E to A and group IV viruses contained a mutation from E to S. The group II viruses also exhibited distinctive amino acid patterns at several amino acid residues (see Figure 9). In most cases the nature of these amino acid changes was conservative.

Evolutionary Analysis of Rabies Virus via P Gene

The nature of rabies virus-host interactions, such as host switching from the Chiroptera to the Carnivora orders (Badrane and Tordo, 2001), across-species transmission (Anderson et al., 1981; Bourhy et al., 1999) and new host species establishment (Nadin-Davis et al., 1994; Tordo et al., 1993), as well as the high RNA replication error rates due to RNA polymerase, together all suggested and supported the possibility of natural adaptive evolution of rabies virus. Consequently, the estimation of selective pressure and mutation rate of rabies virus is of some interest. The positive selection analysis of P gene sequences of isolates circulating in *Eptesicus fuscus* and *Myotis* species of Canada measured a *dN/dS* ratio (ω) range from 0.1874 to 0.2081 and 0.1870 to 0.1955, respectively, under six different program models. Unfortunately, these data did not supply strong evidence of positive selection operating on the rabies virus P gene. Other studies targeting N and G genes of street rabies virus strains also failed to show strong support for positive selection (see INTRODUCTION). Although we already know positive selection occurs occasionally, the limitations of those models for estimating nonsynonymous evolution may be an issue. Protein secondary structure (Fitch, 1971; Simmonds and Smith, 1999) and recombination (Awadalla, 2003) should also be taken account as factors that complicate the use of

phylogenetic approaches to detect the selection pressures, however, recombination of rabies virus is rarely happening.

The average rate of nucleotide substitution as ~ 10^{-3} substitutions per site per year for RNA viruses has been suggested (Jenkins et al., 2002). The substitution rate of bat rabies virus variants estimated from partial N gene sequences in North America was identified to be in the range from 2.32 x 10^{-4} to 1.38 x 10^{-3} substitutions per site per year (Hughes et al., 2005). The substitution rates produced by analyzing partial P gene sequences in this study were 2.545 x 10^{-5} to 1.700 x 10^{-4} and 4.096 x 10^{-4} to 2.146 x 10^{-3} in *Eptesicus fuscus* and *Myotis* species, respectively. Davis et al. (2005) also indicated a low rate of nucleotide substitution of 5 x 10^{-5} substitutions per site per year, in EBLV-1, recently. Iverakk tgese clues indicate that bat rabies viruses exhibit a relatively low substitution rate compared to some other RNA viruses (e.g. HIV).

Bat Host Population Structure Determination

The study of bat populations has always been considered problematic due to lack of direct observational techniques and unique bat mating and dispersal tactics (Dempster, 1975). Molecular genetic techniques, like microsatellite loci and mitochondrial DNA analysis, have contributed to the demonstration of population structure within the order (Burland and Wilmer, 2001). However, to date, only seven of 18 Chiropteran families, including both migratory and non-migratory bats, have been studied for population genetic structure. Migratory species definitely showed very low genetic structure which may not be unexpected given their lifestyle. However, from their biology and some previous population genetic studies of sedentary bat species, well-defined population boundaries or strong genetic differentiation by distance might have been expected.

Although *Eptesicus fuscus* and *Myotis lucifugus* are very common bats in many parts of Canada, no information on their population structure is available. Both species of bats are non-migratory and living in large colonies, such that strong population sub-structures would be expected for a collection of specimens distributed across the country. This study employed microsatellite loci, a widely recognized and utilized tool for population studies, to determine the genetic population structure of *Eptesicus fuscus* and *Myotis lucifugus*.

The population structure analysis of *Eptesicus fuscus* strongly supported two main groups which were clearly segregated geographically when the microsatellite dataset was plotted on a map. The geographic barrier between Ontario and Manitoba, as deduced by population analysis using microsatellites, divided Canadian bats of *Eptesicus fuscus* into two main genetic groups. The physical barrier of lakes between Manitoba and Ontario supported this deduction, for water gaps have been considered as effective barriers to gene flow among bat populations (Burland and Wilmer, 2001). It should be noted however that a lack of bat specimens submitted from Manitoba may be a shortcoming of this study that relied on specimens from a passive surveillance system.

The western group was comprised of bats from British Columbia, Alberta and Saskatchewan, whilst the eastern group consisted of bats from Ontario and Quebec. There is no genetic information of bat species *Eptesicus fuscus* in the Maritime Provinces.

According to the rabies virus P gene phylogeny, six viral variants circulated in *Eptesicus fuscus*, with one group covering samples from different geographic localizations across the country except for the province of Quebec. Using "Structure" software to analyse the microsatellite locus data, the presence of four to five sub-populations of *Eptesicus fuscus* was explored. At K=5, the samples of eastern side were partitioned into three groups with small membership in each group. The result of genetic partition supported the assumption of

K=4; when four genetic groups were identified, the eastern and western samples were clustered into two genetic groups, respectively. Mapping didn't indicate the further subdivision in eastern and western groups.

The seven polymorphic microsatellite loci isolated specifically for bat species *Myotis lucifugus* were also employed for population structure determination. In order to confirm the property of these microsatellite loci, the other DNA samples of *Myotis* species like *Myotis californicus*, *Myotis ciliolabrum*, *Myotis evotis*, *Myotis septentrionalis*, *Myotis volans* and *Myotis yumanensis* were also genotyped at these loci. There was no genetic clustering among different species of *Myotis* (data not shown). Moreover, the co-clustering of a single rabies virus variant from different species of *Myotis* was notable.

High heterozygosity in microsatellite loci reflects the high polymorphism of microsatellites. This would be expected for the repeat type of dinucleotide microsatellites examined in the current study, since microsatellite dinucleotide loci generally have a higher mutation rate than loci with tri- or tetrabase repeats (Chakraborty et al., 1997). At each microsatellite locus of *Eptesicus fuscus* and *Myotis* species, the observed heterozygosity was substantially lower than its associated expected heterozygosity. Such as observation is consistent with population structure and is at odds with the limited population structure predicted here using the "Structure" software. One explanation for this effect might be provided by a "stepping stone" model (Burland, 1998) which proposed that non-migratory bats carry genes that flow continuously via short distances through a large population.

However, not all non-migratory species have displayed evidence of population structure at considerable geographic scales. Among 12 non-migratory species studied for population genetic structure previously, at most four species showed some genetic differentiations among populations with Fst 0.01 to 0.63 (Burland and Wilmer, 2001).

These studies also emphasized certain characteristics of bat biology which significantly impact the identification of bat population boundaries. Seasonal migratory behaviour led to no genetic structure found in migratory species over broad distances up to 1800 km (Wilkinson and Fleming, 1996). Studies on sedentary species didn't support the existence of population structure; furthermore, the occurrence of effective gene flow among populations of vampire bats (*Desmodus rotundus*) was confirmed (Honeycutt et al., 1981). The powerful homogenising force in gene movement was observed by a hierarchical analysis of genetic differentiation among colonies within adjacent regions for the non-migratory species *Plecotus auritus* (Burland, 1998; Peterson, 1996; Rousset, 1997). Although physical barriers have been assumed to limit gene flow, studies on populations of *Artibeus jamaicensis* and *Artibeus lituratus* on Caribbean islands indicated that some bat species could overcome even the most seemingly difficult physical barriers, extensive water channels, to permit gene flow (Phillips et al., 1989; Phillips et al., 1991; Pumo et al., 1988). The mating system of bats, such as multiple mating by females and high levels of skew in male reproductive success has also been blamed for low colony relatedness and low levels of genetic differentiation in most bat species (Burland and Wilmer, 2001).

