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DISSERTATION

THE MOLECULAR EPIDEMIOLOGY OF BLUETONGUE VIRUS IN NORTHERN COLORADO

Submitted by
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In partial fulfillment of the requirements for the degree of Doctor of Philosophy
College of Veterinary Medicine and Biomedical Sciences
Colorado State University
Fort Collins, Colorado
Spring, 2001

Colorado State University

April 4, 2001

WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY DAVID MICHAEL WHITE ENTITLED "THE MOLECULAR EPIDEMIOLOGY OF BLUETONGUE VIRUS IN NORTHERN COLORADO" BE ACCEPTED AS FULFILLING IN PART THE REQUIREMENTS FOR THE DEGREE OF DOCTOROF PHILOSOPHY.

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ABSTRACT OF DISSERTATION

THE MOLECULAR EPIDEMIOLOGY OF BLUETONGUE VIRUS IN NORTHERN COLORADO

Economic losses due to trade restrictions based on BTV have been estimated to exceed $125 million U.S. per year in the U.S., and have been estimated to exceed $3 billion per year worldwide. Despite many years of study, the overwintering mechanism of bluetongue virus (BTV) has never been elucidated to a scientifically satisfactory degree. This is an important area of research because current trade agreements between the United States and its partners allow for the regionalization of disease entities (and their import/export restrictions) based on adequate scientific knowledge of the epidemiology of the disease.

Studies in this dissertation were guided by the hypothesis that BTV overwinters in its arthropod vector, *Culicoides sonorensis*. The overwintering mechanism of BTV was investigated using a variety of traditional virologic methods as well as current molecular methods. *In toto*, the studies were designed to provide a clear picture of the epidemiology of BTV on a microgeographic scale, thereby complementing other studies on a macrogeographic scale. Virus isolates were obtained from the USDA-Arthropod Borne Animal Diseases Research Laboratory (ABADRL) and from collections at the Arthropod-borne and Infectious Diseases Laboratory at Colorado State University (AIDL). Field isolates were characterized by phylogenetic analysis of sequence data from three genome segments (L2, S7, and S10). The viral isolates showed a degree of genetic identity inconsistent with other hypotheses concerning the overwintering mechanism of BTV. In addition, RT-PCR and Northern blot analysis of total RNA from cell-culture models of natural infection, using both mammalian cell lines and insect cells

obtained from ABADRL, were performed to characterize a possible mechanism of persistence of the virus in insect cells. Viral nucleic acid was detected in presumably uninfected cell cultures, as well as in field-collected culicoid larvae by RT-PCR, but not by Northern blot analysis. The previous target of field diagnostics (L2, which encodes the major antigenic determinant VP2) was not detected in insect cell cultures or field-collected larvae; this has obvious implications to the "diagnosis" of endemic and BTV-free regions of the country, as well as surveillance programs based on invertebrate collections.

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Acknowledgements

I would first like to extend my thanks to everyone who made these studies possible. My peace of mind (and occasionally sanity) was sorely tested at several points in the course of my education, and many are responsible for the fact that I still have both.

I am especially indebted to my co-advisors, Drs. Carol Blair and Barry Beaty, for their constant stewardship of my educational and scientific development. Their patience is the stuff of legends.

In addition, I thank the rest of my Committee on Graduate Work: Drs. Bill Black, Mo Salman, Dick Bowen, and Bill Wilson. Their help, advice, and guidance have been invaluable in this work.

Similarly, I would like to thank the scientists at the United States Department of Agriculture Arthropod Borne Animal Diseases Research Laboratory. The supplies and expertise made available to me by Dr. Jim Mecham, Linda McHolland, Lee Thompson, and Dr. Bill Wilson played a vital role in the success of this work.

I would also like to thank Dr. Laura Chandler, Cindy Oray, Pete Justice, Angie Mexas, Dr. Jon Rayner, Dr. Chris Bosio, Erin Searls and everyone else at the Arthropod-borne and Infectious Diseases Laboratory. Special thanks are due Dr. Joel Hutcheson for help with all of the phylogenetic analyses in this work.

I would also like to thank the USDA again for the Biotechnology Training Grant that funded my salary for most of this work, and the College Research Council for funding for materials and supplies.

Finally, I would like to thank my parents and other friends for love and support before, during, and after this work.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

REVIEW OF LITERATURE

HISTORICAL SIGNIFICANCE OF BT DISEASE

Bluetongue disease (BT) was first recognized as a disease entity in the scientific literature by Hutcheon in 1902(59). The disease was designated malarial catarrhal fever at the time, and the etiologic agent was suspected to be an insect-borne plasmodial parasite. However, the agent was shown to be filterable by Spreull in 1905(139). Early vaccination attempts were made by using serum from sheep that had recovered from the disease (passive transfer of immunity) and later by infection with an attenuated strain of the virus (Theiler strain, later serotype 4)(155). Beginning in 1924, BT was recognized outside the African continent, when outbreaks were reported in Cyprus. In 1943-44, a particularly virulent outbreak occurred there(36). This led to the recognition of BT as an emergent disease of sheep outside of Africa, with outbreaks occurring throughout the European continent and Asia. BT was finally recognized in the United States in Texas in 1948(48), and the virus was first isolated in the U.S. in California in 1952(87). Regulation of the movement of animals and the importation of animal products was then initiated by several countries, especially those with a large sheep industry that were BT free, such as Australia and New Zealand(37-39). However, BT eventually emerged in these countries as well(19, 112, 138, 140, 141), and the disease is now considered "pandemic" between the latitudes of 40^{O}N and 35^{O}S, associated with the range of the vector(s)(89).

ECONOMIC IMPORTANCE OF BT DISEASE

Bluetongue virus (BTV) causes BT in sheep and cattle; however, its main importance in the United States is economic. Direct losses (failure to gain weight before

sale, abortion, etc.) can exceed $125 million per year within the food and fiber industry; this figure does not, however, include indirect losses due to import/export restrictions on live animals and germplasm from endemic areas(94, 146). In addition, direct losses (death of adult animals, abortion) occur regularly in second and third world countries, with estimates reaching between $50-100 million to $3 billion per year *in toto*(17, 26, 106, 115, 116, 146).

New trade agreements between the United States and its partners (NAFTA, GATT) allow for the regionalization of disease entities (and their import/export restrictions) based on adequate scientific evidence. A large and growing body of evidence points to the co-localization of BT with *Culicoides sonorensis*. For example, BT is absent from the northeastern United States. *Culicoides variipennis* is the potential vector there, but it is resistant to oral infection when compared to *C. sonorensis*, which is the vector in BT endemic regions of the country (Figure 1.1, (52, 146, 148)). These and other data supporting regionalization of BT within the United States could only be strengthened by knowledge of the transeasonal maintenance mechanism of the virus in endemic foci. However, despite many years of study, the overwintering mechanism of BTV has never been elucidated to such a degree that regionalization could be achieved. The economic effects of regionalization could be profound, removing trade restrictions for a significant portion of the continental United States based on vector prevalence(52, 146) and the absence of a transeasonal virus maintenance mechanism.

MOLECULAR BIOLOGY OF BLUETONGUE VIRUS

Bluetongue is the prototype virus of the genus *Orbivirus* in the family Reoviridae. BTV has a bilayered, icosahedral capsid, which is mainly composed of virion proteins

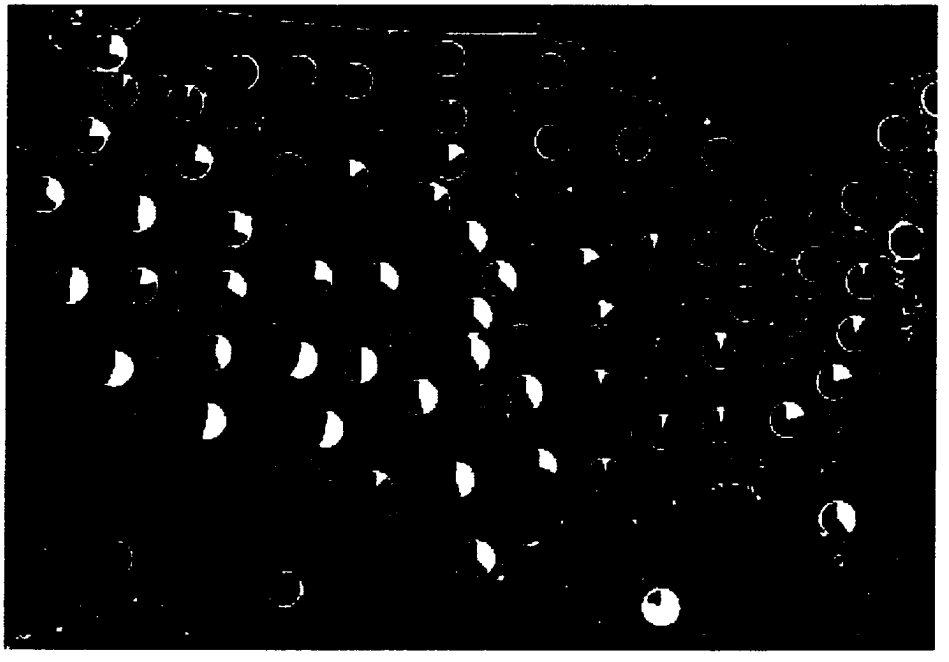

Figure 1.1(a): Seroprevalence of BTV in the United States.

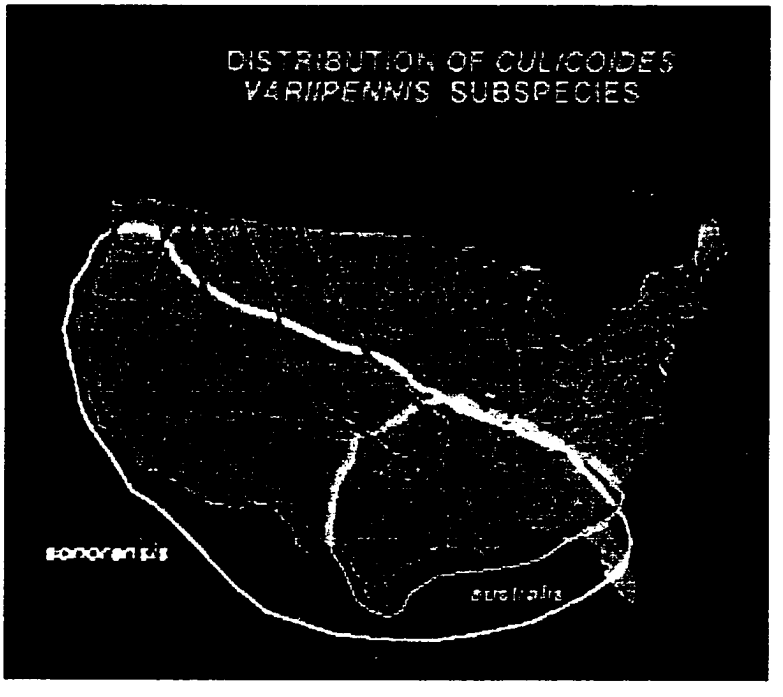

Figure 1.1(b): Distribution of *Culicoides* spp. in the United States. This map was generated before the recent data on the population genetics of *Culicoides*, so it includes some of the old species names. Both figures supplied by kindly by W. Tabachnick, USDA-ABADRL, Laramie, Wyoming.

(VP's) 2 and 5 (outer) and 3 and 7 (inner). It has 10 double-stranded ribonucleic acid (RNA) genome segments that encode a total of 11 proteins. The intact virion is 68-70 nanometers (nm) in diameter, has a sedimentation constant of 550S on rate zonal sucrose gradients, and a buoyant density of 1.36 grams/cubic centimeter in a cesium chloride gradient. There is also a "fuzzy" appearance to the intact virion revealed by electron microscopic examination, which is distinct from other orbiviruses and from other members of the Reoviridae that have a more clearly defined and structured outer layer. The core particle (VP's 2 and 5 removed) has a diameter of 54-58nm and a sedimentation constant of 470S with a buoyant density of $1.40g/cm^3$, and is stable during sedimentation in cesium chloride. The core particle is made up of 32 distinct capsomeres composed of VP7 trimers, each with a circular configuration, arranged in icosahedral symmetry with a triangulation number of T-3. They are tube-like, hollow structures about 8nm long and 10-12nm wide with an axial hole about 4nm in diameter. The characteristic ring-like structure of this assembly accounts for the genus name *Orbivirus* ("orbis" is Latin for ring or circle)(130).

Six hours post-infection, the core particles are further uncoated and subcore particles are exposed(57), which have a characteristic skeleton-like appearance with a hexagonally shaped outline. These subcores have a diameter of 40nm and appear to be made up of the scaffolding for virion assembly (VP3), the genomic dsRNA, and the non-structural VP's 1, 4, 6(57). Purification of subcore particles is possible, but they are very unstable and difficult to study(130).

The intact, infectious virion is composed of about 80% protein and 20% dsRNA. The genome base composition is 42.4% G/C, and there is no evidence of single-stranded

RNA in the intact virion. The outer capsid can be disrupted by appropriate salt and pH conditions, but is stable from pH 5.0 to 9.0 (optimum stability pH 8.0 to 9.0). Virions are also stable in lipid solvents and non-ionic detergents, and are strongly cell-associated in their natural state(130). In fact, there is evidence to suggest that freezing of cell-free virus will result in the loss of infectivity(81), while freezing in association with cultured cells or insects protects the virus(156, 172).

BTV genome segments are numbered according to their size as determined by electrophoretic migration through a polyacrylamide gel. Their gene products (and several relevant points about them) are summarized in the Table 1.1 below.

Bluetongue Virus – Antigenic Relationships

There are 25 serotypes of BTV based on serologic neutralization of infectivity and neutralization cross-reactivity of antibodies(130, 157). These are listed in Table 1.2. In Colorado, BTV serotype 11 is endemic, with occasional isolations of serotypes 10, 13, and 17 (presumably due to importation of infected livestock, but unknown trafficking mechanisms of the virus could exist)(119).

Relevant BTV Gene Product Functions for these Studies

Four genome segments (L2, L3, S7, S10) were selected for the assays in the following studies. These segments and their products will be discussed in depth to outline their relevance to this work. First, segments L3 and S10 were included in this work because they encode proteins that have been shown to be group-specific (reactive with all BT viruses), and have highly conserved nucleotide sequences that make good targets for the simple detection of viral dsRNA. L3 produces VP3, which is the scaffold for virus assembly and the protein to which all of the dsRNA segments bind in the intact

Structure/Function Relationships of BTV Genome Segments

Table 1.1

Gene Product	Characteristics	Segment Designation
Viral Protein 1	RNA-dependent RNA polymerase Non-structural, but required in the infectious virion	Large 1
Viral Protein 2	Mammalian cell receptor ligand Major antigenic determinant Outer shell protein Carboxy terminus is highly conserved (interaction with VP5?)	Large 2
Viral Protein 3	Conserved over time and location and between serotypes Protein scaffold of the inner core	Large 3
Viral Protein 4	mRNA capping function Non-structural, but required in the infectious virion	Medium 4* Medium 1
Viral Protein 5	Not highly conserved Secondary antigenic determinant Outer shell protein	Medium 5* Medium 2
Viral Protein 6	mRNA binding protein (role in viral assembly) Present in virus Inclusion Bodies ATP-dependent helicase activity (double-stranded RNA) Non-structural, but present in the infectious virion	Small 9* Small 3
Virus Protein 7	Tertiary antigenic determinant Not highly conserved Portion of the N-terminus accessible from the virion surface Interacts both with VP3 (inner core) and VP's 2 & 5 Insect cell receptor ligand	Small 7* Small 1
Non-Structural 1	Highly conserved over time and location Highly structured disulfide bonds (high cysteine content) Present in virus inclusion bodies (main constituent of the tubules)	Medium 6* Medium 3
Non-Structural 2	Single-stranded RNA binding activity Present in virus inclusion bodies	Small 8* Small 2
Non-Structural 3	Highly conserved over time and location Expressed at very low levels Membrane protein (role in viral release from cell?)	Small 10* Small 4
Non-Structural 3A	Expressed from an alternative initiation site in S10 Same roles/functions as NS3	Small 10* Small 4

Table drawn from the following references: (15, 48, 60-62, 120, 128, 129, 131, 143, 166, 169, 174, 175). * Some segments have two designations because of older and newer naming schemes in the literature. It is currently correct to number the segments 1-10, with L, M, and S denoting their relative size grouping upon PAGE (e.g. L1 through S10).

Serotypes of BTV
Table 1.2

Serotype	Prototype Strain	Year of Isolation	Location of Isolation
1	Biggarsberg	1958	South Africa
2	Ermelo 22/59	1959	South Africa
3	Cyprus Sample B	1943	Cyprus
4	Vaccine Batch 603	1900	South Africa
5	Mossop	1953	South Africa
6	Strathene	1958	South Africa
7	Utrecht	1955	South Africa
8	Ermelo 89/59	1959	South Africa
9	University Farm	1942	South Africa
10	Ermelo 91/59	1959	South Africa
11	Nelspoort	1944	South Africa
12	Byenespoort	1941	South Africa
13	Mt. Currie	1959	South Africa
14	Ermelo 87/59	1959	South Africa
15	Onderstepoort 133/60	1960	South Africa
16	Hazara	1960	Pakistan
17	Wyoming 2790	1962	United States
18			South Africa
19			South Africa
20	CSIRO 19	1975	Australia
21	CSIRO 154	1979	Australia
22		1982	Australia
23	DDP 90	1982	Australia
24			South Africa
25	I-134	1965	Kenya

Table drawn from the following references: (20, 44, 55).

virion. S10 produces NS3 and NS3a, which are necessary for egress of the virus from the infected cell. S10, however, was included for another reason as well: phylogenetic analysis of its sequence has been shown to group the respective isolates into geographic clusters(7). This "topotypic" quality will be addressed with the analysis; for now, suffice it to say that the geographic distances involved in those analyses could best be described as macrogeographic (greater than 30 miles) so those clusters are most probably not present when analyzed on the microgeographic scale of this work (less than 30 miles). This effect would be most easily explained by an issue of sensitivity – it is expected that the viruses within the natural range of the vector (approximately 30 miles [F. Holbrook, personal communication]) would be homogeneous.