Partial sequences of the mitochondrial cytochrome oxidase subunit I (Cox-1) gene sequences have been successfully used to investigate phylogeographic patterning of the fresh water fish species, *Sida crystallia* (Cox and Hebert, 2001). Segments of these Cox-1 gene sequences provided accurate species and subspecies identification of many mammals (Ward et al., 2005). However, different regions of Cox-1 gene have been studied in different animal species. The direct sequencing of the mitochondrial Cox-1 gene was also applied to DNA samples of *Myotis lucifugus*, and a phylogeny generated using one specific portion of the Cox-1 gene sequence with length of 552 nucleotides was analysed. Unfortunately the

phylogenetic analysis of the mitochondrial Cox-1 gene failed to provide additional genetic evidence to support the existence of population structure for *Myotis lucifugus* in Canada, despite distinguishing certain specimens from British Columbia.

Rabid Bats and Human Rabies

Bat rabies is endemic throughout North America, but only 1% of total bat population are rabid. Most recent human rabies cases in the USA have been caused by bat variant strains of rabies virus. Three deaths of humans infected with rabies virus via rabid bats have also been reported in Canada since 1985. The species and distribution pattern of bats between Canada and the USA are somewhat similar. In addition, migration of bats across the border almost certainly occurs. Tracking human deaths attributed to bat rabies in Canada and the USA by genetic analyses confirmed that most of them were infected by *Lasionycteris noctivagans*, eastern pipistrelle (*Pipistrellus subflavus)* and *Eptesicus fuscus* bat virus strains.

Earlier research on the contribution of rabid bats to human rabies in the USA, identified that several species – *Eptesicus fuscus*, *Lasiurus borealis*, *Lasionycteris noctivagans*, *Tadarida basiliensis* (Brazilian freetail bat) and some *Myotis* bats - were associated with 19 out of 39 human cases and also indicated that rabies isolates circulating in *Eptesicus fuscus* are genetically heterogeneous - multiple clades generated by phylogeny (Smith et al., 1995). A recent study on rabies viruses from bat species in the USA (Shankar et al., 2005), confirmed a similar genetic classification in both the USA and Canada. Rabies virus transmission due to spillover of bat rabies virus to other bat species, to humans and to terrestrial animals and wildlife was clearly observed (Shankar et al., 2005). The last human death in British Columbia of Canada in 2003 was due to the *Myotis* variant (personal communication with Dr. Nadin-Davis, unpublished data). There is no question that Canada and the USA have a similar situation in terms of public health threat of bat rabies.

Correlation of Rabies Virus Variants and Bat Host Distribution

The identification of six rabies viral variants circulating in *Eptesicus fuscus* and one viral variant circulating in *Myotis* species in various locations in Canada indicated virus-host species specificity and specific geographical distribution.

For the six types of rabies viral variants circulating in *Eptesicus fuscus*, viral type I was located in Ontario, while viral type II was distributed in Quebec and Ontario. Viral type III and IV were present in British Columbia. Viral type V was found in the west, including west coast and Saskatchewan. Viral type VI was widely distributed across the country from the west coast to Ontario.

The bat host, *Eptesicus fuscus*, was genetically clustered into two major populations, population I (East) containing bats from the provinces of Ontario and Quebec and population II (West) including bats from the provinces of British Columbia, Alberta, and Saskatchewan.

Rabies viral variants I and II circulated in *Eptesicus fuscus* population I, while rabies viral variants III, IV and V circulated in *Eptesicus fuscus* population II, and rabies viral variant VI circulated in both populations. Although the number and range of samples was expanded using rabies virus negative specimens for microsatellite genotyping of bat hosts, the geographic maps for P gene and microsatellite distribution were similar.

By characterizing of rabies viral variants circulating in different bat hosts (*Eptesicus fuscus* and *Myotis* species), this study has successfully proved one of the hypotheses that the circulation of rabies genetic variants displays host species specificity. A correlation between rabies virus strains and bat host populations was demonstrated in *Eptesicus fuscus* bats. Because population (sub-population) structure was not demonstrated in *Myotis lucifugus*, the population study provided no support of RABV variants segregating within *Myotis* sub-populations. The different outcomes of the study on these two bat species probably resulted

from the different biologies of the two bat species, including such characteristics as mating system and moving distance.

Future Studies/Study Refinement

Population identification is one of the most important components in animal disease management. Bat-related cases of human rabies have been documented and reported recently. How to manage rabid bats is very crucial to prevent human death from rabies. Unfortunately, the determination of bat population structure is still in the dark. Because of the unique biological characteristics of bats, several factors like seasonal migration, paternity and mating system, geographical barriers to gene flow, and colony relatedness and genetic differentiation, together influence the study of bat populations (Burland and Wilmer, 2001).

Generally, microsatellite DNA evolves 20-100 times faster than mitochondrial DNA, therefore, microsatellite DNA markers should be a much more powerful tool for recording recent and contemporary events, while mitochondrial DNA would be more suitable for tracking the historical and past evolutionary patterns (Wan et al., 2004).

Previous study of *Myotis myotis* compared mitochondrial and microsatellite DNA markers and demonstrated a strong male-biased dispersal in bat genetic inheritance (Ruedi and Castella, 2003). mtDNA would be the first selected method to combine with microsatellites for bat population analysis. Despite the use of these established tools for population genetic studies, this study on Canadian *Myotis lucifugus* failed to identify substantial population structure of this bat species. The targeted segment of the Cox-1 gene of mitochondrial DNA does not appear polymorphic enough to conduct a population structure analysis of bat species *Myotis lucifugus*.

The microsatellites isolated and used in this study are all dinucleotide loci with very high genetic diversity. In addition to their confirmed high polymorphism, these

microsatellites should be very useful for bat host studies. Because of the diversity of microsatellites, trinucleotide and tetranucleotide repeat motifs of different microsatellites also can be considered for application together with dinucleotide microsatellites. The inability to determine population structure of *Myotis lucifugus*, suggests that more careful consideration of the DNA templates used for microsatellite isolation might be helpful. In this study, the DNA templates employed for microsatellite library creation of both species were obtained from bats of Ontario. It may be possible to enhance the efficiency of microsatellite markers by isolating microsatellite loci using different sources of DNA templates, such as from bats from British Columbia or the Maritime provinces.

The application of a combination of genetic markers, the use of different base composition of microsatellites, and targeting of mitochondrial DNA sequences of high polymorphism would be a very critical strategy to improve the chances for determining the population structure of these bats, especially, *Myotis* species.

Sampling of bat specimens is very important in any bat population study, thus understanding the distribution of bat colonies and the association between geographic and genetic distances are essential to estimate the bat population differentiation. Using available information for microsatellite loci and mitochondrial DNA fragments, and the handling of excellent population genetic analysis programs will make future studies more cost-effective.

Hopefully, the enhancement of these techniques can make genetic analysis of bat population structure more informative.

CONCLUSIONS

It had been already reported that distinct rabies viral strains were associated with different bat host species in North America, and in Canada in particular. This study addressed the further question of whether discrete rabies virus variants are associated with different subpopulation structure of their bat hosts. Drawing upon extensive material provided from bats for rabies diagnostic submissions, rabies virus positive and negative specimens of *Eptesicus fuscus* and *Myotis* species from across country were employed to examine this question. Phylogenetic analysis identified 7 discrete rabies virus variants associated with these bats, while methods of population structure analysis targeting microsatellite and mitochondrial DNA markers were applied to *Eptesicus fuscus* and *Myotis lucifugus* bats, two species of importance as rabies reservoirs in Canada.

The phylogenetic result of rabies virus P gene, indicating six viral variants circulating in *Eptesicus fuscus* and one viral variant circulating in *Myotis lucifugus*, strongly supported the evidence that bat rabies virus variants circulate in a highly species-specific manner in bat hosts, and *Eptesicus fuscus* and *Myotis* species are two distinct rabies virus maintenance reservoirs in Canada.

The studies comprising of genetic characterization of rabies virus and population structure of bat hosts of *Eptesicus fuscus*, which indicated viral variants I and II circulated *Eptesicus fuscus* population I, viral variants III, IV and V circulated in *Eptesicus fuscus* population II, and viral variant VI circulated in both populations, provided strong evidence that the correlation exits between rabies virus variant transmission patterns and their bat host sub-population structure.

Both nuclear and mitochondrial DNA markers failed to support the evidence of existence of population structure of *Myotis lucifugus* in Canada. The identification of viral

variant subdivision in *Myotis* species in Canada didn't obtain strong support due to small numbers of rabies virus positive specimens.

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APPENDICES

Appendix 1. All Specimens Used in This Study

SUBMISSION NO.	SPECIES	RABIES STATUS	PROVINCE	NOTE
BB89L00672	*Eptesicus fuscus*	positive	SK	2
BB89L00985	*Eptesicus fuscus*	positive	BC	2
BB89L01044	*Eptesicus fuscus*	positive	BC	2
BB89L01656	*Eptesicus fuscus*	positive	BC	2
BB89L01825	*Eptesicus fuscus*	positive	SK	1,2
BB89L01894	*Eptesicus fuscus*	positive	SK	2
BB89L01903	*Eptesicus fuscus*	positive	SK	1,2,4
BB89L02317	*Eptesicus fuscus*	positive	SK	1,2,4
BB89L02496	*Eptesicus fuscus*	positive	SK	2
BB90L01159	*Eptesicus fuscus*	positive	SK	2
BB91L01052	*Eptesicus fuscus*	positive	AB	2
BB91L01056	*Eptesicus fuscus*	positive	AB	2
BB91L01075	*Eptesicus fuscus*	positive	SK	2
BB91L01224	*Eptesicus fuscus*	positive	BC	2
BB91L01485	*Eptesicus fuscus*	positive	AB	2
BB91L01828	*Eptesicus fuscus*	positive	BC	2
BB92L01816	*Eptesicus fuscus*	positive	SK	1,4
LU92L01872	*Myotis lucifugus*	positive	BC	2
BB93L01017	*Eptesicus fuscus*	positive	AB	1,2
BB93L01798	*Eptesicus fuscus*	positive	AB	2
BB93L01833	*Eptesicus fuscus*	positive	BC	2
BB93L01890	*Eptesicus fuscus*	positive	AB	1,2
BB93L01950	*Eptesicus fuscus*	positive	BC	2
BB93L02011	*Eptesicus fuscus*	positive	BC	1
BB93L02111	*Eptesicus fuscus*	positive	SK	1
BB93L02139	*Eptesicus fuscus*	positive	AB	1,4
BB93L02244	*Eptesicus fuscus*	positive	AB	1,2
BB93L02268	*Eptesicus fuscus*	positive	AB	2
BB93L02272	*Eptesicus fuscus*	positive	BC	1,2,6
YU93L02414	*Myotis yumanensis*	positive	BC	1
BB93L02426	*Eptesicus fuscus*	positive	SK	1,2
BB93L02480	*Eptesicus fuscus*	positive	BC	1,2
BB94L00580	*Eptesicus fuscus*	positive	AB	1,2,4
BB94L00843	*Eptesicus fuscus*	positive	BC	1,2

BB94L01039	*Eptesicus fuscus*	positive	AB	1,2
SE94L01295	*Myotis septentrionalis*	positive	BC	1,5,6
BB94L01516	*Eptesicus fuscus*	positive	BC	2
BB94L01536	*Eptesicus fuscus*	positive	BC	1,2,4,6
BB94L01743	*Eptesicus fuscus*	positive	BC	1,2
BB94L01759	*Eptesicus fuscus*	positive	SK	2
BB94L01928	*Eptesicus fuscus*	positive	BC	2
BB95L00723	*Eptesicus fuscus*	positive	BC	2
BB95L00792	*Eptesicus fuscus*	positive	SK	1,2
BB95L00874	*Eptesicus fuscus*	positive	SK	1,2
BB95L00920	*Eptesicus fuscus*	positive	BC	1,2,4
BB95L00952	*Eptesicus fuscus*	positive	SK	1,2,4
BB95L01007	*Eptesicus fuscus*	positive	BC	1,4
YU95L01017	*Myotis yumanensis*	positive	BC	1
BB95L01057	*Eptesicus fuscus*	positive	BC	1
BB95L01103	*Eptesicus fuscus*	positive	AB	1,2
BB95L01305	*Eptesicus fuscus*	positive	AB	1,2,6
BB95L01382	*Eptesicus fuscus*	positive	BC	1,2,4
BB96L01111	*Eptesicus fuscus*	positive	BC	2
BB96L01139	*Eptesicus fuscus*	positive	BC	1,2,6
BB96L01179	*Eptesicus fuscus*	positive	BC	1
BB96L01181	*Eptesicus fuscus*	positive	BC	1,2,4
EV96L01358	*Myotis evotis*	positive	BC	1
BB96L01418	*Eptesicus fuscus*	positive	AB	1,2
BB96L01557	*Eptesicus fuscus*	positive	BC	1,2,4
LU96L01737	*Myotis lucifugus*	positive	BC	1,5,6
BB96L01752	*Eptesicus fuscus*	positive	BC	1,2,4
BB97L01097	*Eptesicus fuscus*	positive	AB	2
BB97L01405	*Eptesicus fuscus*	positive	BC	2
BB97L01771	*Eptesicus fuscus*	positive	AB	1,2,4
BB98L00884	*Eptesicus fuscus*	positive	SK	2
BB98L01072	*Eptesicus fuscus*	positive	AB	2
BB98L01172	*Eptesicus fuscus*	positive	BC	1,2,4
BB98L01202	*Eptesicus fuscus*	positive	BC	2
BB98L01380	*Eptesicus fuscus*	positive	SK	2
BB98L01517	*Eptesicus fuscus*	positive	AB	2
BB98L01766	*Eptesicus fuscus*	positive	BC	1,2,4
BB98L01773	*Eptesicus fuscus*	positive	SK	1,2,4
LU99L00631	*Myotis lucifugus*	positive	BC	1,2,5
BB99L00862	*Eptesicus fuscus*	positive	AB	1,2
BB99L01070	*Eptesicus fuscus*	positive	AB	1,2,4