Second, L2 has been the target of most phylogenetic analyses previous to the work of Bonneau et al.(7), and it was the initial target of these Colorado analyses as well. This genome segment was chosen for that role because most of the other gene segments are relatively conserved, and L2 is acknowledged as the segment that encodes the most phylogenetically relevant data(21). This is most likely due to the fact that its encoded protein, VP2, functions as the vertebrate cell receptor ligand and that it is prominently located on the exterior of the virion. These facts make it the most common target of the vertebrate immune system. However, as will be shown, this genome segment makes a poor target for the detection and/or isolation of viral dsRNA from insects or insect cell cultures.

Finally, S7 encodes VP7, which is the most likely candidate for the insect cell receptor ligand(4, 173). As such, a portion of it is accessible (but not necessarily exposed, *per se*) from the surface of the virion and is subject to similar selective pressures

as L2, although to a lesser degree because of its relatively protected position on the surface. There is evidence that after the intact virion is ingested from the blood of the infected vertebrate host and subjected to proteolysis in the midgut of the vector, VP7 is exposed (the infectious subviral particle is generated) and can then complete its role in the entry of the virus into midgut cells(93). If true, this would indicate that VP7 is not a *primary* target of the vertebrate immune system, as antibodies directed against it are not capable of neutralizing infectivity of the virus (for the vertebrate animal). However, VP7 can be used as a group-specific antigen(1), and as such must be accessible to naturally occurring antibodies. The remaining portion of the sequence (that part which encodes the portion of the protein not exposed on the surface) shows the same conservation seen with the other genome segments. VP7 also varies between certain serotypes, and therefore has the ability to differentiate between some serotypes at the nucleic acid level.

The BTV structure and function information presented above suggest the following hypothesis about the structure of the virion and its life cycle: VP's 2 and 5 represent the outer, "vertebrate" part of the virus, and VP's 3 and 7 represent the inner, "invertebrate" part of the virus. The outer part is composed of those gene products necessary for vertebrate cell infection: VP2 binds to the vertebrate cell and mediates entry, and VP5 provides a scaffold for the correct presentation and orientation of VP2. Similarly, VP7 binds to the insect cell and mediates entry, and VP3 provides the scaffolding for its correct presentation and orientation, as well as binding the dsRNA. This hypothesis is supported by the atomic structure of VP7(4) in that it has a mixture of rigidity and flexibility, which indicate an ability to undergo the conformational changes necessary for receptor binding and mediation of cell entry. In addition, subviral particles,

which lack VP's 2 and 5, are more infective for culicoid cells than the intact virions. In contrast, the subviral particles are significantly less infective than the intact virion for mammalian and mosquito (C6/36) cells(92, 93).

Epidemiology of BTV

BTV is maintained in a cycle of transmission between vertebrate and invertebrate hosts that is interrupted annually by inclement climatic conditions (winter in the temperate regions of the world, and dry seasons in the tropical areas of the world)(43). Many vertebrates can be infected by BTV, and can serve as amplification hosts. These include domestic sheep, cattle, and goats, as well as wild ruminants such as white-tailed deer, bighorn sheep, pronghorn antelope, and elk(51, 130, 144, 159, 171).

As discussed above, the principal invertebrate vector of BTV in the United States is recognized today to be *Culicoides sonorensis*(52, 146). *Culicoides* spp. are the true biological vectors of BTV, although the virus may occasionally be transmitted by ticks, mosquitoes, or sheep keds in epizootics(78, 90, 145). BTV can also be transmitted iatrogenically, as in the case of a contaminated canine vaccine preparation that caused the abortion of litters and acute death in pregnant bitches(30, 76, 110, 170). These minor transmission methods are considered to be inconsequential to the long-term maintenance of the virus, but can be important in outbreaks. On the other hand, the cause of the import/export restrictions noted above is transmission between vertebrates via infected semen or transplacentally(9-12, 144).

BTV Infection and Pathogenesis in Vertebrate Hosts

The natural disease is most severe in domestic sheep, with morbidities ranging up to 75% and case fatality rates up to 100% in lambs and 50% in adults in epizootics.

However, the disease is usually clinically mild (1-2% morbidity, 0% mortality) in sheep in endemic areas(27, 54). The disease is also usually clinically mild in adult cattle (morbidity less than 5%, 0% mortality(53)) with the most serious effect being abortion of fetuses less than 145 days old(130, 158). From roughly 5 months to 7.5 months of gestation, infection of the fetus (either directly or by infection of the cow and transplacental transmission) can lead to immunotolerance later in life(130). However, this is not due to persistent infection of the fetus or neonate, but to a different type and magnitude of immune response as compared to the naïve, immunocompetent animal(79). BTV has been reported to cause severe disease in other vertebrate hosts(127, 130).

The fatal disease in sheep is characterized by the variable appearance of: pyrexia of 1-14 days (average 5-7 days); cracking of the epidermis - especially around the coronary bands, teats, and oral mucous membranes and mucocutaneous border with encrustation along the commisures of the lips and the nasolabial plane (possibly progressing to chronic ulceration with secondary bacterial infection); laminitis; edema of the face, submandibular space, eyes, and eyelids (continuing down along the ventrum of the animal); petechial and/or ecchymotic hemorrhages on the mucous membranes; and a swollen, congested, and/or cyanotic tongue (which gives the disease its name). In addition, there can be generalized stiffness, lethargy, anorexia, and vomiting (with occasional aspiration pneumonia). The final stages of the fatal disease are characterized by ischemic damage to all major organ systems, and eventual death through a variety of pathways depending on which systems are most severely affected; however, the most common cause of death is secondary pneumonia(130).

The target cell type of the virus is the endothelium(18), and the pathogenesis of the virus can be traced to ischemic damage to all body systems, starting with the small capillaries of the dermis and moving inward to the larger vessels and body systems. BTV appears to concentrate in the smaller capillaries of the extremities because of their lower temperature in relation to the interior of the body(130). Initial replication of the virus appears to occur in the lymphoreticular system, spreading to the endothelium (as well as periendothelial cells and pericytes of the capillaries) in close association with erythrocytes. In fact, one of the defining characteristics of BTV infection is its close association with red blood cells (RBC's); the virus can coexist in blood with neutralizing antibody, shielded from neutralization in the rugae of the RBC membrane(130) or within the RBC itself(104). In an evaluation of the vertebrate immune response(124), neutralizing antibodies were first detected in sera collected at day 14 post-inoculation (PI) from BTV-infected calves and lambs. However, infectious virus and specific neutralizing antibody coexisted in peripheral blood of infected lambs and calves for as long as 28 days. Furthering this work, an examination of the specificity and intensity of the humoral immune response found that antisera reacted most consistently with BTV structural proteins VP2 and VP7 and nonstructural protein NS2. In addition, it was found that the intensity of the response was inversely related to the duration of the viremia.

The viremias that develop in the clinical course of BTV infection vary between the species. Viremias in experimentally infected cattle ranged from 1.8-6.6 $\log_{10}$ $CELD_{50}$/ml blood (chicken embryo lethal dose 50%) and lasted from 2-24 days post-infection (p.i.)(80). Peak viremias were from 4-19 days p.i. However, virus could be isolated by inoculation of naïve sheep out to 50 days p.i. by the transfer of 25ml of fresh

blood from the infected cattle, but not past 24 days p.i. by intravenous inoculation of embryonated chicken eggs (ECE). All infected cattle developed neutralizing antibody titers by the 21st day p.i. Only 3 of 10 infected cattle showed any clinical signs, which were minor: a brief period of anorexia for one, and cutaneous lesions for the other two). Viral blood titers were comparable after infection by either intradermal inoculation of the virus (7 animals) or the bite of infected *C. sonorensis* (3 animals).

Similarly, the viremia of BTV20 seen in sheep ranged from 2.33-4.70 $\log_{10}$ $CELD_{50}$/ml blood, and lasted for no more than 10 days. Surprisingly, when infected with BTV17, the viremias ranged from 5.33-5.67 $\log_{10}$ $CELD_{50}$/ml blood and lasted up to 20 days(47). In another study using both sheep and goats, the viremias were not quantified, but they lasted 27-54 days p.i.(74).

In an attempt to characterize the disease in wild ruminants, as well as their potential as reservoir hosts, experimental infection of pregnant elk cows resulted in viremias ranging from 2-3 $\log_{10}$ $CELD_{50}$/ml blood(144) that lasted for no more than 8 days. Infectious virus could not be recovered by allowing naïve culicoids to feed on the elk then feed on naïve sheep after an appropriate extrinsic incubation period for BTV. The sheep did develop lesions that suggested BTV infection, but they did not develop fulminant disease, and attempts at virus isolation from their blood were negative. However, the newborn calves of the elk cows were infected with BTV, which could be transmitted to sheep by naïve culicoids.

BTV Infection of *Culicoides* species

The infection in the invertebrate is non-cytopathic and persistent, which is characteristic of arboviruses in their biological vector species. As far as is known,

culicoids acquire the virus naturally only by feeding upon an infected and viremic vertebrate host. The vector competence of a number of *Culicoides* spp. has been investigated. Vector competence is a measure of the susceptibility to oral infection and subsequent ability of the vector to transmit the virus to a naïve vertebrate host. There are differences in vector competence between vector species (inter-specific) or populations within a vector species (intra-specific)(63). Seventeen species of *Culicoides* have been associated with BTV to date, and of those, only 8 are efficient vectors (*C. sonorensis, C. imicola, C. fulvus, C. actoni, C. wadai, C. mubeculosus, C. bolitinos and C. insignis*)(6, 8, 46, 64, 91, 96, 98, 109, 142, 160-165, 171). Anatomic barriers that condition vector competence include a combination of a midgut infection barrier, a midgut escape barrier, and a dissemination barrier, but not a salivary gland infection or escape barrier(35).

The productive infection of the vector follows a standard pattern of midgut infection and escape, followed by dissemination to other organ systems(16). Infected culicoids have been shown to have a whole-body titer of 5-6 $\log_{10}$ of virus, and to inject between 3 and 20 plaque forming units (PFU's) per bite, which is sufficient to infect a susceptible sheep(89). The extrinsic incubation period in competent vectors is 10-15 days, and infected vectors remain infected for life(34). Oral susceptibility to infection ranges from 0% to 51.6% within a species(63). This variance is presumably due to several factors including, but not limited to, the titer of the bloodmeal, the genetic susceptibility of the individual insect, and a constellation of environmental factors (temperature, etc.)(69). While some evidence exists that the titer of virus within an infected insect decreases with time(68), studies have shown that infection can last at least 35 days, and transmission can be accomplished up to 21 days after infection(34, 82). The

decrease in titer with age is probably not relevant in a natural setting, where the lifespan of the insect is estimated to be 14-21 days(58, 99).

BTV Overwintering/Transeasonality

BTV is maintained in nature in a cycle involving haematophagous insects and their vertebrate hosts. The virus can be maintained in year-round transmission cycles in tropical parts of the world, and even possibly in Southern California(40). However, such cycles are impossible in some years and in many locations outside the tropical belt due to environmental conditions(43). The virus must have a mechanism(s) that allows it to survive periods of inclement climatic conditions and vector diapause in temperate regions that experience an overt winter, as well as in tropical regions that experience a significant dry season. Three principal hypotheses have been proposed for BTV transeasonal maintenance: 1) high altitude, air-current based, reintroduction of infected insects from year-round cycles to endemic foci on a yearly basis, 2) survival in the vertebrate host, either through persistent infection of adults or transplacental transmission to fetuses, and 3) vertical transmission from the infected insect to its progeny. These hypotheses will be discussed in order.

Annual Reintroduction of BTV-infected Culicoids to Endemic Foci

The reintroduction of infected insects from continuous, tropical transmission cycles to endemic, temperate foci is mentioned here only for the sake of completeness(134). This mechanism was shown to be highly unlikely in several studies. Investigators have consistently been unable to show a positive link between weather patterns and BT outbreaks(13, 133, 136, 137). However, not all areas of the world have a "winter" (a time of inactivity for insects); indeed, several areas of the world have low-

level BTV activity year-round(102). Additionally, the introduction of BTV-infected culicoids into previously BTV-free, local areas on air currents could represent an important mode of transmission on a small geographic scale(107). However, this hypothesis is less tenable for annual reintroduction of BTV on a large-scale into temperate regions of the world. When considering the distances and other variables involved, it seems unlikely that the same genotype of BTV would be reintroduced to the same endemic focus each year. At the very least, reintroduction by infected culicoids would not seem to be relevant to the year-to-year maintenance of the virus in endemic foci.

Overwintering of BTV in Vertebrate Hosts

The second hypothesis – overwintering in the vertebrate host – is a more plausible mechanism for BTV transeasonal maintenance in endemic foci. However, an examination of the evidence also makes this an unlikely mechanism for the overwintering of BTV. Persistent infection of immunotolerant cattle by BTV has been recognized since 1975(53, 83), but this has been shown to be a rare event, which would be unlikely to be important in the year-to-year maintenance of the virus in a natural cycle(3, 41, 42, 84, 158). Long-term viremia following an acute infection would be more likely to be responsible for the reintroduction of the virus into a transmission cycle in a subsequent year. However, infectious virus cannot be isolated from infected cattle past 50 days, and viral RNA cannot be detected by RT-PCR past 160 days(71). In between those times, samples are RT-PCR-positive but virus isolation (VI) negative. Apparently, non-infectious virus or viral RNA exists in close association with (or inside) erythrocytes(104).

Studies were also conducted to determine if the bite of an uninfected insect could rescue virus that had eluded attempts at virus isolation(149). Naïve *Culicoides sonorensis* fed on cattle whose blood samples were positive by RT-PCR for viral RNA, but were negative for BTV by VI, did not become infected, nor did they transmit BTV to uninfected sheep upon taking subsequent blood meals after an appropriate extrinsic incubation period for the virus.

Assuming that animals with a long-term "viremia" (as above) cannot directly infect vectors, they would then have to be able to infect other vertebrates that could then serve as the reservoir for naïve vectors. The potential for venereal transmission of BTV has been investigated. Virus is shed in the semen of infected males (bulls or rams), but it can only be detected shortly after peak viremia (3-10 days post-infection) and is limited to the time of the peak alone. In addition, heifers impregnated with BTV infected semen seroconverted in only 4 of 9 cases, and none of the fetuses showed any pathology or sign of infection(11, 12). Viremias in infected calves have been shown to last as long as 20 weeks (by RT-PCR detection), but in the critical experiment, those viremias were shown to be infective for naïve *Culicoides sonorensis* for only 2 weeks(85). Thus, transplacental infection of BTV could represent a theoretical transeasonal maintenance mechanism for BTV; however, it would not reach the level essential for reliable, yearly maintenance of an arbovirus(154).

Finally, pharmacologic (hormone-induced or other), infectious (ovine lentivirus-induced), or stress-induced immunosuppression could be responsible for the establishment of persistent BTV infection in sheep(14) and as such could have implications in the transeasonal maintenance of BTV. While this is an intriguing

possibility, its importance in the year-to-year maintenance of the virus cannot be fully assessed at this stage. At first glance, it would seem to be a fruitful area of research; however, it has already been revealed that the bite of a naïve vector cannot "recover" latent virus from a "persistently infected" vertebrate host(149). Tabachnick et al. ((149), as described above) performed the critical experiment that closely approximated the natural situation (albeit in cattle). Hence the stress of the bite(s) and/or the immunosuppressive qualities of saliva are probably not factors in relation to this hypothesis.

On the other hand, the nature of a possible persistent infection in sheep, and its relation to the virus' ability to be transmitted to naïve culicoids would have to be investigated further before this possible overwintering mechanism of BTV could be discounted completely. In this regard, the bite of naïve culicoids caused a "'showering' of BTV into the blood stream" of a persistently infected bull(83). This possible overwintering mechanism of BTV has been discounted for various reasons (W. Tabachnick, personal communication); however, it could be re-investigated using newer techniques given these new data and hypotheses.

In toto, the evidence does not support the hypothesis that the virus overwinters in the vertebrate host. The few reports of transeasonality in the vertebrate host are probably inconsequential to the long-term, year-to-year maintenance of the virus in a natural cycle(71, 85).

Overwintering of BTV in the Invertebrate Vector

The final hypothesis – that BTV overwinters through vertical transmission of the virus by infected adult *C. sonorensis* to their overwintering, fourth instar larval progeny –

would seem to be the most likely of the three. In fact, preliminary studies support the validity of this idea: BTV has been isolated from adult midges (reared from overwintering larvae) from an endemic focus in northern Colorado before the start of the transmission season(23), BTV dsRNA has been detected in *C. sonorensis* larvae collected from the same focus (see Chapter 3 and (119)), and a persistent infection has been observed in a *C. variipennis* (or, most probably, *C. sonorensis*) cell line that was initiated from larvae collected from the same area((167, 168), W. Wilson, personal communication). There is some evidence to indicate that transovarial transmission (TOT) of BTV does not occur in *C. sonorensis*(68, 105). BTV antigen was detected in the vitelline membrane of infected, adult females and proteoid yolk bodies of their oocytes but not in the ovarian tissue itself – which would be necessary for ovarian transmission of BTV to progeny(TOT). However, this study did not preclude the possibility of vertical transmission of the virus through an anatomic structure other than the ovaries to the progeny. In fact, demonstration of BTV antigen that was "dense" in the reproductive structures in which it was found (as above, yolk bodies and vitelline membrane)(105) would suggest that such an event could occur. For example, vertical transmission of flaviviruses in mosquitoes occurs via the micropyle as the egg passes through the oviducts during oviposition(126). A similar mechanism could function for vertical transmission of BTV in *C. sonorensis*.