EV99L01301	*Myotis evotis*	positive	BC	1,5,6
BB99L01395	*Eptesicus fuscus*	positive	BC	1,4
BB99L01477	*Eptesicus fuscus*	positive	BC	1,2
BB99L01514	*Eptesicus fuscus*	positive	BC	2
BB99L01518	*Eptesicus fuscus*	positive	BC	2
LU99L01631	*Myotis lucifugus*	positive	BC	1,2,3,5,6
BB99L01771	*Eptesicus fuscus*	positive	BC	1,2,4
BB99L01936	*Eptesicus fuscus*	positive	BC	1,2,4,6
BB99L02084	*Eptesicus fuscus*	positive	SK	1,2
BB00L00835	*Eptesicus fuscus*	positive	BC	1,2,4
BB00L01013	*Eptesicus fuscus*	positive	AB	2
SE00L01396	*Myotis septentrionalis*	positive	BC	1,5
BB00L01397	*Eptesicus fuscus*	positive	BC	1,2
BB00L01509	*Eptesicus fuscus*	positive	SK	1,2
BB00L01594	*Eptesicus fuscus*	positive	BC	1,2
BB00L01756	*Eptesicus fuscus*	positive	BC	1,2,4
BB00L01762	*Eptesicus fuscus*	positive	AB	1,2
BB00L01800	*Eptesicus fuscus*	positive	SK	1,6
BB00L01836	*Eptesicus fuscus*	positive	BC	1,2
YU00L01855	*Myotis yumanensis*	positive	BC	1
BB00L01869	*Eptesicus fuscus*	positive	SK	1,2,4
BB00L01966	*Eptesicus fuscus*	positive	SK	1,2
BB00L02097	*Eptesicus fuscus*	positive	SK	1,2
BB00L02266	*Eptesicus fuscus*	positive	SK	1
BB02L00393	*Eptesicus fuscus*	positive	BC	1
BB02L00586	*Eptesicus fuscus*	positive	BC	1,4,6
CI02L00604	*Myotis ciliolabrum*	positive	BC	1,5
BB02L00681	*Eptesicus fuscus*	positive	BC	1,4,6
SE02L00687	*Myotis septentrionalis*	positive	BC	1
BB02L00827	*Eptesicus fuscus*	positive	BC	1,4
BB02L00926	*Eptesicus fuscus*	positive	BC	1,2,6
EV02L01055	*Myotis evotis*	positive	BC	1,5
BB03L00468	*Eptesicus fuscus*	positive	BC	1,2
BB03L00506	*Eptesicus fuscus*	positive	BC	1,2,4
KE03L00671	*Myotis Keenii*	positive	BC	1,5
VO03L00692	*Myotis volans*	positive	BC	1
BB03L00770	*Eptesicus fuscus*	positive	BC	1,2,4,6
BB03L00771	*Eptesicus fuscus*	positive	BC	1,2
BB03L00781	*Eptesicus fuscus*	positive	BC	1,2,6
EV03L00798	*Myotis evotis*	positive	BC	1,5
BB03L00897	*Eptesicus fuscus*	positive	BC	1,2,6

BB03L01081	*Eptesicus fuscus*	positive	BC	1,2,4
EV03L01088	*Myotis evotis*	positive	BC	1,5
BB03L01097	*Eptesicus fuscus*	positive	BC	1,2,6
BB03L01217	*Eptesicus fuscus*	positive	BC	1,2,6
BB03L01422	*Eptesicus fuscus*	positive	BC	1,2
BB03L01524	*Eptesicus fuscus*	positive	SK	1,2
BB04L00025	*Eptesicus fuscus*	negative	AB	2
BB04L00213	*Eptesicus fuscus*	negative	SK	2
BB04L00215	*Eptesicus fuscus*	negative	BC	2
BB04L00227	*Eptesicus fuscus*	negative	BC	2
BB04L00266	*Eptesicus fuscus*	negative	BC	2
BB04L00290	*Eptesicus fuscus*	negative	BC	2
BB04L00291	*Eptesicus fuscus*	negative	BC	2
BB04L00298	*Eptesicus fuscus*	negative	BC	2
BB04L00327	*Eptesicus fuscus*	negative	BC	2
BB04L00372	*Eptesicus fuscus*	negative	BC	2
LU04L00374	*Myotis lucifugus*	negative	AB	2,3
BB04L00411	*Eptesicus fuscus*	negative	BC	2
LU04L00429	*Myotis lucifugus*	negative	AB	2,3
BB04L00438	*Eptesicus fuscus*	negative	BC	2
LU04L00457	*Myotis lucifugus*	negative	BC	2
BB04L00466	*Eptesicus fuscus*	negative	BC	2
LU04L00496	*Myotis lucifugus*	negative	BC	2,3
BB04L00546	*Eptesicus fuscus*	negative	BC	2
BB04L00548	*Eptesicus fuscus*	negative	BC	2
LU04L00550	*Myotis lucifugus*	negative	BC	2,3
LU04L00563	*Myotis lucifugus*	negative	BC	2,3
BB04L00564	*Eptesicus fuscus*	negative	BC	2
BB04L00579	*Eptesicus fuscus*	positive	BC	1,2
BB04L00590	*Eptesicus fuscus*	negative	BC	2
LU04L00610	*Myotis lucifugus*	negative	BC	2,3
LU04L00612	*Myotis lucifugus*	negative	BC	2,3
LU04L00615	*Myotis lucifugus*	negative	BC	2,3
LU04L00618	*Myotis lucifugus*	negative	BC	2
LU04L00646	*Myotis lucifugus*	negative	BC	2,3
LU04L00651	*Myotis lucifugus*	negative	AB	2,3
BB04L00676	*Eptesicus fuscus*	negative	BC	2
LU04L00679	*Myotis lucifugus*	negative	BC	2,3
LU04L00685	*Myotis lucifugus*	negative	BC	2,3
LU04L00699	*Myotis lucifugus*	negative	SK	2,3
LU04L00734	*Myotis lucifugus*	negative	BC	2,3

LU04L00742	*Myotis lucifugus*	negative	BC	2,3
LU04L00743	*Myotis lucifugus*	negative	BC	3
BB04L00745	*Eptesicus fuscus*	positive	BC	1,2,4
LU04L00749	*Myotis lucifugus*	negative	AB	2,3
LU04L00750	*Myotis lucifugus*	negative	AB	2,3
BB04L00764	*Eptesicus fuscus*	positive	BC	2
LU04L00773	*Myotis lucifugus*	negative	BC	2,3
LU04L00785	*Myotis lucifugus*	negative	BC	2
LU04L00796	*Myotis lucifugus*	negative	BC	2
BB04L00816	*Eptesicus fuscus*	negative	BC	2
BB04L00833	*Eptesicus fuscus*	negative	BC	2
BB04L00834	*Eptesicus fuscus*	negative	BC	2
LU04L00837	*Myotis lucifugus*	negative	AB	2
BB04L00843	*Eptesicus fuscus*	negative	BC	2
LU04L00848	*Myotis lucifugus*	negative	BC	2
BB04L00856	*Eptesicus fuscus*	positive	BC	1,2,4,6
BB04L00863	*Eptesicus fuscus*	positive	BC	1,2
LU04L00867	*Myotis lucifugus*	negative	BC	2,3
LU04L00872	*Myotis lucifugus*	negative	MB	2,3
LU04L00873	*Myotis lucifugus*	negative	BC	2,3
BB04L00884	*Eptesicus fuscus*	negative	BC	2
BB04L00892	*Eptesicus fuscus*	negative	SK	2
BB04L00898	*Eptesicus fuscus*	negative	BC	2
LU04L00908	*Myotis lucifugus*	negative	BC	2,3
LU04L00914	*Myotis lucifugus*	negative	BC	2,3
BB04L00926	*Eptesicus fuscus*	positive	BC	1
LU04L00945	*Myotis lucifugus*	negative	BC	2,3
LU04L00951	*Myotis lucifugus*	negative	AB	2,3
LU04L00952	*Myotis lucifugus*	negative	AB	2
LU04L00957	*Myotis lucifugus*	negative	BC	2,3
LU04L00958	*Myotis lucifugus*	negative	BC	2,3
BB04L00959	*Eptesicus fuscus*	negative	BC	2
BB04L00961	*Eptesicus fuscus*	negative	BC	2
LU04L00982	*Myotis lucifugus*	negative	BC	2
BB04L00989	*Eptesicus fuscus*	negative	BC	2
LU04L00993	*Myotis lucifugus*	negative	BC	2,3
LU04L01005	*Myotis lucifugus*	negative	BC	2,3
BB04L01010	*Eptesicus fuscus*	negative	BC	2
LU04L01011	*Myotis lucifugus*	negative	AB	2,3
BB04L01013	*Eptesicus fuscus*	negative	AB	2
LU04L01014	*Myotis lucifugus*	negative	AB	2,3

LU04L01021	*Myotis lucifugus*	negative	AB	2,3
BB04L01069	*Eptesicus fuscus*	negative	SK	2
LU04L01085	*Myotis lucifugus*	negative	SK	2,3
LU04L01091	*Myotis lucifugus*	negative	MB	2
BB04L01160	*Eptesicus fuscus*	positive	BC	2
BB04L01183	*Eptesicus fuscus*	positive	BC	2
BB04L01212	*Eptesicus fuscus*	positive	AB	1,2
EV04L01219	*Myotis evotis*	positive	BC	1,5
BB04L01228	*Eptesicus fuscus*	negative	BC	2
BB04L01263	*Eptesicus fuscus*	negative	SK	2
LU04L01276	*Myotis lucifugus*	negative	BC	2,3
BB04L01286	*Eptesicus fuscus*	negative	BC	2
LU04L01298	*Myotis lucifugus*	negative	BC	2,3
LU04L01303	*Myotis lucifugus*	negative	AB	2,3
BB04L01335	*Eptesicus fuscus*	negative	AB	2
LU04L01320	*Myotis lucifugus*	negative	BC	2
SE04L01384	*Myotis septentrionalis*	positive	ON	1,5
BB01N00308	*Eptesicus fuscus*	positive	ON	1,2
BB01N01402	*Eptesicus fuscus*	positive	ON	1
BB01N04119	*Eptesicus fuscus*	positive	ON	1
BB01N04120	*Eptesicus fuscus*	positive	ON	1,2
BB01N05594	*Eptesicus fuscus*	positive	ON	1,2
BB01N05669	*Eptesicus fuscus*	positive	ON	1
BB01N06144	*Eptesicus fuscus*	positive	ON	1,6
BB01N06900	*Eptesicus fuscus*	positive	ON	1,2
LU01N06956	*Myotis lucifugus*	positive	ON	1,2
BB01N06959	*Eptesicus fuscus*	positive	ON	1,2
BB01N07428	*Eptesicus fuscus*	positive	ON	1
BB01N07843	*Eptesicus fuscus*	positive	QC	1,2
BB01N08035	*Eptesicus fuscus*	positive	ON	1,2
BB01N08041	*Eptesicus fuscus*	positive	ON	1,2
BB01N08124	*Eptesicus fuscus*	positive	ON	1,2
BB01N08184	*Eptesicus fuscus*	positive	ON	1
BB01N08440	*Eptesicus fuscus*	positive	ON	1,2,6
BB01N08445	*Eptesicus fuscus*	positive	ON	1,2
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LU04N06731	*Myotis lucifugus*	negative	ON	2,3
BB04N06782	*Eptesicus fuscus*	positive	ON	1,2

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Note: 1: characterised at the rabies virus P gene locus; 2: used for microsatellite analysis; 3: applied for Cox-1 gene sequence analysis; 4: included in *Eptesicus fuscus* dataset for virus evolutionary analysis, 5: employed in *Myotis* species dataset; 6: samples compared with rabies virus reference samples