BTV Evolution in Nature

As a general rule, RNA viruses exhibit a high degree of genetic diversity when compared with DNA viruses. BTV possesses considerable evolutionary potential – the genome is both RNA and segmented, so BTV can evolve by intramolecular changes

(antigenic drift – e.g. point substitutions, etc.) and by intermolecular changes (antigenic shift – e.g. reassortment).

Antigenic shift contributes to the evolutionary potential of BTV. Indeed, genome segment reassortment has been demonstrated in the laboratory in vertebrate cells and in vectors (see below) and apparently occurs frequently in nature. For example, it is believed that the BTV-13 "prototype" virus is a reassortant virus produced from a dual infection with an original (natural) BTV-13 and the BTV-10 vaccine strain(111). However, the reassortment potential of the virus is limited by a time-dependent barrier to superinfection that is established by the primary virus. While there is evidence that such a barrier occurs in vertebrate cells after 4 hours(121), there is also evidence that the barrier in the insect vector occurs very late in the course of the infection (4-5 days after infection with the primary virus)(28). In addition, the insect vector is infected for life, whereas vertebrate animals could best be described as having transient infections (of various lengths, depending on the animal). This suggests that while reassortment can occur in both the vertebrate and invertebrate hosts, the insect vector is more permissive for dual infection and subsequent segment reassortment, and is therefore more likely to be the primary site for generation of viral diversity in natural populations of BTV(5, 28).

Antigenic drift is a catch-all phrase that includes a constellation of intramolecular modifications, including point mutations, translocations, insertions, deletions, etc. This evolutionary potential is most likely due to the lack of a proofreading function (exonuclease activity) in all identified viral RNA-dependent RNA polymerases (RDRP's). This error-prone replication system leads to the accumulation of point mutations in every generation of viral replication, with a nucleotide substitution

frequency as high as 10^{-2}(29). This frequency of mutation results in populations of RNA viruses that can best be described as "quasispecies". In a quasispecies, there are typically very few viruses in a replication cycle that are completely genetically alike(25). One of the best representations of this idea is that of a "master" or "consensus sequence" that is surrounded by a "cloud" of minimally divergent sequences – some very close to the master sequence, and some quite a number of mutations away from it. With every viral replication cycle yielding a new set of mutations with the mathematical certainty of a standard frequency (1 mistake in every hundred replicated bases), it is easy to imagine how such a cloud can be generated from even a single input master sequence.

However, there are very few data concerning the rate of genetic mutation in dsRNA genomes. The rate of genetic drift for the BTV-1 L2 was assessed in a cell culture experiment(45). In 20 serial passages, only 10 nucleotide changes occurred in the roughly 3000 base pairs of the genome segment, six of which were silent, and two of which resulted in conservative changes in the amino acid sequence. This rate is significantly lower than the 10^{-2} nucleotide substitution rate for some other RNA viruses(29). Nonetheless, both this work (see Chapter 2) and other studies have found adequate genetic polymorphisms in viruses from natural populations for phylogenetic studies(21, 22, 73, 85, 111, 125).

Finally, both genetic drift and genetic shift evolutionary events are constrained by the relative fitness of the newly evolved progeny viruses versus the "consensus sequence" in the viral population. In fact, most new members of the quasispecies cloud are removed through the action of purifying selection (they are out competed by the more fit consensus sequence). On the other hand, although some reassortment events are random,

there is evidence that some are non-random(97, 121). While defined selective advantages have not been ascribed to the "linked" pairs of genome segments, the observation of non-randomness raises the question of a possible selective advantage(s) of specific genome segments (or constellations of segments). This situation would represent an occurrence of positive selection, where the progeny is more fit than the parent or the consensus sequence through random-chance reassortment. Additionally, relative fitness considerations may account for the lack of point mutations in L2 observed during continual passage on cell culture versus that seen in wild type, natural populations. Continuous passage *in vitro* may select for VP2's that maximize cell entry, where viruses with mutations are less fit and therefore out-competed by more efficient viruses with fit sequences (an example of purifying selection). In contrast, in natural populations, the viruses must continually adapt to vertebrate host and invertebrate vector host cells, and interact with their respective immune responses (an example of diversifying selection)(21, 22, 73, 85, 111, 125).

CULICOIDES SONORENSIS ECOLOGY AND MOLECULAR BIOLOGY

After its implication in the transmission of BTV in 1954(117), *Culicoides variipennis* was first colonized in 1957 by R. Jones(65). This colony failed after 12 consecutive generations; however, *C. variipennis sonorensis* was again reported to have been colonized in 1960(66) with great success. These events allowed the study of the vector and the virus separately, or the interaction between the two. These important events laid the groundwork for both the current research.

C. sonorensis Population Genetics

We now know that those studies on *C. variipennis* in Colorado were more likely studies on *C. sonorensis*. *C. sonorensis* was considered a subspecies of *C. variipennis* (*C. variipennis sonorensis* and *C. v. variipennis*), but *C. sonorensis* was elevated to a distinct species in 2000(52), as well as being synonymous with *C. v. albertensis* and *C. v. australis*. Studies of the genetic basis of vector competence within *C. sonorensis* revealed that a single control locus (blu) determined bluetongue virus susceptibility in *C. variipennis* (presumably *C. sonorensis*)(147). Studies also demonstrated that *C. v. sonorensis* was more susceptible to oral infection by BTV than *C. v. variipennis* – making it more likely to be the primary vector of BTV in the United States(148, 150).

In addition, two laboratory colonies of *C. sonorensis* differed in vector competence when orally challenged with 5 different serotypes of BTV (considered individually by serotype). However, this difference became statistically insignificant when an average of all serotypes between the two strains was taken(88). This would seem to indicate a role for viral determinants of vector competence, as well as a role for vector genetics. Similarly, the effect of the environment on vector competence cannot be underestimated: differences in the rate and success of virogenesis in insects resulted from simply holding the different experimental groups at different temperatures(100).

Ecology of larval *C. sonorensis*

After the association of BTV with *Culicoides* spp., it was thought that the insect could represent a critical control point in the life cycle of the virus. Studies were undertaken to characterize the ecology of the insect more fully. One of the first was the characterization of the larval habitat of 29 species of culicoids(67). The larval habitat of

C. variipennis (Coq.) was described as "small, wet areas – nonvegetated, polluted with manure, and exposed to direct sunlight". *C. variipennis* preferred the sunlight, and was the most tolerant of differences in the environment such as the salt and/or acidity of the water. Most species also preferred stagnant water to running water; in fact, water running too swiftly precluded the site as a breeding location of the insects. In addition, while some species could breed in "plant environments" (tree holes, etc.) *C. variipennis* seemed to prefer manure as the source of organic nourishment for the growing larvae. This would make that species more likely to reside in close proximity to the vertebrate host of BTV, and was the first suggestion that species was the principal vector.

Feeding Behavior of *C. sonorensis*

Drop trap studies were instigated to investigate the feeding behavior of *C. sonorensis* in northern Colorado. A sheep and a calf were staked out and insects were collected from them periodically. *C. crepuscularis* preferentially selected and fed upon sheep, whereas *C. sonorensis* preferentially selected and fed upon cattle(118). This may be explained simply by the increased amount of the chemo-attractant carbon dioxide (CO_2) that cattle produce over sheep, but may be due to other factors as well.

Overwintering of *C. sonorensis*

Investigations into the actual overwintering state of *C. sonorensis* in Colorado revealed that it overwinters as a fourth instar larva in diapause, living as deep as 5 centimeters in the mud of its larval habitat(2). The following conditions have been proposed to be sufficient to induce diapause in *Culicoides* spp.: maximum temperatures less than or equal to $13^{O}C$ on 45% of days or more in any given winter month; a weighted "degree day" value of less than or equal to 1.35 of 6 and an average daily maximum

temperature of less than or equal to 12.5°C (both for the coldest month); together with a total of 40 days or more with maximum temperatures 13°C and/or 10 consecutive days with maximum temperatures 13°C for the whole winter(135). A search of the literature revealed no information concerning a light cycle/photoperiod signal that would induce diapause. In contrast, it was found that adult *C. sonorensis* could undergo a rapid "cold-hardening" response(103). In this situation, the insect can adapt itself to sudden drops in temperature, with a brief period of acclimation: when temperatures were reduced from 20°C to –10°C for 2 hours, there was 100% mortality. However, with a 1 hour period of acclimation at 5°C, then the rapid reduction to –10°C, 96% of the insects survived. On the other hand, exposure to –20°C for 2 hours caused 100% mortality despite the initial conditions, showing us the limitations of this response. While cold hardening may condition survivorship of insects during brief, sudden cold periods at the beginning of winter, it is probably not related to the survival of the adult insect over the course of a full winter. The effect of BTV infection and/or replication in the insect during diapause is unknown at this time, but represents an interesting area of future research.

SINGLE STRAND CONFORMATION POLYMORPHISM (SSCP) ANALYSIS

Single-strand conformation polymorphism (SSCP) analysis was first alluded to in the work of Fisher and Dingman(24, 33), but was first overtly described in 1989 by Orita et al.(108). There, it was found that SSCP analysis could detect a single base substitution in a human gene exon, which had been recovered from a restriction endonuclease digestion of whole, genomic DNA(108). This demonstrated the theoretical sensitivity of the procedure as compared to the standard of the day – restriction fragment length polymorphism analysis (RFLP). This work also demonstrated that the mutation could be

detected in the presence of a large number of unrelated DNA fragments, and that point mutations could be detected at various positions in the DNA fragment analyzed. This had obvious implications for the detection of point mutations in DNA fragments amplified by PCR, and therefore significant implications in the field of molecular diagnostics.

Most of the factors that can contribute to different patterns upon SSCP analysis have been addressed in a large body of literature. For example, Orita et al. state that "conformation of single-stranded nucleic acid is expected to be affected by environmental factors such as the temperature of the gel during electrophoresis, the concentration of [components of the] electrophoresis buffer, and the presence of denaturing agents in the gel"(108). Additionally, the effects of length of the fragment, the ionic strength of the denaturing solution, the amount of DNA loaded onto the gel, the percent concentration of acrylamide in the gel(49) and the inclusion of glycerol or polyethylene glycol in the reaction(86) have been studied or reviewed in various papers.

Perhaps most relevant to this dissertation is work done previously in this laboratory concerning the initial characterization of dengue virus (DEN) isolates from all over the world(31). There, it was not only found that SSCP analysis was capable of distinguishing distinct genetic groups both within and between topotypes of DEN, but that the positive predictive value for distinguishing genetic differences was 100%. However, it was also found that the positive predictive value for similarity was 26%. While it is evident that SSCP represents a very sensitive and powerful technique, it is also evident from this body of literature that any new SSCP technique requires optimization and validation to a sequence analysis.

PHYLOGENETIC ANALYSIS OF VIRUSES

The term "phylogenetics" refers to the mathematical comparison of two or more sequences, with the endpoint being the inference of the evolutionary relationship between them. As noted before, a molecular epidemiological approach is being pursued in this work. Phylogenetic analysis is one of the most robust methods with which to show similarity (genetic identity) between two virus isolates. This robustness stems from the fact that it is a direct examination of the most basic unit of inheritance: the primary sequence of the nucleic acid genome.

For the sake of this discussion, the comparison of DNA sequences only will be considered. Because of the necessity of RT-PCR, RNA is considered as DNA for the purposes of these analyses. A, C, G, T, and a gap make up the 5 character states of nucleic acid sequences. Most of the following methods can also be applied to amino acid sequences, however. They are simply more complex as the number of character states increases to 22 (21 naturally occurring amino acids and gap).

The most simple comparisons include but are not limited to those concerning nucleotide composition, codon usage, nucleotide pair frequencies and/or gap frequencies, and plots of constant vs. variable regions of sequence. To perform these simple comparisons, the differences between two sequences are enumerated and compared as a simple percentage. These comparisons are mathematically and intellectually easy to understand. The intricacy of phylogenetic analyses comes in the estimation of genetic distance between two sequences, and includes a set of assumptions that can dramatically change the outcome of that comparison.

Sequence Alignment

All of the following methods require alignment of the sequences to be compared. The sequences are lined up and a direct comparison on a site to site basis is performed. Gaps are introduced at this point to keep the most similar motifs together and offset the effects of insertions or deletions. This is usually accomplished by examining the sequences as continuous k-tuples, which are usually doublets (k=2). The similarity of each k-tuple to the one before and after it is calculated, and this information is used to determine when to introduce gaps and what size they should be. The alignment is a very important part of the overall comparison, as distance measures will be directly related to the quality of the alignment. Therefore, most are hand-corrected by the researcher to achieve the biological relevance that most computer algorithms are incapable of incorporating into the alignment(50).

It is important to note that the alignment assumes that the sequences compared are homologous – that is, that they arose in a common ancestor. For example, an alignment of all of the L2 sequences from several samples of BTV satisfies this assumption, whereas an alignment of the sequences of L2 and L3 from two of the samples would be erroneous from the outset. As an aside, the opposite condition to homology is similarity, an indication of the overall "likeness" of two sequences without an overt indication of their evolutionary history. For example, if two genes arose independently and came to resemble each other through convergent evolution, they would be considered similar but not homologous(77).

Finally, the order in which sequences are entered for alignment can influence the outcome. Hence, it is important to do several alignments in different orders and use the most common outcome in subsequent steps(50).

Genetic Distance Estimation

After alignment, the genetic distance between the aligned sequences is estimated using any of a number of distance matrix building algorithms (for distance analysis). All of these methods have an inherent set of assumptions that must be understood in order to adequately interpret the result; however, it is also true that the assumptions become less important as the number of data points increases. In fact, all of the methods discussed below give similar results when the dataset is large (greater than 500 informative sites(56)). Consequently, they are usually chosen for ease of use rather than actual biological relevance. In fact, none is relevant to most of the analyses in the literature today because they were designed for non-coding sequences, while most of the phylogenetic analyses seen in the current literature are performed on gene coding sequence, as are the analyses presented in this work.

Along with an assumption of homology, all genetic distance estimators assume a relatively constant rate of evolution – that is, viruses with a generation time of days cannot be compared to humans with a generation time of years. All of the algorithms include a time factor, which is cancelled out by the assumption of a constant rate of evolution. If two very dissimilar organisms were to be analyzed together, time factors would have to be input, greatly complicating the mathematical analysis and increasing the computational time exponentially(77).

On its most basic level, genetic distance is measured by the number of nucleotide substitutions in the two sequences being considered. This leads to a proportion (p) of nucleotide positions that are different, the simplest of the distance measures (p-distance). By adding layers of assumptions about which substitutions are more important than others and/or what those substitutions mean, the higher-level comparisons that are most commonly used today can be made.

One of the first levels of complexity is added in the Jukes-Cantor distance method(70). This method assumes that the rate of substitution is the same for all 4 nucleotides, and gives a maximum likelihood estimate of the genetic distance between the two sequences. This assumption is obviously biologically false, but it can give a good estimate of genetic distance for short sequences that have roughly equal proportions of the nucleotides.

An increasing level of complexity is added by the Tajima-Nei distance measure(151). In this method, algorithmic correction of nucleotide frequencies to those that more closely approximate the natural situation where frequencies are often unequal was attempted, moving away from the assumption of 0.25 under Jukes-Cantor). This was shown to be robust in computer simulations of sequence analyses.

The Kimura 2-parameter model(72) builds on previous assumptions by correcting for the difference in the rates of transitional substitutions (from one purine or pyrimidine to the other) over transversional substitutions (from purine to pyrimidine or vice versa). However, this model also assumes nucleotide frequencies of 0.25 throughout evolutionary history (as in Jukes-Cantor), so Tamura attempted a correction of both problems in the new maximum likelihood distance estimator(152).

Finally, Tamura and Nei(153) tried to correct a last failing of all previous methods – the assumption of constant rates of substitution at every site in the sequence. The biological reality of mutational "hot-spots" has been recognized since the early days of bacterial genetics, and is the rule in all biological systems. Their correction adds a factor (gamma distances) to the previous calculations. This factor follows the observation that mutational rates at different sites roughly follow a gamma distribution for large numbers of nucleotides (greater than 500), and can be estimated directly from that distribution. However, the factor is usually set to any number that has been observed to be biologically relevant for the sequences being studied.

Separately, Nei and Gojobori(101) have created an estimator that incorporates factors to account for the relatively higher number of synonymous over non-synonymous substitutions. Synonymous substitutions are those substitutions occurring at the nucleotide level that have no effect on the amino acid sequence of the encoded gene product, and are therefore not subject to negative selection pressures (i.e. they are evolutionarily silent except in the case of secondary and tertiary structure). Non-synonymous substitutions are removed from the gene pool by purifying selection. After accounting for these factors, the Nei-Gojobori distance estimator generates a genetic distance estimate with the Jukes-Cantor formula. This is a good choice because of the limited amount of data – the dataset being comprised only of a few positions with 2 character states (synonymous vs. non-synonymous).

When selecting a method to use, the algorithm with more parameters would seem to be the better choice. However, with increasing complexity comes an increasing number of assumptions, as well as an increase in computational time and difficulty.

Additionally, extra assumptions increase the error inherent in estimation, as well as increasing the sampling error – which both decrease the validity of the resultant distance matrix. On the other hand, sampling error decreases with a larger dataset. All of these factors must be taken into account when selecting a method for the analysis. Other genetic distance estimators exist and are being written every day. The above were presented here as the most commonly used.