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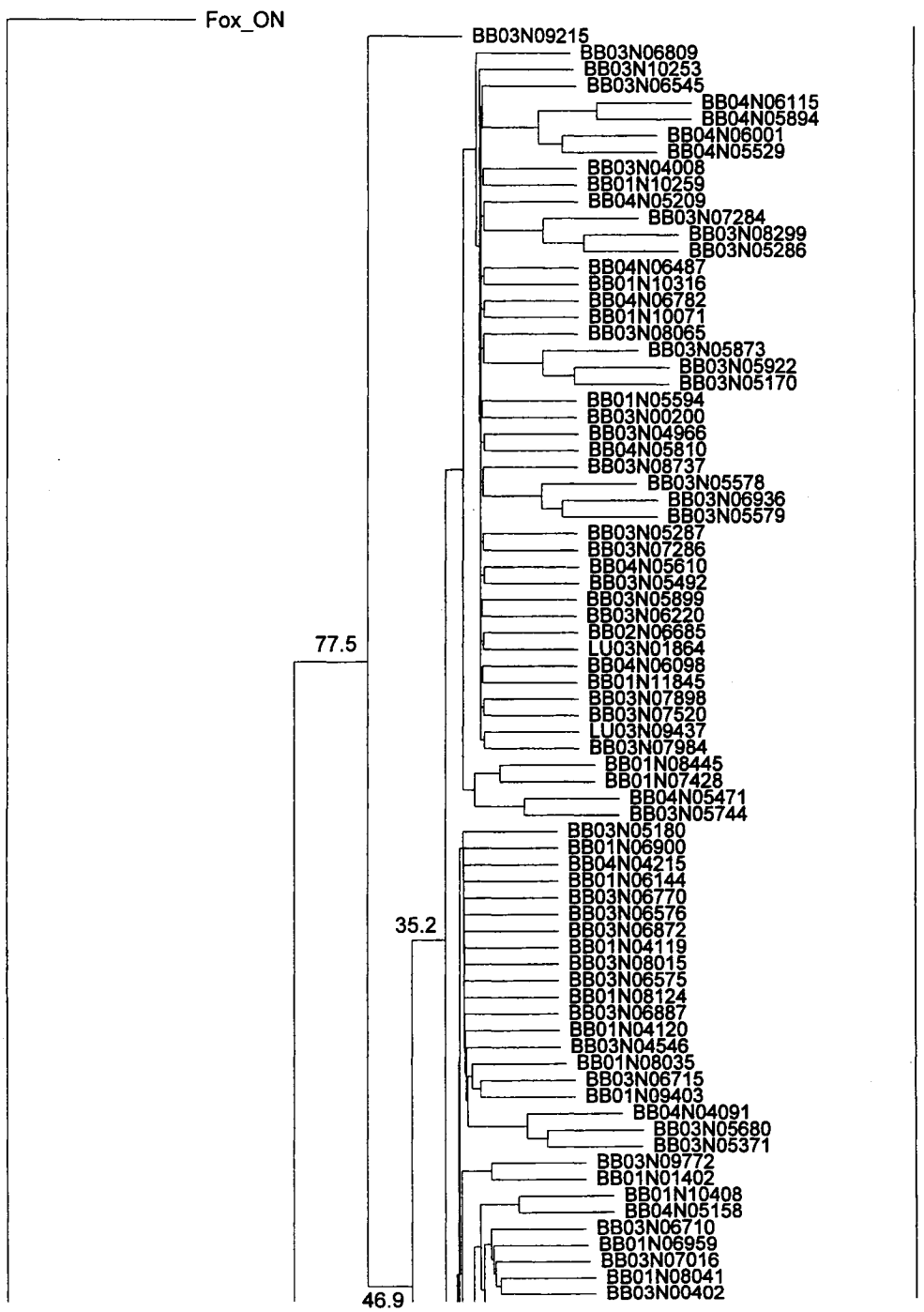
Appendix 3. Phylogenetic tree of 231 rabies viral isolates from *Eptesicus fuscus* and *Myotis* bat species (see Appendix 1). Analysis was by a NJ algorithm using 1000 bootstrap replicates of a 597 bp sequence of the central region of the P gene. Numbers to the left of a branch indicate the bootstrap value supporting the branch. Numbers on the right-hand side of the figure indicated the main phylogenetic groupings. Ontario fox rabies virus P gene sequence (Fox_ON) was the outgroup.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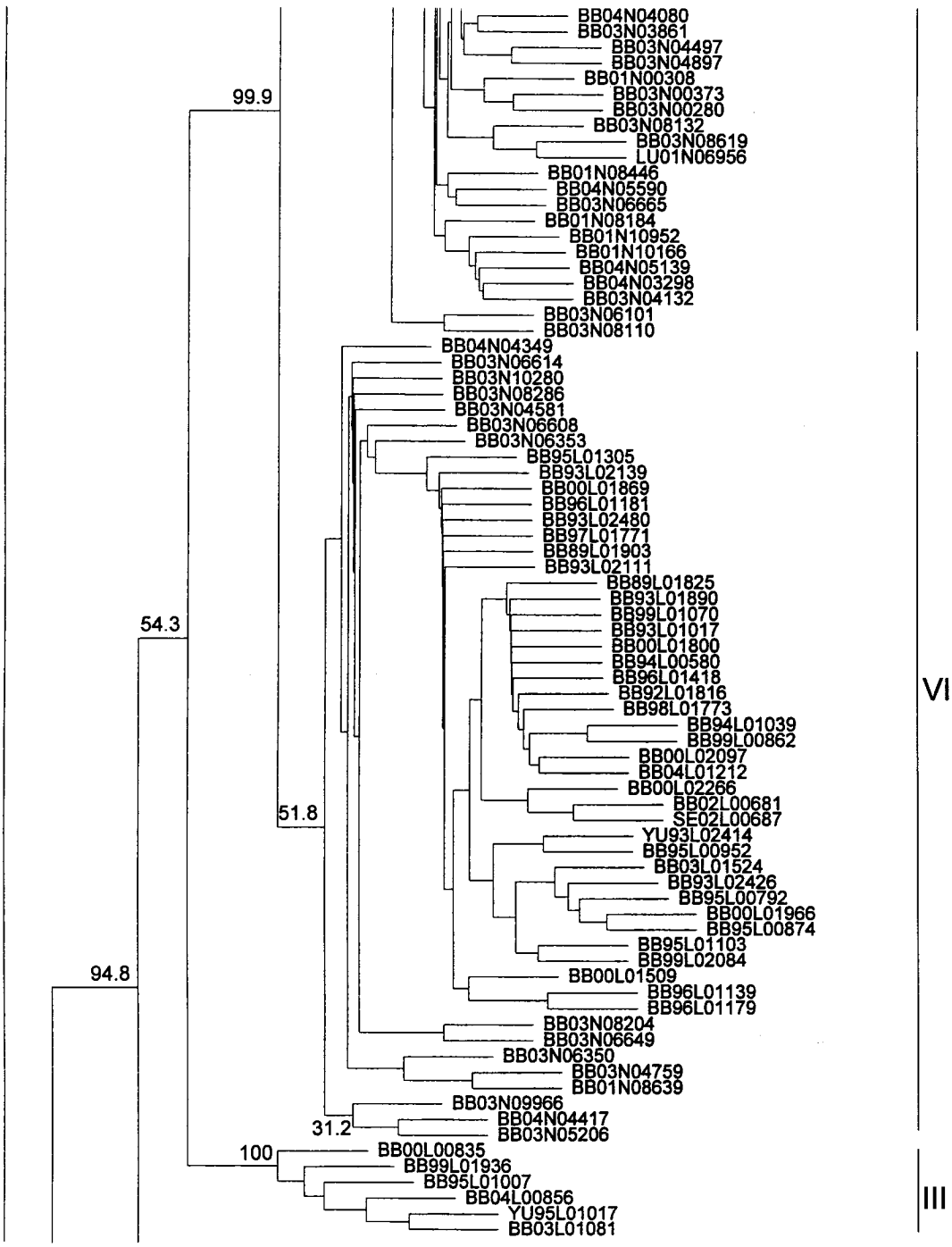
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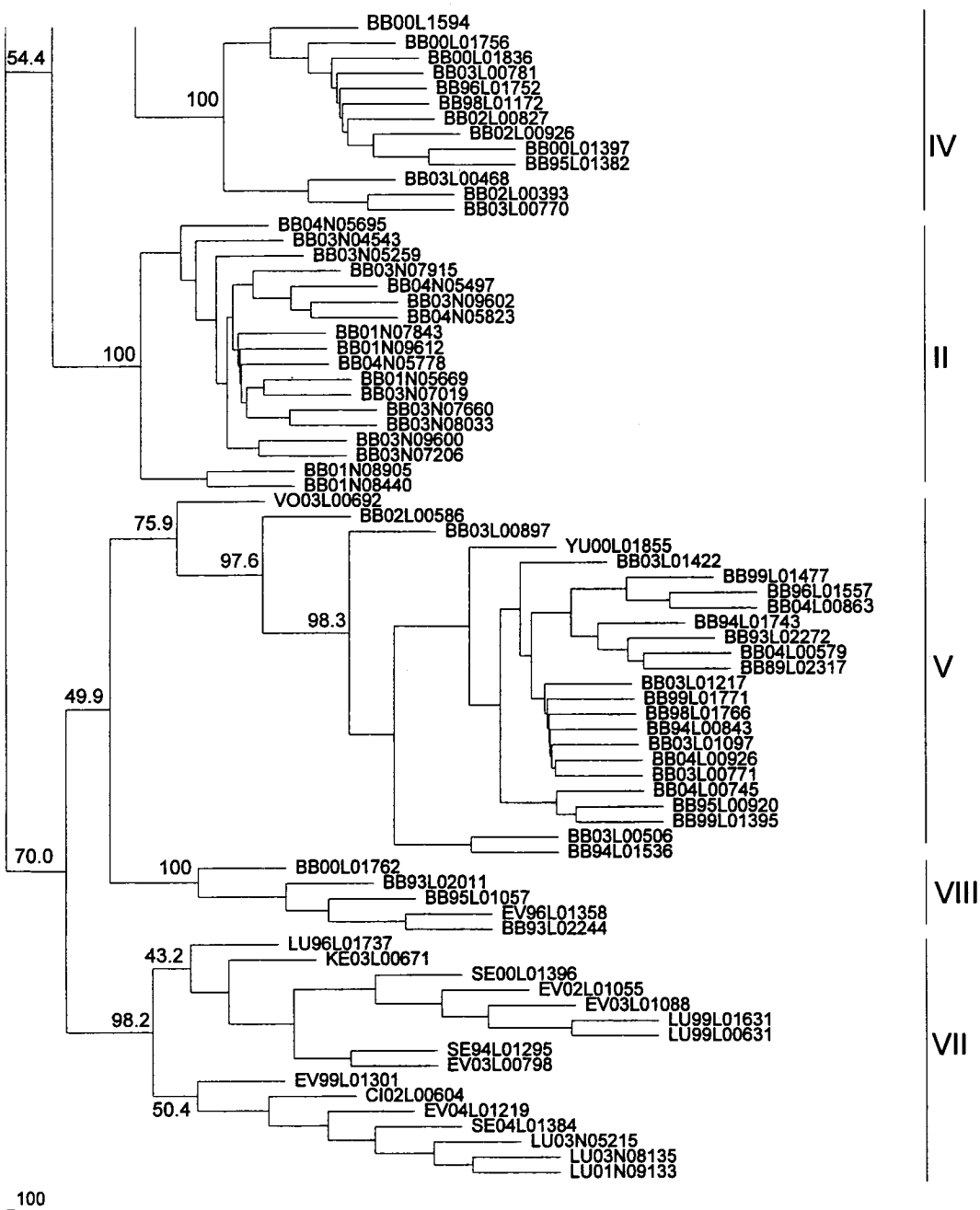
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IV