Phylogenetic Inference

Distance analysis requires that a distance matrix is calculated ("built") first, then the actual phylogenetic inference can take place. The other two methods – maximum parsimony (MP), and maximum likelihood (ML) – use heuristic search algorithms to construct phylogenetic trees straight from the aligned sequence file. An additional, important distinction occurs here: distance methods measure genetic distance without the explicit inference of evolutionary relationship. Both of the other methods try to infer evolutionary history; however, a significant proportion of scientists (pheneticists) feel this cannot be done adequately and should not be attempted. Phenetic methods assume strict mathematical concepts only. Therefore, they choose outgroups to root their trees only with objective, scientific data that proves a direct lineage between the analyzed sequences and the root. Unrooted trees, calculated with phenetic methods, consequently result in a measure of similarity, which may or may not indicate homology. Cladists, on the other hand, feel good and true inferences of ancestor-descendant relationships can be made, and design their mathematical methods with that in mind. Therefore, they can choose their outgroups more subjectively, inferring evolutionary history that may or may not have been proven in the literature(77).

Distance Methods

Distance methods are a set of methods that take distance information from the matrix building algorithms described above, and display it using an algorithm that assumes some sort of functional relationship among the distance values. Two discussed here are the unweighted pair-group method with arithmetic mean (UPGMA) and neighbor-joining (NJ)(77).

UPGMA is the simplest method to construct taxonomic phenograms; however, because of ever increasing computer speeds it is not commonly used today. It will be discussed here simply as a logical starting place for other discussions. Here, all operational taxonomic units (OTU's – usually one of the sequences for analysis) are compared, and the most similar pair is grouped together. These two then represent a baseline for all further comparisons. The tree is built sequentially by finding the third OTU that is the most similar to the composite OTU represented by the most similar pair. These three are then "collapsed" into a second composite OTU, and the next most similar OTU is identified and subsequently incorporated(77). This collapse, however, requires a set of assumptions that are often not true: first and foremost, the assumption of equal rates of evolution is made for all OTU's. This allows assignment of equal branch lengths from a common ancestor, which we know now is not the biological reality dictated by evolution. In addition, because the third OTU is compared to the average of the distance between the first, most similar pair, the assumption of additivity of evolutionary rates is made. We also know this to be a false assumption when dealing with the natural world, which is why this method works well for large, similar sequences, but can break down when comparing genes from more dissimilar organisms. For example, when two

sequences are equidistant from a composite OTU, the computer or the researcher must make a decision as to which to incorporate into the composite OTU first, or to include them both at the same time.

Layers of complexity can be added as with the distance matrix calculations. For example, adding outgroups to calibrate the tree as it is built against a reference point from a different organism (e.g. the L2 sequence from other BTV serotypes is used for the tree building in this work) and/or basing the tree upon an Ocham's razor-like assumption that the shortest branch lengths are the most correct (the shortest branch length representing the fewest number of evolutionary steps, and therefore the simplest way to explain the differences between the sequences). Then, many possible trees are iterated with as few assumptions placed upon them as possible, and the tree with the fewest branches and/or the shortest branch lengths is chosen. Another source of error in this process is in the choice of outgroup, however. Outgroups are assumed to have been ancestral sequences from which the OTU's currently under study have evolved. If this is not the case (if a related organism is chosen for an outgroup, for example) then the tree will not adequately represent the true relationship between all OTU's. However, because of limited database size and money, this limitation is often overlooked, and related organisms are used to provide a baseline for comparison of the sequences under study.

One method where outgroups can be used is the neighbor-joining method(132), where the initial tree is simply made up of all OTU's spaced equidistant from a single, common internal node (a "star" pattern). Then, two of the OTU's are grouped as "neighbors", and set apart from the previous internal node as a separate cluster with their own common node. This is done many times in a random fashion until the initial cluster

of two that has the shortest branch length from the common node of all the others is found. These two are then placed in a composite OTU (as above). Then, to correct the assumption of equal rates of evolution inherent in UPGMA, a transformed distance matrix is constructed that adjusts the branch lengths between each of the nodes (the center of the star pattern) based on the mean divergence from all other nodes. This adjustment has the net effect of removing the distance between the two initially grouped OTU's from all future calculations of distance. In effect, each successive addition to the composite OTU is calculated free from the effects of all other distance calculations in the matrix. This allows for outgrouping because distance from the outgroup is calculated for each OTU separately, allowing an adequate representation of their real-world relationship. After the adjustment, the composite OTU is tested as a neighbor with all of the remaining OTU's in a stepwise fashion until the shortest branch is again found. These three are the collapsed into a composite OTU, and the testing is again iterated until all OTU's are incorporated into the tree.

Maximum Parsimony (MP) and Maximum Likelihood (ML)

MP and ML represent two alternatives to the distance calculation and cluster analyses described above. In these approaches, assumptions are made about the relationships of the different character states directly from the alignment, and phylogenetic trees are calculated based on those assumptions(77).

MP methods are those that first infer the ancestral sequence at each nucleotide site based on a simple comparison of "parsimony-informative sites" – those sites that have at least two different nucleotides that are each represented twice (or those that favor one tree over the others). One of the weaknesses of this method is that other sites are not

used, while they are used in both distance and maximum likelihood analysis. After generation of the ancestral sequence, all possible tree topologies for the current data are drawn based on a simple distance calculation (number of substitutions) of the experimental sequence from the ancestral sequence. The final tree is chosen based simply on the tree that requires the fewest evolutionary changes at parsimony-informative sites to explain the observed differences in the experimental OTU's. This method also assumes that the shortest distance between two points will most closely represent the actual evolutionary history of the genes or sequences being studied(75, 77).

An additional problem is that, in practice, there are often many "equally parsimonious trees", which have the same calculated number of steps from the ancestral sequence. This problem can be resolved by the construction of a "consensus" tree. All the candidate trees are examined and attempts at a resolution of the conflicts between them are made. The researcher simply needs to decide what level of consensus is desired. In the case of strict consensus, all conflicts are resolved by multifurcating the conflicting branch points to include all sequences. On the other hand, majority-rule resolves conflicts by multifurcating if a certain number of the total trees supports the conflict – 50% and 70% are common "decision levels". For example, assume three trees were generated that have a similar branching pattern in two cases, and a conflicting pattern in the third. The strict and 70% majority consensus trees would multifurcate the disputed branch point to include all downstream sequences, where the 50% majority tree would simply use the branching pattern dictated by the two sequences over the third(75).

A final problem with this method is the fact that, when the number of sequences to be compared exceeds 10, massive computational power is required to generate and

examine all possible tree topologies. With less than 10 sequences, an "exhaustive search" of all possible tree topologies is performed; however, mathematical "short cuts" exist to ease the burden of calculating all possible trees for larger datasets. There are two main short-cut methods – the branch-and-bound method and the heuristic search method. The branch-and-bound method is where a maximum probable genetic distance is calculated from an initial core of trees. Genetic distances are calculated as trees are built, and those with obviously longer genetic distances than the set already generated are disregarded. Shorter trees are generated and examined, leading to a "semi-exhaustive search" of the possible trees. The heuristic search method functions similarly to the branch-and-bound method except that it minimizes genetic distance at each stage of tree construction. This leads to the possibility that some equally parsimonious trees will not be constructed because their anticipated distance was not estimated correctly(75).

The other method to be discussed here is maximum likelihood (ML). The basic method here is the same as for MP. However, instead of the distance from the ancestral sequence being calculated based on number of substitutions, it is calculated based upon the likelihood of the occurrence of a particular replacement of a nucleotide from a pool of nucleotides at each site in the sequence over a certain period of time. As stated above, one of the strengths of this method is that it considers all sites in the sequence. This is done because there is a small likelihood that a replacement and reversion to the original state occurred at each site in the past, so each site has a small probability of being "likelihood-informative". Another strength is that, because it uses all sites, a set of assumptions concerning the rate of mutation at any given site, a molecular clock can be attached to this algorithm to calculate real divergence times for the compared sequences.

The final tree is chosen based on the total likelihood of direct descendence from the inferred ancestral sequence. That total is based on the product of all the individual likelihoods calculated for each position in the sequence, given independent evolution of all nucleotides(77, 95).

Tree validation (Bootstrap Analysis)

Finally, all trees must be validated statistically by one of several applicable methods. The first of these methods is simply the generation of standard errors at each individual step of tree construction and overall by examination of the variance of the various factors that went into the computation of the tree. These will provide standard statistical measures of significance, including p-values (α) or confidence probabilities (CP, 1-p)(75). The specifics are beyond the scope of this discussion, but it should be clear that sources of error will include but not be limited to sampling error (in small datasets), the inference of the ancestral sequence for initial comparison in MP or ML, as well as the estimation of the upper limit of genetic distance in the branch-and-bound method, or the individual likelihood estimates in the total maximum likelihood calculation.

One of the main methods for testing the consistency of deriving the final tree from any given dataset is the bootstrap analysis(32). The basic principle here is that the original dataset is resampled, changing some of its data randomly. This could include actual changes in primary sequence, or changes to the order of input, to exclusion of whole sequences. One ingenious (and the most common) method takes one site of the original dataset and makes it the first site of the new dataset, and then repeats the process until a similarly sized dataset is created. For example, the eighth site of all the original

OTU's is made the first site of the new, resampled dataset. Then, the 100th site is made the second, and the 123rd site is made the third, etc. All sites are sampled in a random fashion, and sites can be sampled many times; thus, some sites may be included multiple times, and some may not be included at all. The net effect of this is to remove the effect of small differences while upholding the effects of large differences(77).

The same phylogenetic inference methods are then used on the new dataset, and each branchpoint is evaluated for its presence in the full tree and all subsequent trees. The idea is that tenuous groupings of sequences will be easily disrupted by the resampling method, whereas robust groupings will hold up to the changes in the original data. These permutations are repeated 500-1000 times (the more replications the better the result) and values at the branchpoints of trees are the percentage of times clades remained in the resampled data; that is, the consistency of any clade is equal to the percentage of times that the clade occurred in the new analysis (BCL = bootstrap confidence level: 70-100 is considered significant, 50 or less is no more than random chance)(95).

Phylogenetic Analyses of BTV

Each method must be chosen for its biological relevance to the data, as well as the individual strengths and weaknesses of the technique in relation to the type of sequence to be analyzed. In the case of BTV, the situation is one of limited variability in genome segment sequence over time(21, 22, 73, 85, 111, 125). This necessitates an analysis of long sequences of many isolates to ensure the acquisition of an accurate picture of the natural evolution of the virus. MacLachlan and others (7, 21, 22, 114) used the Kimura 2-parameter model in their phylogenetic analyses of BTV. In this dissertation, the

Tamura-Nei distance matrices were thought to be more representative of the natural situation on a microgeographic scale (several viral isolates from one site) versus the macrogeographic scale (sites at least 30 miles apart). However, as is usually the case with large datasets, recomputation of the trees from the data provided by MacLachlan and colleagues using our methods yields essentially the same result (See Figure 2.4, Chapter 2). In addition, the neighbor joining tree construction method was chosen because of the size of the dataset and its relative computational ease versus the more complex MP and ML methods. This choice was validated by recomputation of the data presented here with both MP and ML methods, which yielded essentially the same result as the simpler distance analysis (see Figures 2.55, 2.65, 2.75, Chapter 2).

WOUND TUMOR VIRUS

Wound tumor virus (WTV) is another member of the Reoviridae, but is classified in the genus *Phytoreovirus*. WTV is a pathogen of plants transmitted by *Culicoides* spp. The molecular biology of this relative of BTV is relevant as a model of a possible mechanism of transeasonality for BTV. WTV can "lose" genome segments during long-term infection of plants (the lytic infection system comparable to sheep and cattle in the BTV life cycle) without alternate passage in insects(122, 123). After plant infection for a number of years, certain WTV segments were not detectable upon polyacrylamide gel electrophoresis (PAGE), which has been reported to detect segments that are present in only 0.1% of the total virions analyzed(123). However, RT-PCR and/or Northern blot analysis were not attempted in the works cited. Interestingly, the segments that became undetectable were those necessary for insect cell infection. It is presumed that without the selective pressure of alternate passage in insects, maintenance of "extra" gene

segments apparently became a negative selective force on the virus. These facts, when taken together, lead to the hypothesis that genome segments that are unnecessary to the propagation of the virus in a particular host or vector can either be lost or, more likely, are down-regulated in absolute number or activity (either transcriptional or translational) in the virus population as a whole. This may be particularly significant for BTV transeasonality in larval culicoids.

Since WTV or BTV are unlikely to be propagated in nature without passage in vectors, the "loss" of genome segments would seem to be a laboratory artifact. An alternative hypothesis would stem from an examination of WTV in insect cells. There, the segments are present (detectable by PAGE) but exist in a state of decreased activity. In addition, modification of all WTV mRNA's occurs in persistent infection of vector (invertebrate) cell cultures, making them less translationally active than those produced during the acute phase of the infection(113).

Both of these features of the viral life cycle may hint at a mechanism of "hypovirulence" of the virus, which conditions WTV persistence in the infected vector and, consequently, improved survival of the virus. Either or both mechanisms may function to promote efficient BTV overwintering in insects. They may also represent family-wide mechanisms of establishing persistent infections in one or both hosts in the life cycle. This model system is the basis for the design of the experiments outlined in Chapter 3.

RESEARCH GOALS

The principal goal of this research was to test the hypothesis that BTV overwinters in its invertebrate vector, *Culicoides sonorensis*. This entailed extensive

field work attempting to isolate virus from endemic foci, and characterizing field isolates electrophoretically, serologically, and phylogenetically. Conceptually, these studies were divided into three main categories:

- The Molecular Epidemiology of Bluetongue Virus in Northern Colorado
- The Molecular Basis of Persistence of Bluetongue Virus in the Vector *Culicoides sonorensis*
- The Development of a strategy for a Third-Generation Vaccine Construct for Bluetongue Virus. Studies were initiated to develop such a vaccine, but were discontinued because of lack of time. The development strategy is presented in Appendix I in the event that this work will be pursued in the future.

The information presented here is critical for all future surveys of BTV in natural settings and populations of insects. In addition, these studies could provide new information concerning the epidemiology of BTV, which could then lead to the designation of regions of the country as "BTV-free" or "BTV-endemic".

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CHAPTER 2 - PHYLOGENETIC ANALYSES OF BLUETONGUE VIRUS

INTRODUCTION

Bluetongue virus has been an object of study for many years. Much is known about the molecular biology and epidemiology of the virus, but the overwintering mechanism has eluded researchers to date. This information is vitally important to our understanding of the epidemiology of BTV, as well as in the planning and execution of control and/or eradication strategies. Of the possibilities outlined in the Review of Literature (Chapter 1), vertical transmission of the virus from adult, infected culicoids to their overwintering progeny is the most likely mechanism for transeasonal maintenance of the virus. This was the guiding hypothesis for this work. Previous studies in this laboratory presented evidence in support of this hypothesis in the form of detection of viral nucleic acid or antigen in overwintering larvae(10, 11, 42, 53); however, virus was never isolated directly from overwintering larval insects, satisfying the "Gold Standard" for this type of work. To clarify, all viral isolates from AIDL are from adult culicoids reared from larvae collected at the locations and dates shown in Table 2.1. This necessity of rearing the insects, however, allows for the possibility of laboratory contamination of the adult insects in the processing steps, as do the molecular detection methods cited above. Because of the failures of the past, and the expectation of similar problems in the future, a different strategy was adopted to determine if BTV overwinters in the juvenile stages of the vector.

Molecular epidemiology is a powerful tool for tracking pathogens through populations (on either a regional, national, or world-wide scale) and even in monitoring genetic changes in a pathogen through the course of a disease in a single patient. One

famous example of this approach was the molecular determination that an entire group of HIV patients had been infected by a single individual – their dentist(7). These techniques are now widely used in the field of epidemiology, one of the most prominent uses being the tracking of hepatitis C throughout the world(29, 50).

Perhaps more relevant to this work, phylogenetic analysis of dengue virus nucleic acid sequences at the E/NS1 gene junction from throughout the world revealed that there was a previously unrecognized sylvatic cycle of dengue virus in West Africa, where the virus is probably endemic(46). Another analysis of the same dataset by Blok and Gibbs(4) provided additional insight: an analysis of the amino acid sequence provided much the same result as that seen by Rico-Hesse(46), but recalculation of the phylogeny of the nucleotide sequence indicated that isolates outside West Africa had a larger number of silent mutations, indicating a relatively unburdened evolutionary environment outside of the endemic focus of West Africa. In addition, Rico-Hesse et al.(48) also described a major risk factor for the emergence of dengue hemorrhagic fever (DHF) in the Americas. First, it was shown that a 240nt fragment from the E/NS1 gene junction of the dengue genome used in phylogenetic analysis was a valid topotypic and evolutionary determinant for dengue 2 virus, which drew from and extended the original oligonucleotide fingerprinting (ONF) work of Kerschner et al.(28) and phylogenetic analyses of Lanciotti et al.(31, 32). This validation allowed for the recognition of the association between introduction of strains of dengue from Southeast Asia to the Americas and the emergence of DHF in the New World in the 1980's(48). Then, this same fragment was used as a predictor of DHF in a group of isolates from Thailand(47), as well as to show that all isolates that were clinically associated with the more severe disease segregated out into a

distinct genotypic cluster. In an even further extension of this work, Leitmeyer et al.(34) associated specific amino acid changes in the envelope (E) protein, as well as nucleotide changes in both the 5' and 3' non-translated regions (NTR), with DHF.

Finally, molecular epidemiological techniques quickly revealed the geographic origin of the strain of West Nile virus (WNV) responsible for recent outbreaks in New York City(26, 33). This virus seems to have originated in Israel, as it differed from viruses isolated there in previous years by only a few nucleotides, whereas it differed from viruses isolated from other parts of the world more substantially. Molecular epidemiological data have also demonstrated a role for migratory birds in the spread of WNV around the world(43).