II
V
VIII
VII
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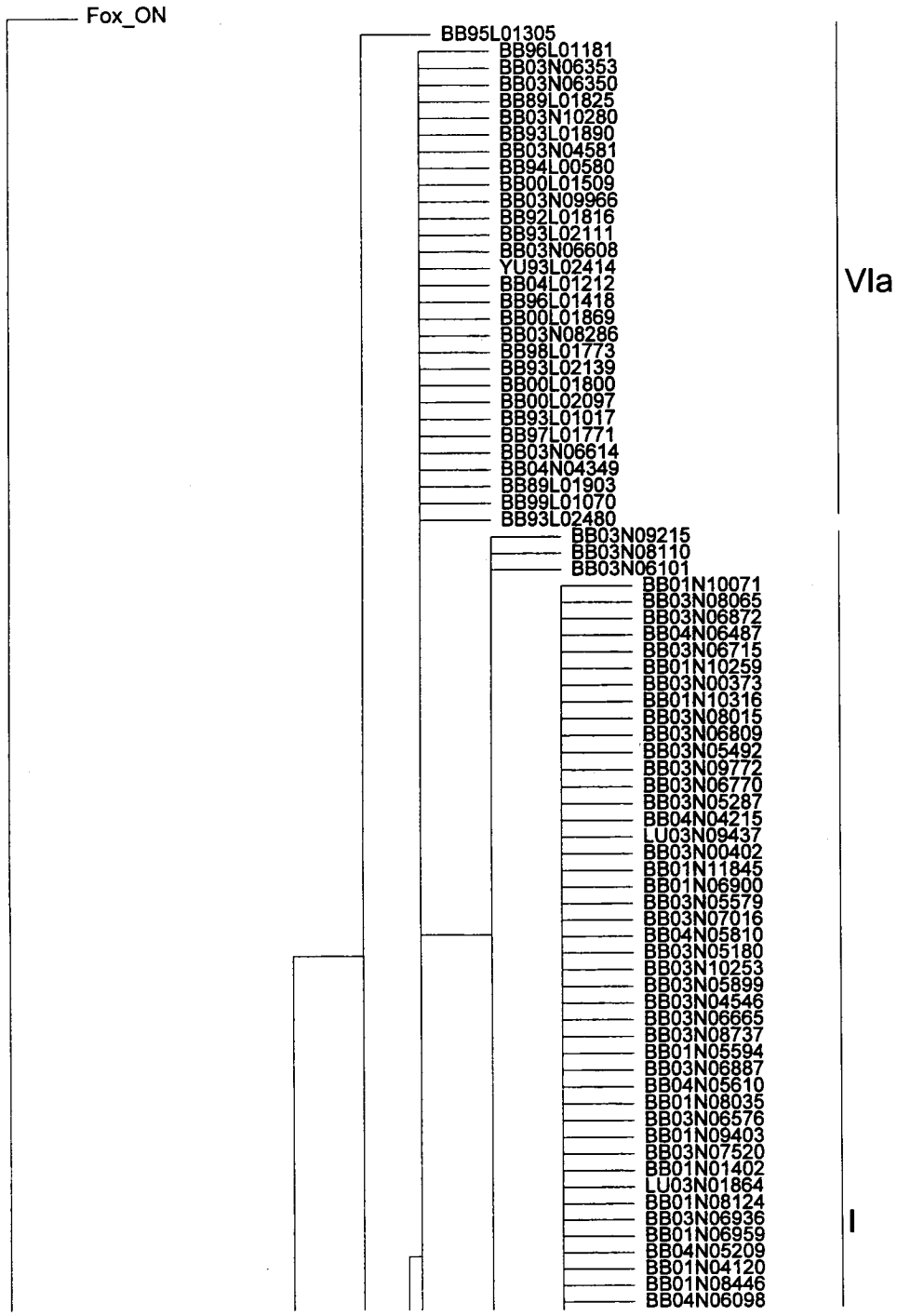
Appendix 4. Phylogenetic tree of 231 rabies viral isolates from *Eptesicus fuscus* and *Myotis* bat species (see Appendix 1). Analysis was by a MP method using a 597 bp sequence of the central region of the P gene. Numbers on the right-hand side of the figure indicated the main phylogenetic groupings and subgroupings, which correlated with the groupings predicted by NJ analysis (Appendix 3). Ontario fox rabies virus P gene sequence (Fox_ON) was the outgroup.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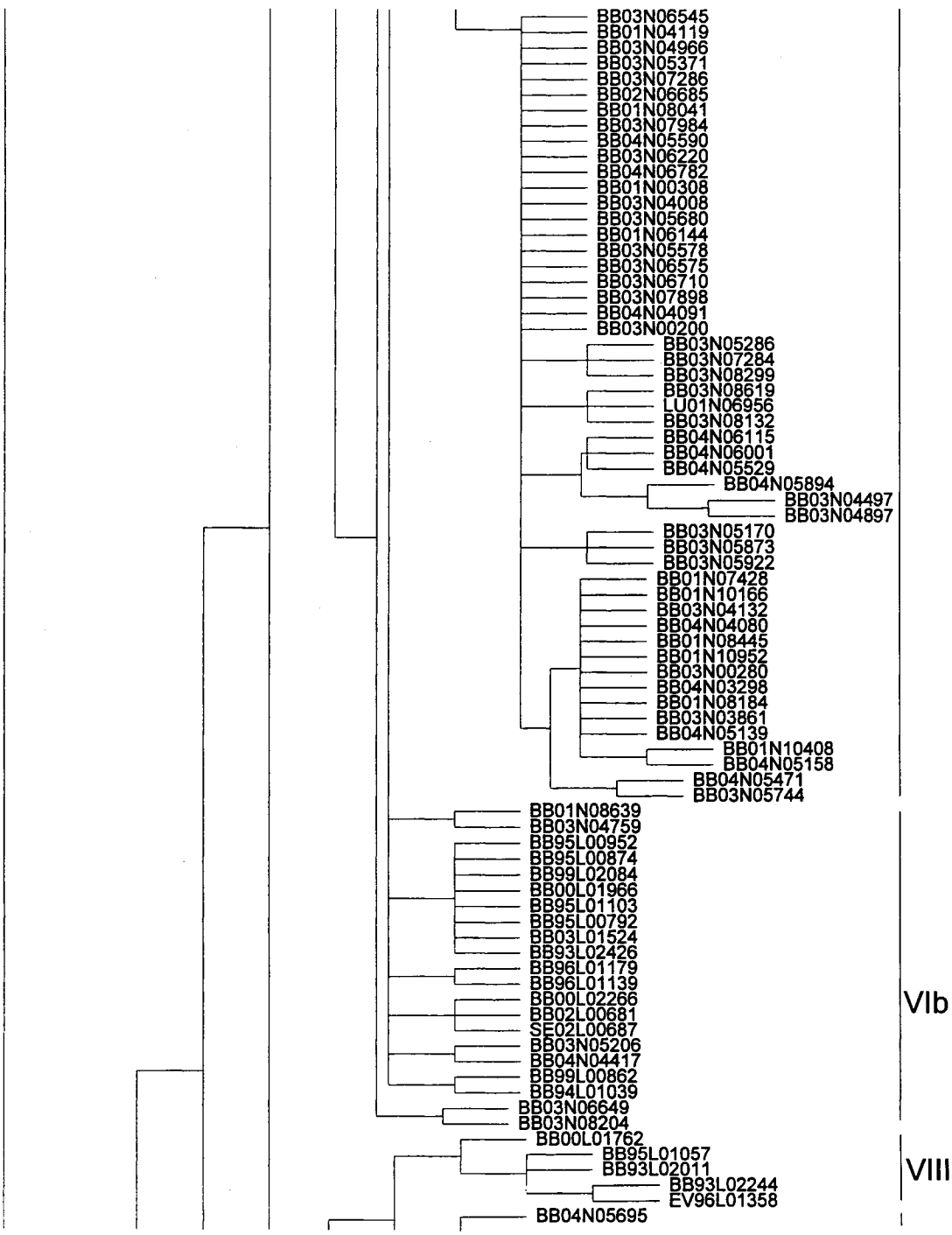
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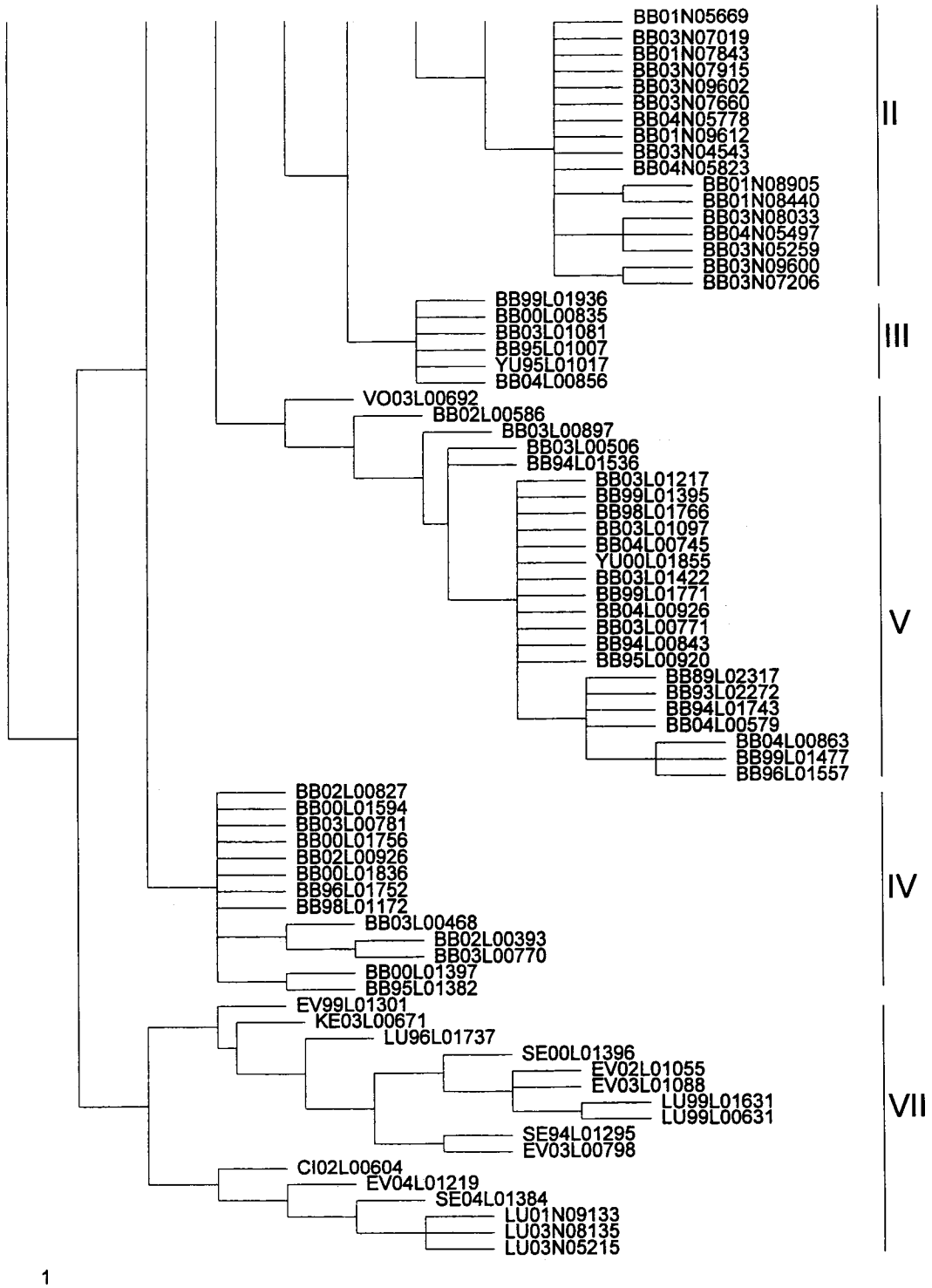
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VII
1