As can be seen by these and other examples, molecular epidemiological techniques are powerful tools to investigate the trafficking potential of arboviruses. To investigate the epidemiology of BTV in Northern Colorado, a molecular epidemiological approach was used to characterize the phylogenetic relationships of field isolates of BTV. This library of virus isolates was collected both by AIDL and ABADRL in long-term longitudinal studies of BTV in endemic foci. When it was determined that single strand conformation polymorphism analysis (SSCP) was not sufficiently sensitive for the characterization of BTV field isolates (see pg.71), an analysis similar to that used by de Mattos et al. was performed(8, 9). There, the L2 genome segment of numerous BTV isolates from vertebrates was sequenced in order to examine the evolution of the virus over time in the San Joaquin Valley in California. In those studies, the sequences were between 96.8-99.1% similar to each other, and between 93.8-95.1% similar to the prototype strain of the virus. Analysis of the amino acid substitution pattern indicated

some were conserved through time, while others were not correlated with time of isolation, and some were unique to individual isolates. It was hypothesized that demonstration of genetic identity over time in the most variable genome segment, L2(16, 17, 30, 57, 58), would be an indicator of genetic identity between all segments of the virus.

A second genome segment not under the same selection pressures as the gene encoding the outer capsid protein was also analyzed to verify the L2 gene analysis as well as to avoid potential problems associated with cell-culture adaptation of the virus, which could reduce genetic polymorphisms(19). An inner capsid protein gene was selected as the "internal control" for the analysis. The rationale was that such a gene would be removed from the selective forces placed upon genes encoding an outer capsid protein (receptor binding, immune selection), but would still be under the same structural/virus-assembly constraints as the other segments. The L3 segment was thought to be too conserved over time, serotype, and geographic location(18, 25) to provide informative phylogenetic results. The S7 segment, which encodes VP7, was chosen because it was thought likely to be more polymorphic and thus more useful for phylogenetic analyses (Table 1.1). In its role as the insect cell receptor ligand, VP7 represents a structural and functional analog in the subviral particle to VP2 in the intact virion, and thus the S7 segment would seem to be the perfect candidate for an internal control of the phylogenetic analysis of L2 sequences. The sequence encoding the portion of VP7 that is accessible to the surface of the virion was chosen for the analysis(35) because the remaining S7 sequences show similar conservation over time to that seen in the L3 segment(25). As discussed in the Review of Literature, this protein is subject to the

selective pressures of the vertebrate immune system as it can be (and is) bound by antibodies. Since L2 is the vertebrate cell receptor ligand, antibodies directed against VP7 would not be expected to neutralize the vertebrate infectivity of the virus(45); however, they would allow any immune cell with an F_C receptor to bind the virus and process it for presentation to and activation of the acquired immune response. In addition, these antibodies could neutralize the invertebrate infectivity of the virus by preventing the binding of the virus to insect cells in the midgut of the naïve gnat ingesting a bloodmeal, and could therefore have a significant effect on the survivorship of the virus. However, it is also clear that these pressures are much less than those focused on VP2 because of its relatively protected position in the intact virion. Selection of the sequence coding for the exposed portion of VP7 for analysis represented a balance between the sequence conservation shown by BTV over time and the need for phylogenetically informative characters in the analyzed gene fragments.

The phylogenetic analyses of L2 and S7 sequences were performed in 1997, before the publication of two important papers in 1998 and 1999(5, 39) concerning the molecular epidemiology of BTV. These papers revealed that phylogenies based on L2 segment analysis segregate based on serotype, whereas phylogenies based on the S10 segment segregate based on geographic location independent of serotype. The supposition is that S10, which encodes NS3 and NS3a, is subject to genetic drift and genetic shift (reassortment) independent of immune selection by the vertebrate host or tropisms conditioned by the cellular receptors of the L2 and S7 gene products. Thus, analysis of S10 can define topotypes of BTV, and phylogenetic "clusterings [of BT viruses are] independent of the host species of isolation"(39). It must be noted that those

results were based upon isolates obtained from vertebrate hosts; conclusions concerning clusterings that may occur based on vertebrate versus invertebrate origin of isolation remain to be determined (see Figure 2.5). However, in its role as a gene encoding a non-structural protein, the S10 segment must function efficiently in both its vertebrate and invertebrate hosts, and presumably is not subjected to significant directional selection pressure(s) in either. However, any actual selection pressures (or lack thereof) on S10 during replication in different phyla (Chordata versus Arthropoda) remain to be experimentally determined. In any case, it became evident with the publication of those papers that any interpretive analysis of the identity of BTV with relation to geography and/or time would be incomplete without a concurrent analysis of the S10 genome segment.

It should be noted that BTV had been previously topotyped based on the sequence of L3(24, 41). In fact, this very important work was responsible for the discovery of the Carribean origin of BTV2 in the United States, and the U.S. origin of BTV17 in the Carribean(41). However, the significance of the newer work for these studies was the fine detail shown in the phylogenies of the S10 segment. Instead of the previous scale of geographical regions of the United States available with an L3 phylogeny, distinctions were possible within a state as well as across whole country borders(5, 39).

Similar molecular epidemiological techniques (as above) were applied to test the hypothesis that BTV overwinters *in situ* in endemic foci. If true, BTV would most probably exist in a genetically stable state in endemic locations(37, 49). The rationale was that this genetic identity over time could be demonstrated and characterized by sequence analysis, and would be incompatible with annual reintroduction from different

areas of the country or world (which would have different serotypes, different topotypes, and much more variation than that seen locally). Genetic stability of BTV over time, in conjunction with the detection of BTV antigen and/or dsRNA from larval culicoids early in the transmission season(11, 53) would suggest that BTV overwinters in endemic foci.

It is recognized that proving a negative is scientifically impossible. However, given the difficulties with virus isolation and the lack of success in identifying the overwintering mechanism of BTV previous to this work, it was felt that an epidemiologic approach could substantiate or refute the hypothesis. Such an approach would also provide data that could be used in other areas of BTV experimentation, such as the designation of suitable vaccine targets and/or the elucidation of the mechanism of transeasonality in *C. sonorensis.*

EXPERIMENTAL DESIGN

Preliminary Studies

Preliminary characterization of BTV Isolates

The electrophoretic mobility of dsRNA segments in a polyacrylamide gel has been used in the characterization of BTV field isolates since 1970(55). While the technique was not expected to be sensitive enough as an endpoint in the characterization of the viral isolates outlined in Table 2.1, it was felt that it represented a good starting place to validate their identity as BTV by showing the co-migration of the isolated dsRNA with that of a known BT virus. However, this technique was not revealing in the differentiation of field isolates, and was therefore dicontinued.

A pilot study was then conducted to assess the ability of SSCP analysis of a selected sequence fragment of the L2 gene segment to reliably detect genetic

polymorphisms. As discussed in the Review of Literature, SSCP analysis is a technique designed to detect genetic differences among short fragments of amplified DNA. However, there were previous reports of a lack of sensitivity of this method for some sequences(21). In addition, the fragment chosen for analysis was 358bp long, which is longer than the length reported to be optimal for SSCP of 300bp or below(21). This length was necessary in order to design primers to a conserved region of the L2 segment while still including one identified variable region(17) in the fragment. The isolates from AIDL were subjected to RT-PCR-SSCP, then certain isolates were sequenced to check the validity of the SSCP analysis against the actual, primary sequence.

Preliminary Phylogenetic Analyses

After the sensitivity concerns brought up in the SSCP analyses (pg. 71), a study was conducted to determine if a fragment of L2 (instead of the entire sequence) would provide enough phylogenetically informative characters for a reliable analysis (see Results, Figure 2.4). A 1304bp fragment (bp 1183-2463) was chosen for analysis because it includes three of five identified variable regions of L2, which were thought to have biologically important phylogenetic characters(16, 17). This fragment was compared to the full length sequence, and to the 358bp fragment which had been used for SSCP analysis (bp 1770-2104). Phylogenies of this fragment, and the full-length sequence of the genome segment, were constructed from an existing dataset downloaded from Genbank, originally submitted by de Mattos et al.(9).

For the S7 segment analysis, a 620bp fragment (bp 18-638) of each viral isolate was analyzed. This sequence represents a highly conserved area of the genome segment

for primer binding, and includes the coding region for the portion of the protein that is displayed on the surface of the virus in the interior of the fragment(20, 35).

For the S10 segment, the full length of the segment for each viral isolate (822bp) was analyzed.

MATERIALS AND METHODS

Preliminary Studies

Electrophoretic Migration (PAGE)

The electrophoretic mobility of dsRNA segments in a polyacrylamide gel has been well described(55). Briefly, dsRNA is isolated from infected cell cultures as described later in this chapter. This preparation can be quantified, or just passed on to the next steps based on experience with the procedure. The dsRNA is appropriately aliquoted and mixed with 0.05% Bromophenol blue/0.05% Xylene cyanol and loaded onto a 6% non-denaturing polyacrylamide gel containing 5% glycerol. The gel is then subjected to electrophoresis in standard tris-borate-EDTA buffer(15) for 16-18 hours at room temperature at a constant voltage of 350V (approximately 16 mA). Gels were then silver stained following standard protocols for polyacrylamide gels(15) and bands were visualized on a light box.

SSCP Analysis

SSCP analysis was performed following standard protocols developed in Dr. William Black's laboraory(6). Briefly, a 6% non-denaturing polyacrylamide gel was poured as above and loaded with a mixture of 5µl of RT-PCR product (direct from the reaction tube) and 1.5µl of loading buffer (10 mM NaOH, 95% formamide, 0.05% Bromophenol blue, 0.05% Xylene Cyanol) that had been heated to 95^{O}C for 5 min. and

plunged into a ice-cold slurry mixture of ice and water. This cold mixture was then loaded directly onto the gel, and subjected to electrophoresis as above. Gels were then silver stained and visualized as above.

Virus History and Propagation

Isolation from invertebrates

Viruses were isolated and propagated as described by Deines(10) were used in this work. Optimal larval habitat was identified at endemic foci that had been the object of longitudinal studies by AIDL and ABADRL for many years (see Figure 2.1, A-D). Briefly, mud was collected from 6 inches below the waterline (under the water), at the waterline, and 6 inches back from the waterline, to try to obtain a representative sample of larval culicoids in the area. This mud was brought back to the lab and mixed with de-ionized water. No organic substrates were added for nourishment – it was felt that the organic content of the mud would last through the emergence of larvae from eggs for several weeks before being depleted. The mud slurries were checked daily for the presence of 4^{th} instar larvae and/or pupae, which were then collected and placed into emergence cages. These cages consisted of a small cup of water inside a one-pint cardboard carton with a screen (organdy) cover, which allowed the larvae to pupate and then emerge as adults. Adults, when present, were fed with raisins and wet cotton wicks placed on top of the cages.

After approximately 7 days, the adults were collected and pooled into groups of 30-50 based on the date of the collection, not the date of their emergence or collection from the mud. These adults were then frozen until they were pooled and subjected to a

Figure 2.1(A): Example of larval habitat of *C. sonorensis*. Picture provided by T. Raich.(42).

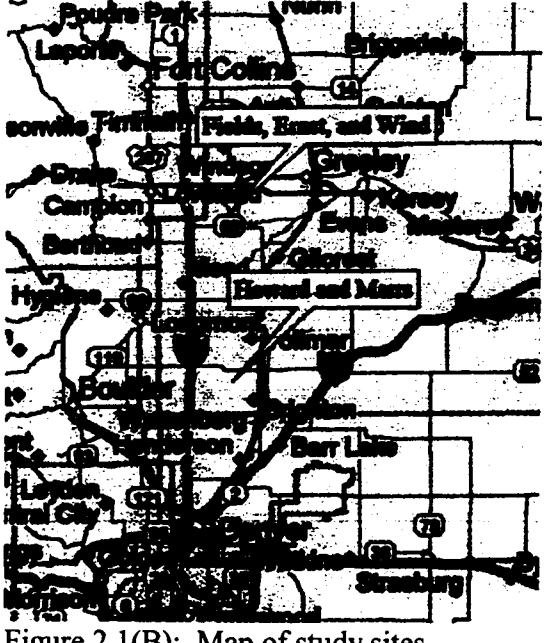

Figure 2.1(B): Map of study sites.

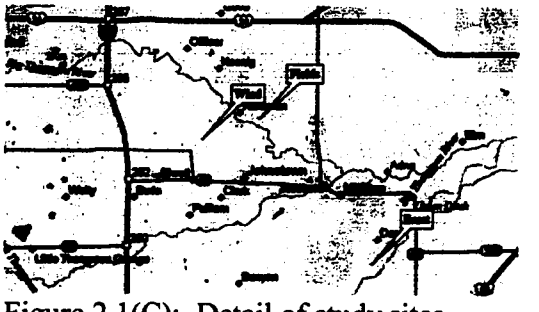

Figure 2.1(C): Detail of study sites.

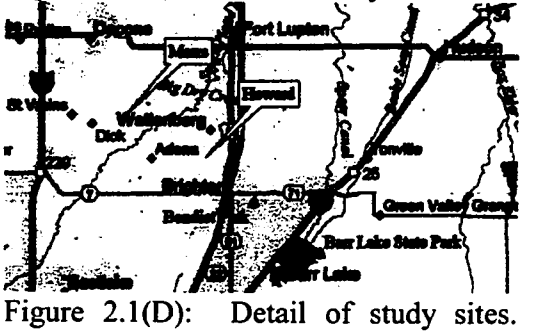

Figure 2.1(D): Detail of study sites.

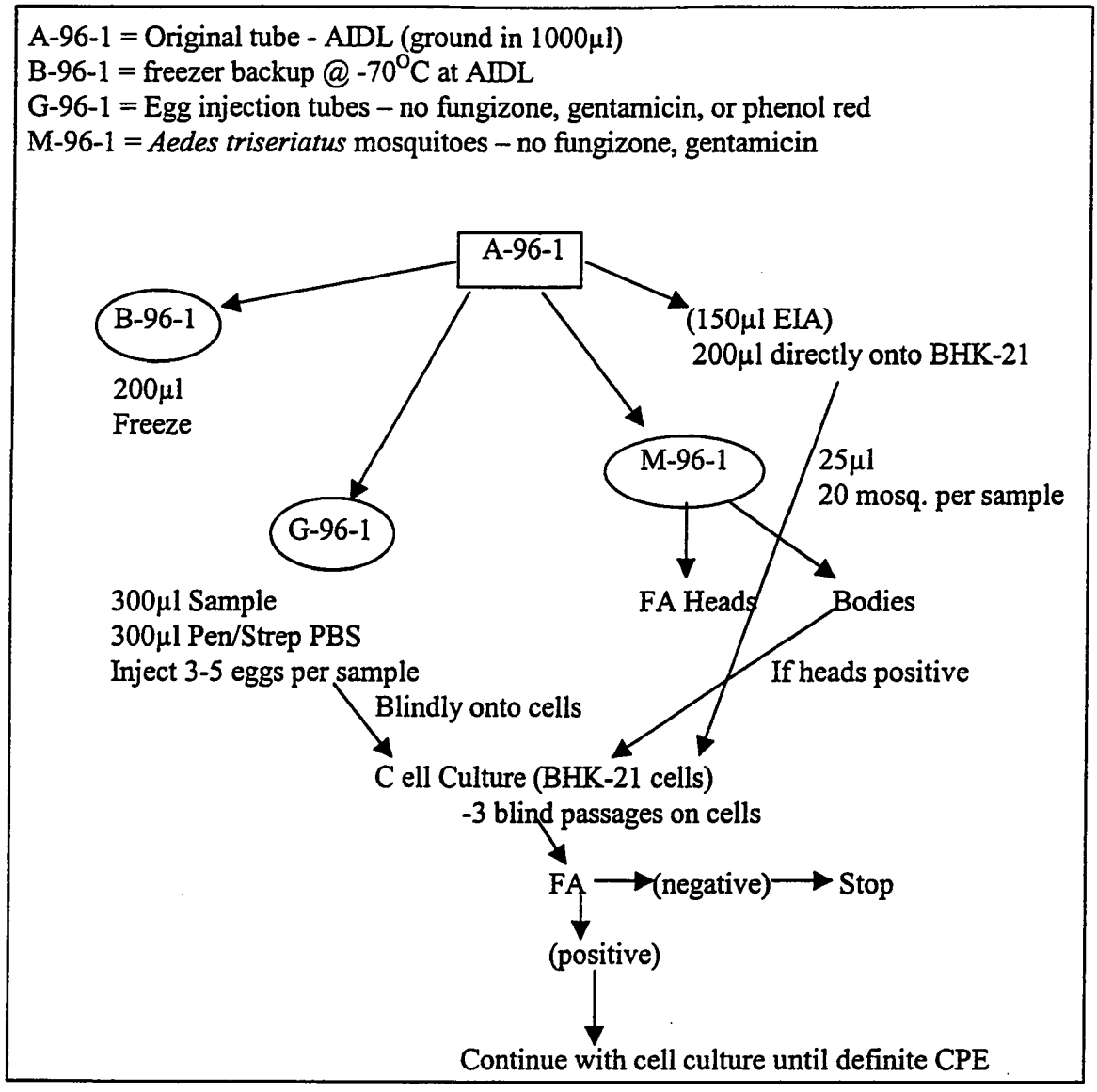

Figure 2.2: Virus isolation flowchart. A, B, G, M = Technique designation. 96 = Year of isolation. 1 = sample number.

surface wash with 10% ethanol (EtOH), 10% fetal bovine serum (FBS), and 80% sterile phosphate-buffered saline (PBS). They were then triturated in 1.7ml microcentrifuge tubes in a mixture of 10% fetal bovine serum, 5X penicillin/streptomycin (Pen/Strep = 250,000units penicillin and 250,000μg streptomycin total/500ml medium), and sterile PBS (1 ml total). The suspensions were then aliquoted into four portions – one for each of the three viral isolation methods, and one to reserve for possible future use (stored at $4^{O}C$, as per the recommendations of Luedke and Jones(36) – See Figure 2.2). Gentamicin (50mg gentamicin total/500ml medium) and fungizone (2.5mg fungizone total/500ml medium) were then added to the aliquots destined for cell culture and storage. Any remaining material from virus isolation was also stored for future use.