Appendix 5. Solutions Used in This Study

Ampicillin (100 mg/ml)

Dissolve 1 g of Ampicillin in 10 ml dH2O.
Sterilized by passage through a 0.45 micron filter and store aliquots at -20°C.

DEPCH_2O

Add 0.1 ml of DEPC (diethylpyrocarbonate) to 100 ml of dH2O in a glass bottle. Incubate at 37°C over night. Autoclave at 121 °C for 20 minutes.

0.5 M EDTA

For 100 ml
Dissolve about 1.4 g NaOH pellets in abut 60 ml of dH2O then add 18.61 g Na2EDTA. Continue adding NaOH pellets until the pH is about 8.0. Bring volume close to 100 ml then adjust the pH to 8.0 with 10 M NaOH. Autoclave at 121°C 20 minutes and store at room temperature.

Ethidium Bromide

5 mg/ml in dH_2O.

SOC Media

For 250 ml

Mix	5.00 g	bacto-tryptone
	1.25 g	bacto-yeast
	0.13 g	NaCl

Autoclave at 121°C for 20 minutes. Add 0.90 g glucose and 625 µl of 4M MgCl2, then sterilize by filtration through a 0.22 micron filter.

5 M NaCl

For 100 ml
Dissolve 29.22 g NaCl and dilute to 100 ml in dH_2O. Autoclave at 121°C for 20 minutes and store at room temperature.

3 M NaOAc

For 100 ml
Dissolve 40.81 g of NaOAc (sodium acetate.3 H_2O) in 80 ml of dH_2O and adjust the pH to 5.5. Bring volume of the solution to 100 ml and autoclave at 121°C for 20 minutes and store at room temperature.

20 % SDS

For 100 ml
Dissolve 20 g SDS and dilute to 100 ml in dH_2O.

20 X SSC (3 M NaCl, 0.3 M NaCitrate)

For 100 ml
Dissolve 17.55 g NaCl and 8.8 g NaCitrate (294.1 g /Mole) in 100 ml dH_2O.
Autoclave at 121°C for 20 minutes and store at room temperature.

1 M Tris

For 100 ml

Dissolve 12.11 g Tris-base MW=121.1 in 70 ml of dH_2O, then adjust the pH to 8.0 using concentrated HCL (around 1.5 ml). Dilute to 100 ml with dH2O. Autoclave at 121°C for 20 minutes and store at room temperature.

TLE (Tris-Low-EDTA) (10 mM Tris, 0.1 mM EDTA)

For 100 ml

Mix 1.0 ml Tris pH = 8
20 µl 0.5 M EDTA

Bring to final volume of 100 ml with dH_2O. Autoclave at 121°C for 20 minutes and store at room temperature.

TE (Tris-EDTA) (10 mM Tris, 1.0 mM EDTA)

For 100 ml

Mix 1.0 ml Tris pH = 8
200 µl 0.5 M EDTA

Bring to final volume of 100 ml with dH2O. Autoclave at 121°C for 20 minutes and store at room temperature.

X-Gal (40 mg/ml)

Dissolve 0.4 g of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) in Dimethyl Formamide in a sterile polypropylene conical tube to a final volume of 10 ml. Wrap the tube in aluminum foil to protect from light.

Direct DNA Purification Buffer

50 mM KCl
10 mM Tri-HCl (pH 8.8 at 25°C)
1.5 mM MgCl2
0.1 % Triton® X-100

Hybridization Solution

2 x Hyb Solution (12 x SSC, 0.2 % SDS)

For 100 ml

Mix 60 ml 20 x SSC
1 ml 20 % SDS

Bring to final volume of 100 ml with dH_2O.

1 x Hyb Solution (6 x SSC, 0.1 % SDS)

For 100 ml

Mix 30 ml 20 x SSC
500 µl 20 % SDS

Bring to final volume of 100 ml with dH_2O.

Washing Solution

2 x SSC, 0.1 % SDS

For 100 ml

Mix	10 ml	20 x SSC
	500 µl	20 % SDS

Bring to final volume of 100 ml with dH_2O.

1 x SSC, 0.1 % SDS

For 100 ml

Mix	5 ml	20 x SSC
	500 µl	20 % SDS

Bring to final volume of 100 ml with dH_2O.

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