The field-collected insect samples were then assayed for BTV using three isolation methods: intravenous inoculation of embryonated chicken eggs (ECE), intrathoracic inoculation of *Aedes triseriatus* mosquitoes, and direct inoculation of baby hamster kidney clone 21 (BHK-21) cells (see Figure 2.2). Samples were then passaged blindly three times on BHK-21 cells, and monitored for cytopathic effect (CPE) on a daily basis.

Positive isolates were passaged one additional time in BHK-21 cells to provide enough virus for use in future experiments and storage, thus making all AIDL samples a minimum of passage number 4 (inclusive of the 3 blind passages necessary for isolation). At the time of sequencing, these viruses had been further passaged an additional 1-3 times in BHK-21 cells. Thus, all the AIDL isolates had been passaged an average of six times.

Isolation from Vertebrate Animals

All samples from ABADRL were initially isolated from the blood of infected vertebrates, obtained by venipuncture. Blood was diluted, washed, and injected into 10 day old ECE following standard procedures(14). After three blind passages, passage numbers similar to the viral isolates from AIDL (4-7) would be expected.

RNA Analyte Preparation

At the beginning of these studies, isolates were passaged one additional time in order to obtain viral nucleic acid. Viral isolates from Northern Colorado (Table 2.1) were propagated on BHK-21 cells until 4+ CPE was noted, and viral RNA was then isolated from the infected cells.

Isolation was accomplished using the PUREscript kit (Gentra Systems), which involved scraping off any remaining adherent cells and pelleting the cell fraction by centrifugation at 2500 RPM (500xg) in a microcentrifuge for 15 minutes. The cells were then lysed using a proprietary citric acid, ethylenediamine tetraacetic acid (EDTA), and sodium dodecyl sulfate (SDS) mixture. Protein and DNA were selectively precipitated with a proprietary citric acid and sodium chloride (NaCl) mixture, leaving the total RNA in solution. The RNA was then washed with RNAse free 70% EtOH and resuspended in 20-50μl diethyl pyrocarbonate (DEPC)-treated water.

Reverse Transcription – Polymerase Chain Reaction

Reverse transcription was performed using the technique of Wilson and Chase(56) in the standard reaction buffers that came with the enzyme. Moloney murine leukemia virus reverse transcriptase (MMLV-RT, Gibco-BRL) was used to ensure hydrolysis of the RNA and prevent interference of the dsRNA with the subsequent steps (MMLV-RT has

BTV isolates for phylogenetic analysis

Table 2.1

Designation	Date of Isolation	Site of Isolation	Species of Isolation	Serotype
BTV-11 Station Strain	1944	South Africa	Vertebrate	BTV-11
270	7/26/93	Howard	*C. crepuscularis*	BTV-11
A-94-5	3/23/94	Howard	*C. variipennis*	BTV-11
A-94-42	5/17/94	Fields	*C. variipennis*	BTV-11
A-94-64	5/24/94	Fields	*C. crepuscularis*	BTV-11
M-94-368	10/27/94	Howard	*C. variipennis*	BTV-11
A-94-389	10/27/94	Howard	*C. variipennis*	BTV-11
M-94-389	10/27/94	Howard	*C. variipennis*	BTV-11
E-94-393	10/27/94	Howard	*C. variipennis*	BTV-11
M-94-430	10/27/94	Howard	*C. variipennis*	BTV-11
201400	7/30/81	Fields	Cattle (Beef)	BTV-11
201767	6/1/82	Howard	Cattle (Beef)	BTV-11
201773	6/1/82	Howard	Cattle (Beef)	BTV-11
202020	6/10/82	Howard	Cattle (Beef)	BTV-11
202894	8/18/82	Ford	Cattle (Dairy)	BTV-11
203732	6/24/83	Fields	Cattle (Beef)	BTV-11
203733	6/24/83	Fields	Sheep	BTV-11
203734	6/24/83	Fields	Sheep	BTV-11
203794	6/29/83	Marrs	Cattle (Dairy)	BTV-11
203826	6/30/83	Wind	Cattle (Dairy)	BTV-11
203843	6/30/83	Ernst	Cattle (Dairy)	BTV-11
203853	7/1/83	Ernst	Cattle (Dairy)	BTV-11

RNase H activity). After denaturing 1-2µl of dsRNA at 95°C for 5 minutes in the presence of 0.5-1µl 37% formamide(final concentration = 12.3% formamide), the tubes were plunged into ice to prevent re-annealing, and the RT reaction mixture was added. The RT reaction mixture was prepared according to the directions accompanying the enzyme, with 100 units of RT and the primers to a final concentration of 10 picomoles/reaction for each primer. Reactions were incubated at 42°C for 60 minutes, then held at 65°C for 5 minutes to denature the enzyme. One microliter of that 20µl reaction was then used for the subsequent polymerase chain reaction (PCR), which was conducted using standard buffers for the Taq DNA polymerase (ProMega Corp.), 10 picomoles of each primer per reaction, 2.5 units of enzyme, and a 10 millimolar (mM) concentration of each dNTP (A, G, C, T). PCR reaction mixtures minus the enzyme were added to the cDNA from the RT. Those mixtures were heated to 95°C for 5 minutes, then reduced to 80°C for addition of the Taq polymerase (a hot start procedure). Reactions were then cycled through 35 steps of 95°C for 1 minute, 56°C for 1 minute, and 72°C for 2 minutes. The optimal annealing temperature of 56°C was calculated using the nearest neighbor method(51) and was close enough to the optimal temperature calculated for each to be used for all primer sets. Reactions were then held at 72°C for 10 minutes for final extension, and then stored at 4°C until agarose gel electrophoresis was performed. Samples were subjected to electrophoresis through a 1.5% agarose gel in standard tris-acetate-EDTA (TAE) buffer(15) at approximately 120 volts for 20-30 minutes. Resolved bands were evaluated for size using a 1 kilobase marker set (Gibco-BRL Life Technologies). Positive samples were either TA-cloned into Invitrogen's pCR 2.1™, or subjected to SSCP analysis. TA-cloned RT-PCR products were used to

transform competent *Escherichia coli* INVαF' cells provided in the TA cloning kit, and grown in an overnight culture. Plasmid was isolated using the Mini-Prep system (Qiagen Inc.).

Plasmids from TA-cloning were tested for successful integration of the gene fragment with PCR using the standard M13(-20) and M13reverse primers. Plasmids containing an insert of the correct size were sent in triplicate to be sequenced by automated cycle sequencing (Dye-deoxy method, Automated Biosystems Inc.), at either the Colorado State University Macromolecular Resources facility or the Davis sequencing facility. Alternatively, PCR products were purified with a Qia-spin column (Qiagen Inc.) and sent directly for automated sequencing. While interesting conclusions about the quasispecies nature of the virus could possibly be derived by an analysis of multiple clones, this was outside the purview of this work. A consensus sequence for comparison to other sequences was desired; hence, when direct sequencing from PCR products became a technically viable option, that course was pursued.

Analysis of Sequence Data

Sequences were assembled into a consensus sequence using Seqman™ (DNA Star) and aligned using ClustalX (v1.8, European Molecular Biology Laboratory). Distance matrices were constructed using the Tamura-Nei algorithm in PAUP (v4.0b4, Sinauer Assoc.). Trees were constructed using the neighbor joining method(52), and bootstrap values were obtained using the same program. Trees were displayed using Drawgram (PHYLIP v3.5c, Univ. of Washington) and corelDraw (v.8.232, Corel corp.), where labels and bootstrap values were added to the final display. Distance matrices

were displayed using Molecular Evolutionary Genetics Analysis (MEGA v 1.02, Kumar, Tamura, Nei, and the University of Pennsylvania).

RESULTS

Preliminary Characterization

A typical polyacrylamide gel eletrophoresis of dsRNA segments of field isolates of BTV is shown in Figure 2.3, A. The electropherotypes of the field isolates and BTV11 (Station Strain) were very similar. Also, those electropherotypes taken as a group differed from the known BTV2 used as a control. However, the types were not revealing in terms of the molecular epidemiology of the virus isolates.

The results of SSCP analysis of a 358bp fragment of the L2 segment from selected viral isolates from AIDL are shown in Figure 2.3, B. Since this 358bp fragment is included in the sequence of the 1304bp fragment that was eventually used for these analyses, the sequences were not included in this dissertation. Very similar patterns are seen between some isolates (BTV11, A94-5, M94-389, etc.) and different groupings are seen as well. Sequence analysis of BTV11 (Station Strain) and isolate A94-5, which have very similar SSCP signatures, showed a single difference between the two on the level of the primary nucleic acid sequence (an insertion in BTV11 Station Strain with a gap in A94-5). Additionally, there are two separate gaps in M94-389, which have bases in the other two samples. In contrast, isolates 270 and E94-393 had the same sequence, but a difference in their SSCP patterns is readily evident.

Finally, isolates A94-389 and M94-389 had very different SSCP patterns. This difference probably resulted from being isolated using two different methods: A94-389 is an isolate from an adult culicoid pool that was isolated directly in cell culture, and M94-

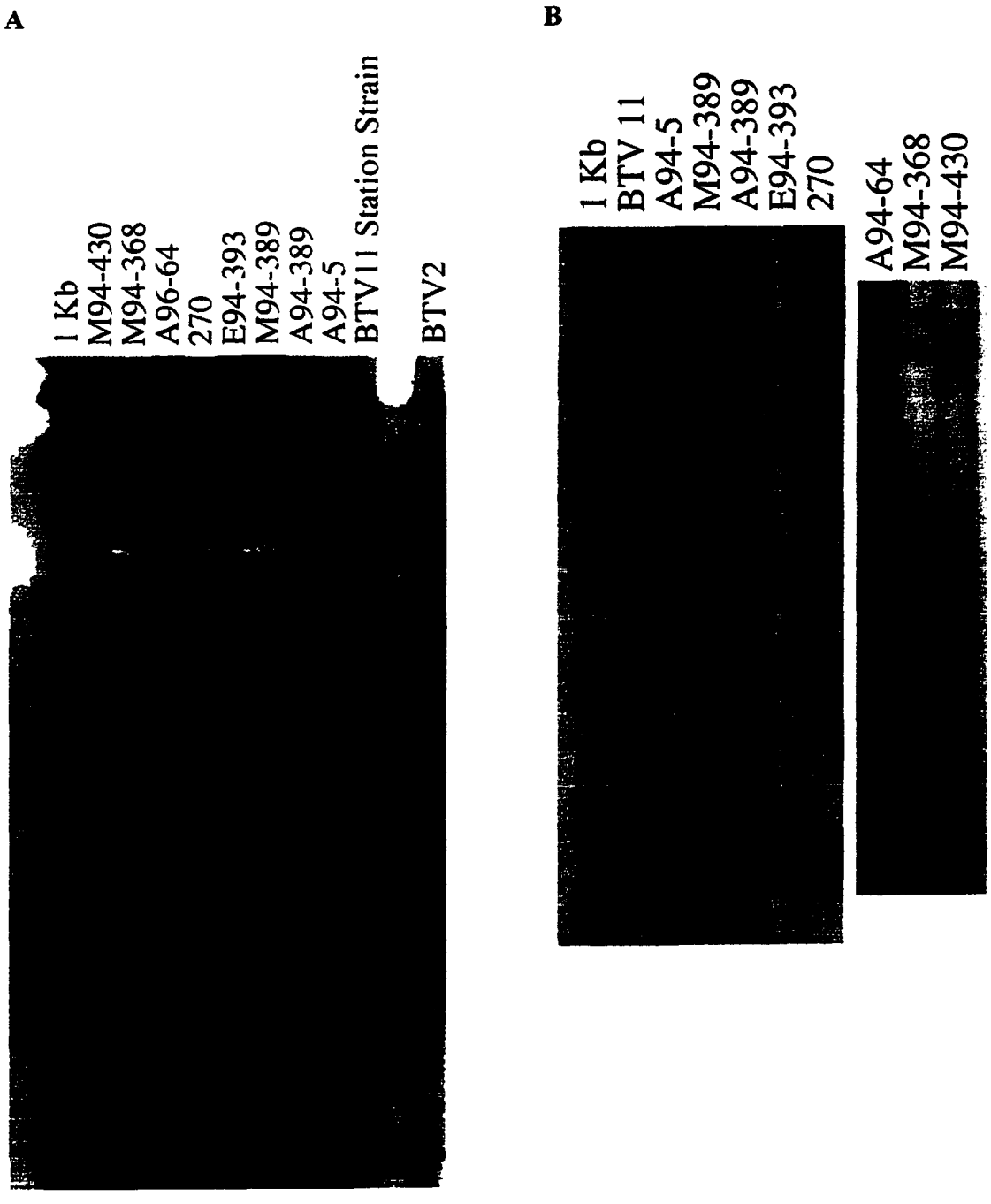

Figure 2.3 A and B: PAGE(A) and SSCP(B) analysis of viral isolates.

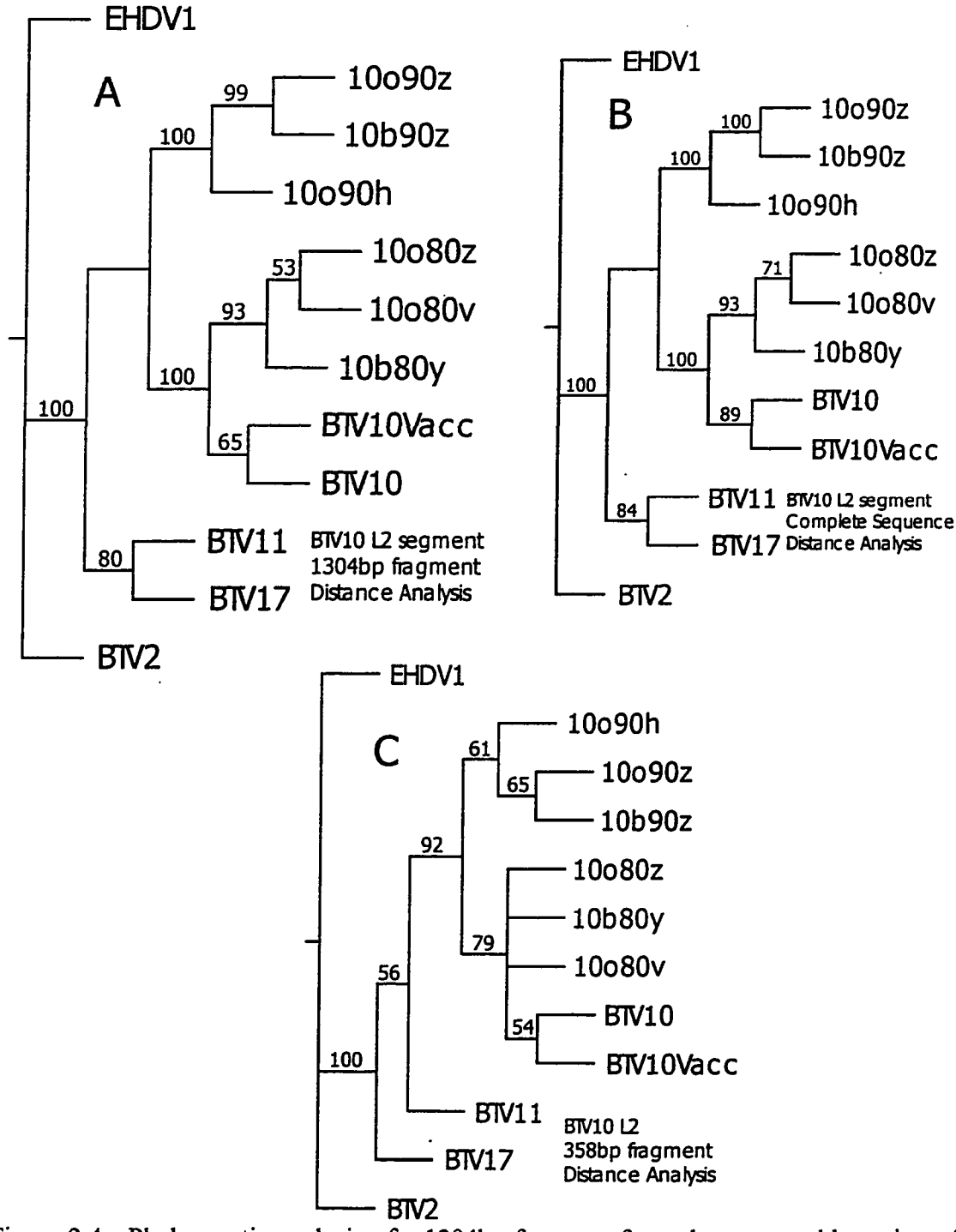

Figure 2.4: Phylogenetic analysis of a 1304bp fragment from the comparable region of the L2 segment of BTV10 (A) compared to analysis of the sequence of the entire L2 segment (B) and a 358bp fragment (C) used for SSCP analysis. All of the sequences were obtained from de Mattos, et al(8), which also describes the sequence labels. This preliminary study was conducted using pre-existing sequences to validate the use of the 1304bp fragment, negating the need to sequence the full length of the fragment (over 2000bp). See text for a full explanation.

389 is a virus from the same sample which was intrathoracically inoculated into mosquitoes, and then amplified in cell culture. There are small sequence differences between the two samples that were correctly identified by the SSCP analysis – 2 separate insertions in M94-389, and one insertion in A94-389. However, the SSCP analysis placed them into two different categories – a situation which is known to be false biologically.

Figure 2.4 shows the results of the preliminary analysis using existing BTV10 sequence data as outlined in Experimental Design. It is important to note that while the overall tree topology of the three phylogenies (A, B, C) looks the same, a lack of statistical support for the topology of the tree analyzing the 358bp fragment is demonstrated by the lack of bootstrap support (Figure 2.4, C).

Phylogenetic Analyses of the L2 Segment of BTV Isolates from Northern Colorado

Figure 2.5 shows the results of the analysis of the 1304bp fragment of the L2 genome segment of all viral isolates described in Table 2.1, of which 509 were parsimony informative characters (see pg. 35). Those samples from the 1980's are shaded for ease of identification. The overall tree topology represents either temporal groupings, or clusters based on species from which the isolates were made. Isolates are between 98.85-100% similar to each other, and are 97.93-98.85% similar to the prototype virus (see Appendix A for distance matrix, including transition and transversion matrices, and alignment files).

Phylogenetic Analyses of the S7 Segment of BTV Isolates from Northern Colorado

Figure 2.6 shows the results of the analysis of the 620bp fragment of the S7 genome segment of all viral isolates described in Table 2.1, of which 53 were parsimony

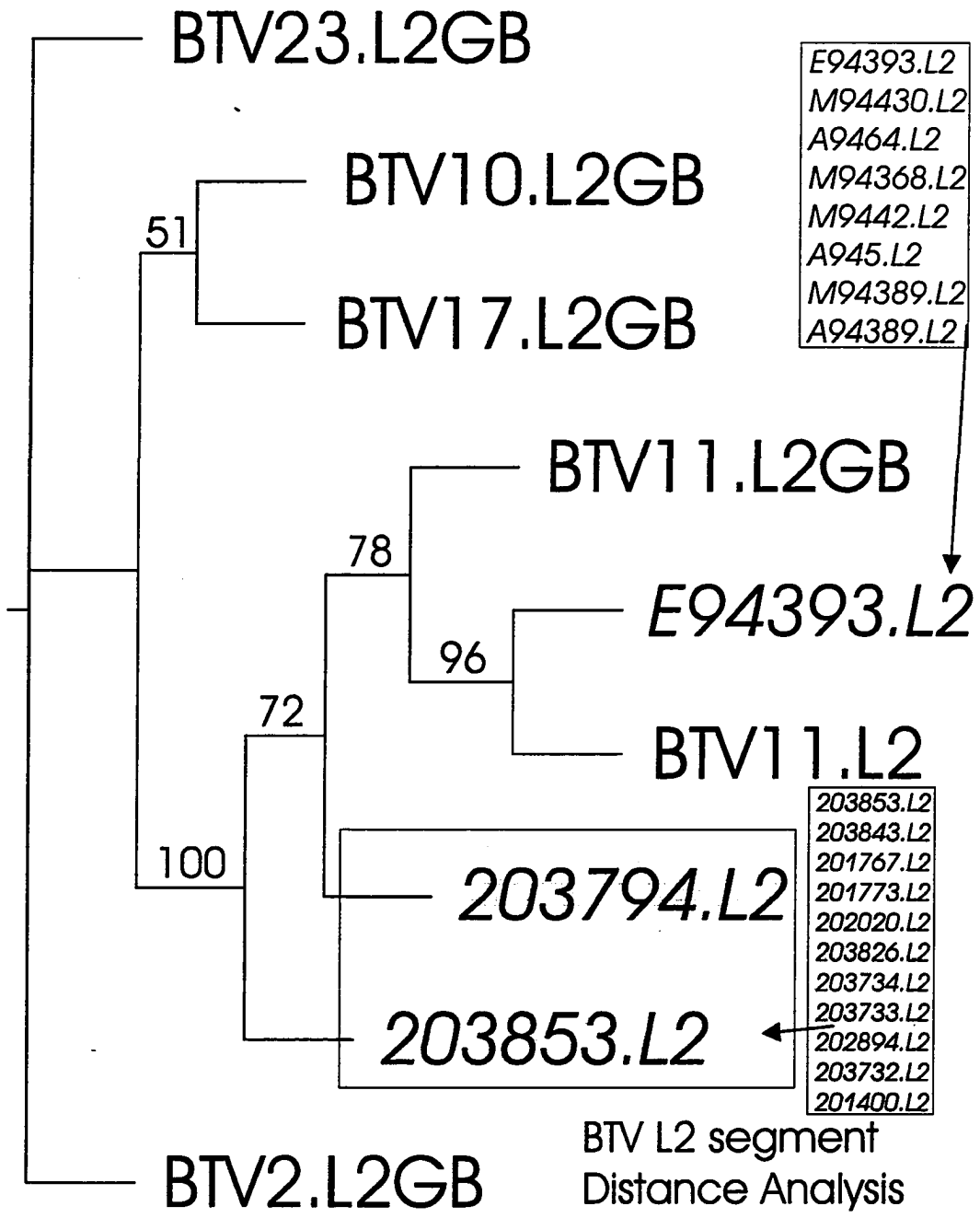

Figure 2.5: Phylogenetic tree derived from sequence data from a 1304bp region of segment L2 of BTV isolates from Northern Colorado, as described in the text. The suffix "GB" indicates a sequence from Genbank for comparison. Samples from 1981-1983 are shaded for easy identification, and represent either a temporal clade or a clade based on the species of origin of the isolate. Bootstrap values are the percentage of times that branch appeared in 1000 bootstrap replicates. Italicized viruses were isolated from the same microgeographic location as in Table 2.1. A representative of 100% identical isolates was included for ease of calculation – the group of identical sequences is shown in the box with a line indicating the representative used in tree construction.

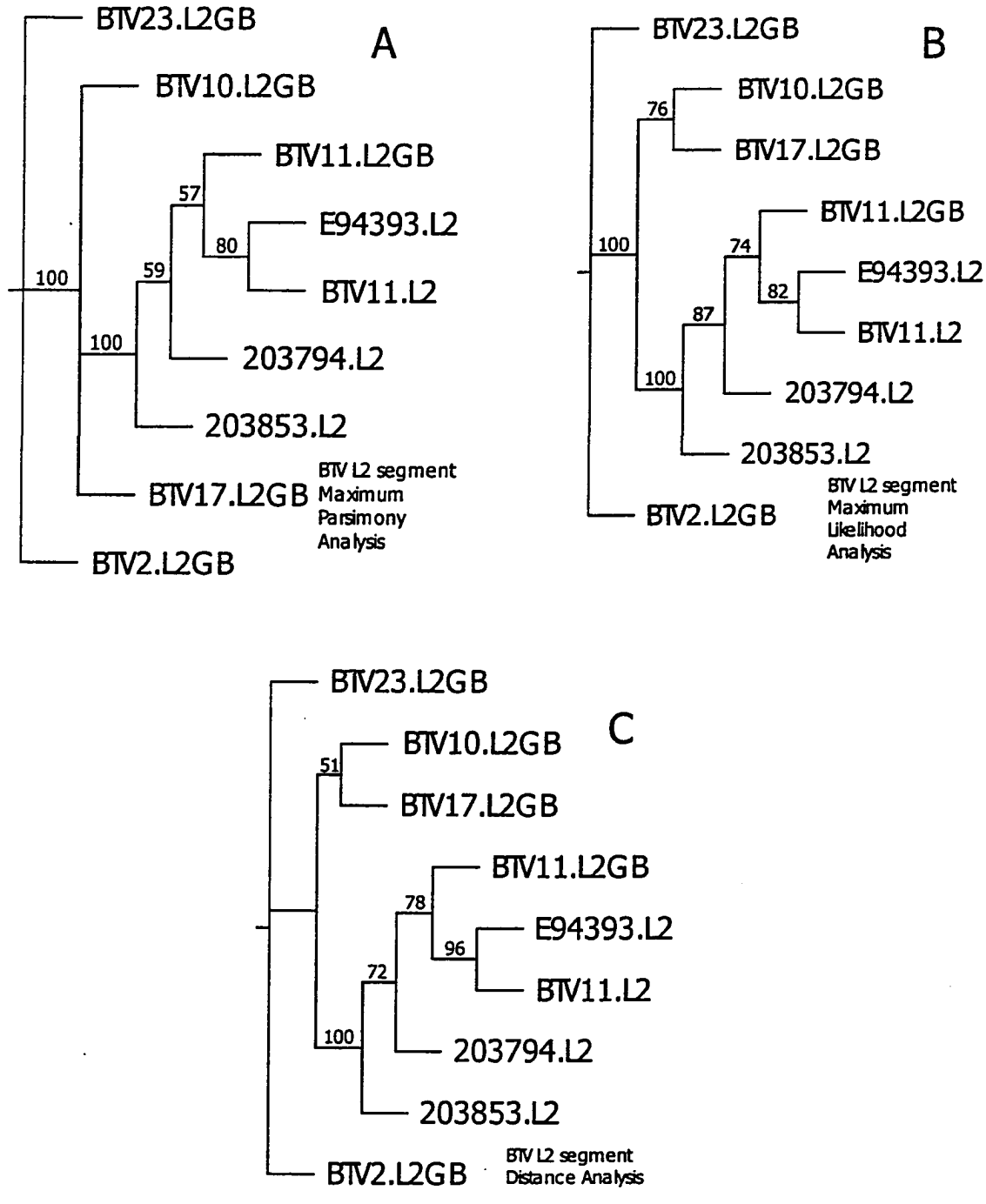

Figure2.55: Comparison of the different phylogenetic inference methods for the sequence data from the L2 segment of BTV isolates. Recomputation of the data shown in Figure 2.5 (C) by maximum parsimony (A) and maximum likelihood (B) methods. The more complex methods yielded essentially the same result as the simpler distance analysis.

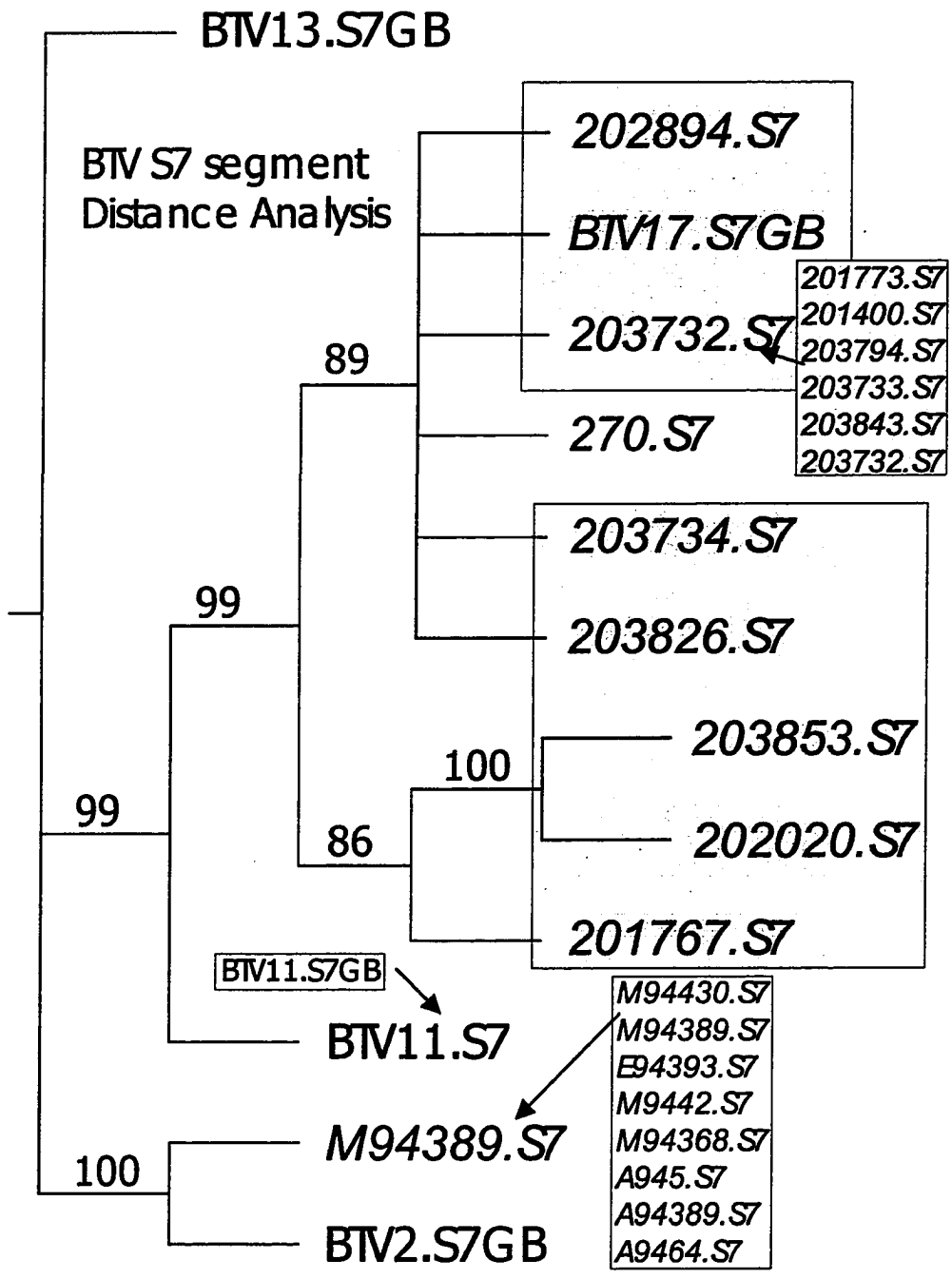

Figure 2.6: Phylogenetic tree derived from sequence data from a 620bp region of segment S7 of BTV isolates from Northern Colorado, as described in the text. The suffix "GB" indicates a sequence from Genbank for comparison. Again, samples from 1981-1983 are shaded for easy identification, and represent either a temporal clade or a clade based on the species of origin of the isolate. Bootstrap values are the percentage of times that branch appeared in 1000 bootstrap replicates. Italicized viruses were isolated from the same microgeographic location as in Table 2.1. A representative of 100% identical isolates was included for ease of calculation – the group of identical sequences is shown in the box with a line indicating the representative used in tree construction.

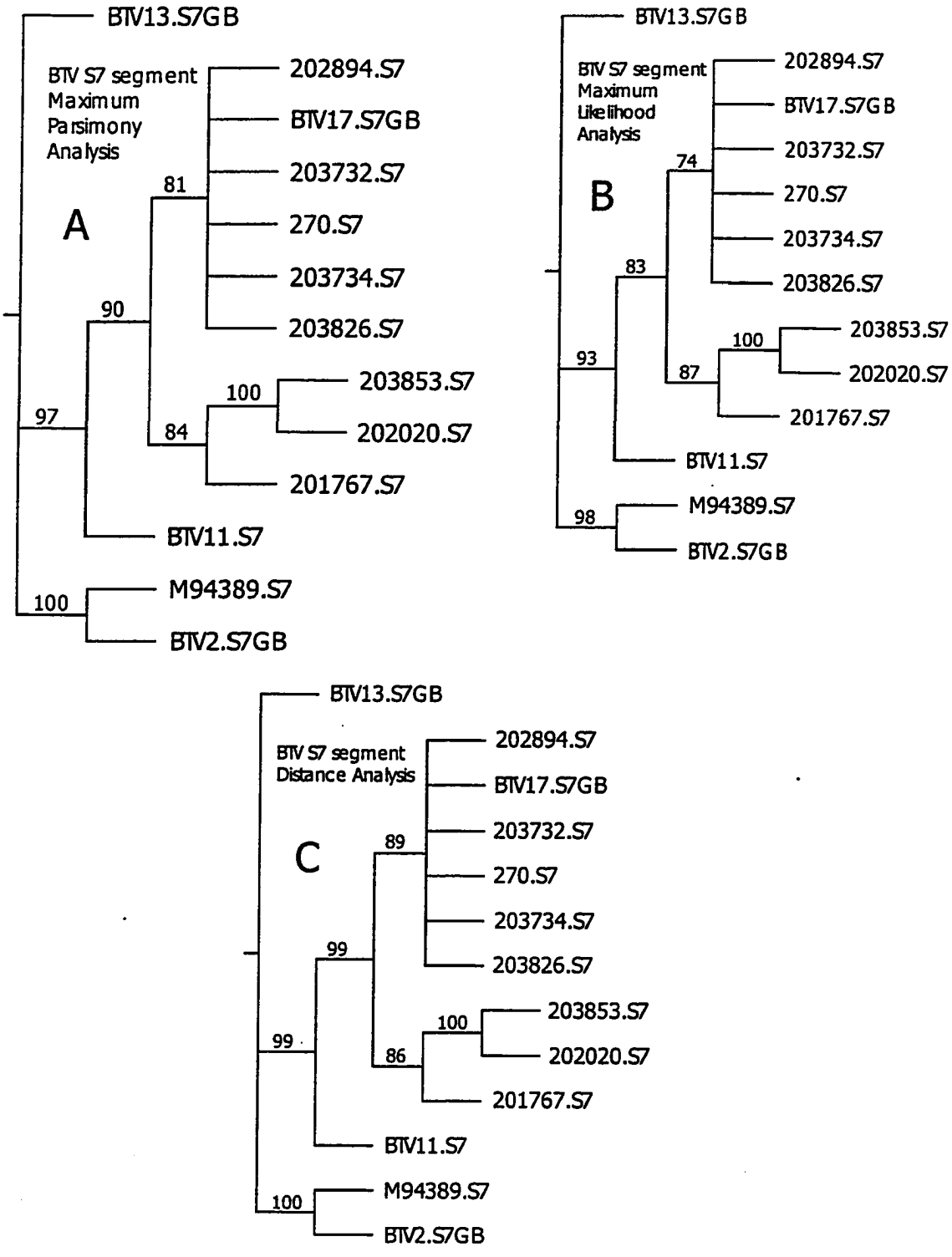

Figure2.65: Comparison of the different phylogenetic inference methods for the sequence data from the S7 segment of BTV isolates. Recomputation of the data shown in Figure 2.6 (C) by maximum parsimony (A) and maximum likelihood (B) methods. The more complex methods yielded essentially the same result as the simpler distance analysis.

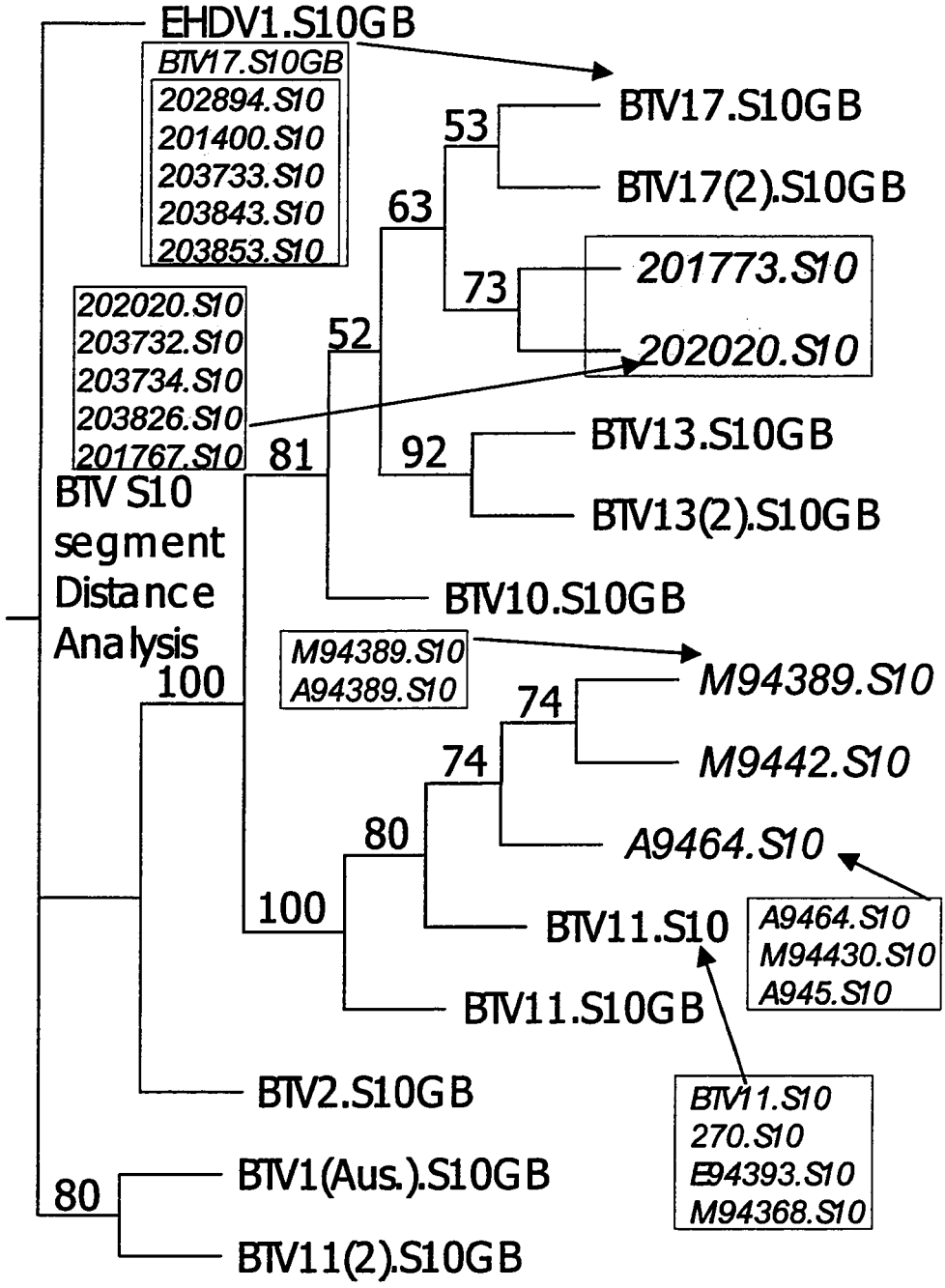

Figure 2.7: Phylogenetic tree derived from sequence data from genome segment S10 of BTV isolates from Northern Colorado, as described in the text. The suffix "GB" indicates a sequence from Genbank for comparison. Again, samples from 1981-1983 are shaded for easy identification, and represent either a temporal clade or a clade based on the host from which the isolation was made. Bootstrap values are the percentage of times that branch appeared in 1000 bootstrap replicates. Italicized viruses were isolated from the same microgeographic location as in Table 2.1. A representative of 100% identical isolates was included for ease of calculation – the group of identical sequences is shown in the box with a line indicating the representative used in tree construction. Isolate 203794 was not included because of sequencing difficulties.

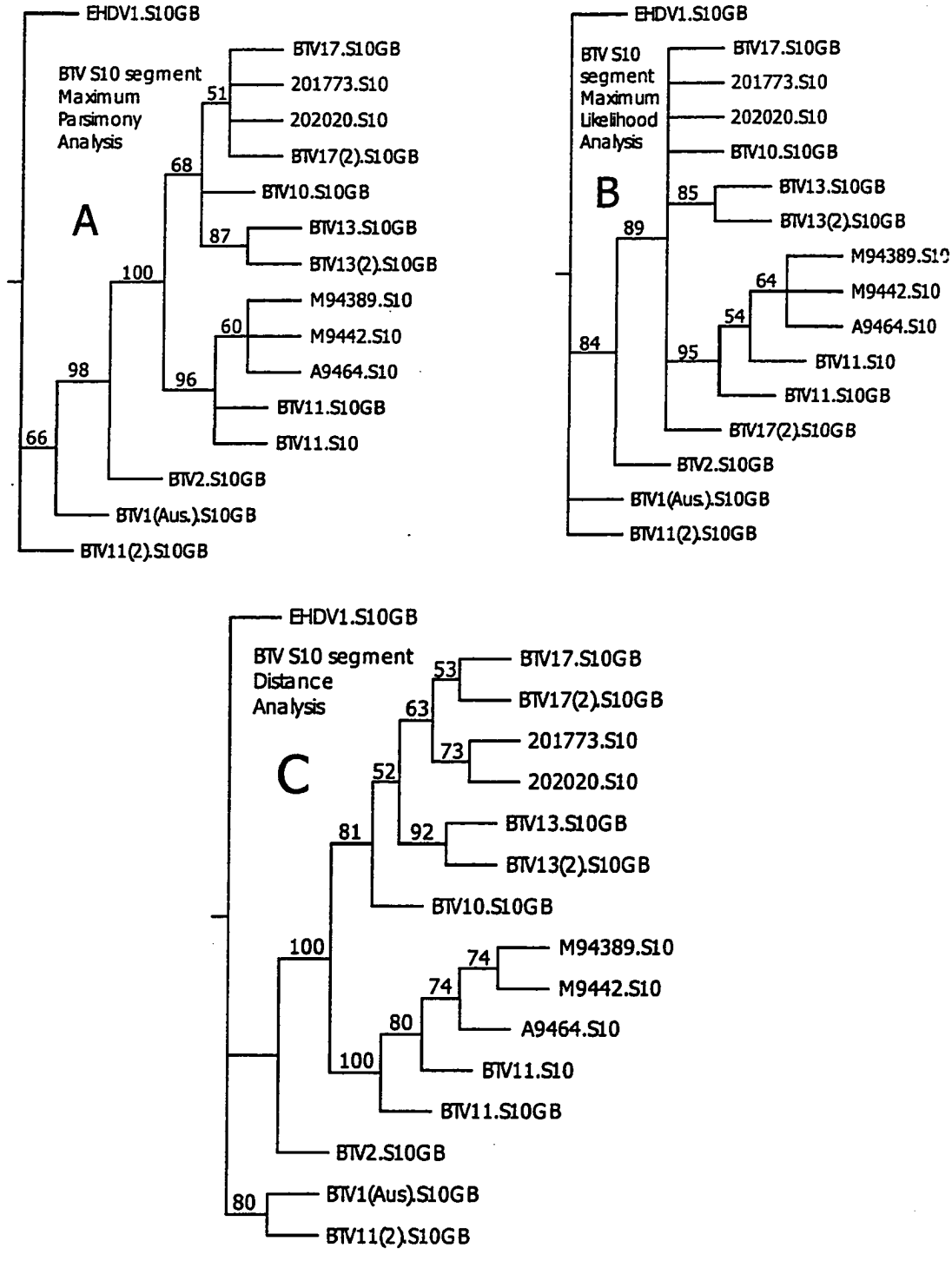

Figure2.75: Comparison of the different phylogenetic inference methods for the sequence data from the S10 segment of BTV isolates. Recomputation of the data shown in Figure 2.7 (C) by maximum parsimony (A) and maximum likelihood (B) methods. The more complex methods yielded essentially the same result as the simpler distance analysis.

informative characters (see pg. 35). Samples from the 1980's are shaded for ease of identification. The overall tree topology again suggests either temporal groupings, or clusters based on the species of the host from which the isolate was obtained. However, a third possibility exists in this analysis: a reassortment event. The analysis revealed that almost all the 1990's samples had an S7 segment of BTV2-like origin (100% support), and all the 1980's samples had an S7 segment of BTV17-like origin (99.9% support). See the Appendix C for distance matrix, including transition and transversion matrices, and alignment files.

Phylogenetic Analyses of the S10 Segment of BTV Isolates from Northern Colorado

Figure 2.7 shows the results of the analysis of the full length sequence of the S10 genome segment of all viral isolates described in Table 2.1, of which 131 were parsimony informative characters (see pg. 35). Samples from the 1980's are shaded for ease of identification. In this analysis, conclusions about the relationships of the isolates are clouded by the similarity of all S10 segments, as evidenced by the grouping of a BTV11 sequence with a BTV1 sequence, and the BTV13 sequences with the BTV17 sequences. Hence, the overall tree topology could suggest temporal groupings, clusters based on host, or the reassortment suggested in Figure 2.6. However, it is clear that the isolates do group in a way consistent with that seen in Figures 2.5 and 2.6, as well as being consistent with the hypothesis that BTV overwinters in endemic foci. See the Appendix E for distance matrix, including transition and transversion matrices, and alignment files.

DISCUSSION

Preliminary Characterization

The SSCP analysis was consistent with the previous finding for this method of a high positive predictive value for differences in sequence, but a low positive predictive value for similarity(12). While this technique may be useful in screening a large number of BTV isolates, the sensitivity required for this work was not provided by SSCP analysis. SSCP is insensitive for the detection of some primary sequence differences in field isolates of BTV, and overly sensitive to others. These results suggested that SSCP analysis of the L2 genome segment was not appropriate for this work; therefore, optimization efforts were not continued. Coincidentally, the price of automated sequencing came down dramatically at this time, so SSCP analysis was abandoned in favor of sequence analysis of field isolates.

Preliminary Phylogenetic Analyses

A preliminary study was conducted using existing segment L2 sequence data(9) from BTV10 to determine the minimum gene fragment size necessary for sequence analysis. Sequences were obtained from Genbank, and subjected to phylogenetic analysis either as full-length sequences (2953bp), or as gene fragments as described in Materials and Methods. The 1304bp fragment contains three variable regions, whereas the 358bp (bp 1770-2104) segment is internal to the larger fragment, and contains one variable region(16, 17).

As can be seen in Figure 2.4, an analysis of the 1304bp gene fragment of the BTV10 L2 segment gives almost identical results to an analysis of the full-length of the genome segment. In contrast, the analysis of the 358bp fragment (Figure 2.4) yielded a

phylogenetic tree that may appear similar, but has much less support for the overall tree topology. Thus, any conclusions drawn from this representation of the phylogenetic history of the genome segment would not necessarily mirror reality. For example, support for the separation of the BTV11/BTV17 clade from the BTV10 isolates and prototype sequence is absent, as is interior support for the separation of the prototype and vaccine sequence from the viral isolates. This lack of support is disturbing when considered in light of the sequence conservation of BTV relative to other RNA viruses. In fact, the analysis to be performed on the field isolates from Northern Colorado depends on the small distinction between the viral isolates as a group when compared to the prototype strain, as seen in the work of de Mattos et al.(8). Therefore, the lack of resolution in the analysis of the 358bp fragment rules out the analysis using the smaller fragment. Similarly, the demonstration of resolution similar to the full-length sequence in the analysis of the 1304bp fragment validates use of the larger fragment in place of the full-length sequence. Thus, all further phylogenetic analyses of the L2 segment utilized the 1304bp gene fragment.

Phylogenetic Analyses of the L2 Segment of BTV Isolates from Northern Colorado

The BTV isolates from Northern Colorado segregated into two different clades with a common root (Figure 2.5). A higher level of similarity between isolates than that seen by de Mattos et al.(8) was observed; however, this was expected considering the smaller dataset used and the microgeographic scale within which the isolates were collected. The grouping pattern may either represent temporal clades (1981-1983 vs. 1993-1994) or clades based on the host species from which the isolate was obtained (vertebrate host vs. invertebrate vector). It is even possible that it is some combination of

the two as all of the 1980's samples are from the vertebrate host, and all of the 1990's samples were isolated from the invertebrate vector. However, the results presented in Figure 2.5 support the main hypothesis: BTV overwinters in endemic foci. Genetic variation over time, while present, is not great enough to support the hypothesis of random reintroduction of virus from either continuous tropical cycles (culicoids carried on high-altitude wind patterns) or movement of infected animals into the endemic focus. In the case of random reintroduction, different genotypes within a serotype, and possibly even different serotypes of BTV, would be expected to be detected. These would include but not be limited to other U.S. genotypes (or serotypes) in the case of infected vertebrate animals, or tropical serotypes in the case of infected, tropical culicoids (e.g. serotypes 1, 3, 6, 12 (22)). In the context of the genetic variation seen within a single serotype in different parts of the country(23), these isolates from Northern Colorado appear to represent the evolution of a single cohort of viruses with little outside influence. This is similar to the results of de Mattos et al.(8) and is inconsistent with a viral population constantly in flux due to immigration of new virus genotypes.

Phylogenetic Analyses of the S7 Segment of BTV Isolates from Northern Colorado

The analysis of the S7 genome segment of all isolates in the viral library confirmed the results of the L2 segment analysis, but also provided an unexpected result (Figure 2.6). As seen in the analysis of the L2 segment (Figure 2.5), the isolates segregated into 2 clades that are either based on date of isolation or on host species from which the isolate was obtained, some combination thereof, or based on the reassortment seen by the grouping of the isolates from the 1980's with the BTV17 S7 segment, and the grouping of the 1990's isolates with the BTV2 S7 segment. The serotypic determinant in

BTV is VP2, the gene product of the L2 segment. As was seen in Figure 2.5, all BTV's from Northern Colorado segregated with BTV11 when the analysis concerned only segment L2, confirming the results of traditional serological tests. In contrast, the evidence of reassortment can be seen easily in Figure 2.6. Without further information, it is impossible to tell whether this reassortment occurred in the field or in the course of laboratory passage. Laboratory contamination is unlikely as it would require that two separate laboratories contaminated two separate sets of isolates in such a fashion that their S7 segments reassorted in all isolates. All samples were low passage – averaging 4-6 cell culture passages for the samples from ABADRL, and 6-8 insect and cell culture passages for the samples from AIDL. At the very least, if this finding represents a laboratory contamination, it is provocative in that it suggests increased relative fitness of different S7 segments in vector and/or vertebrate cell cultures.

It is more likely that these reassortments occurred naturally. However, BTV2 has never been isolated in Colorado, so this hypothesis requires either the trafficking of viruses containing a BTV2-like S7 to Colorado, or the unrecognized presence of a BTV2-like virus in Colorado at some point in the recent past. While it is impossible to distinguish between these possibilities given the current information, what can be said is that the ranges of BTV11 and BTV2 barely overlap in the United States (the overlap is in Florida, where BTV2 was first introduced). However, the origin of S7 has never been investigated in BTV isolates throughout the country. In fact, VP7 has been considered a group-specific antigen for many diagnostic tests, and has therefore not been considered suitable for phylogenetic analysis(1). Until the study by Bonneau et al.(5) in 1999, most sequence analyses of BTV were confined to the L2 segment, or analysis of single genome

segments without concurrent analysis of others. In any case, there is no record of a concurrent S7 and L2 segment analysis within a serotype in the scientific literature – and therefore, no record of different S7 segments within a single virus population in a natural setting. Thus, it is possible that viruses with S7 segments of different origins from the L2 segments have been circulating within the endemic populations of BTV's in the United States for many years without detection. These reassortments would never have been detected because all S7 segments code for proteins that react as a group-specific antigen. Positive samples are then serotyped based on the antigenic characteristics of VP2. Thus, serotypic designation does not address the possibility that one or more of the other genome segments could be from different serotypes.

If we first assume that a complete BTV11 virus existed in northern Colorado at some time in the past, we are led to the conclusion that the "new" S7 segments of different origin replaced the previously existing, BTV11 origin S7 segment. This reassortment then suggests that there is some selective advantage accorded to the S7 segment from BTV17- and BTV2-like viruses over the S7 segment of BTV11 origin. This advantage could be due to a number of factors, including relative environmental conditions (e.g. ambient temperature affects insect body temperature) to insect and/or vertebrate genetics and/or immune status. For whatever reason, conditions existed that favored the BTV17-like S7 segment during the early 1980's to 1993, and conditions existed that favored a BTV2-like S7 segment during the 1994 transmission season. These conditions may have occurred during the latter part of 1993 as the virus and vector were entering the overwintering phase of their life cycles.

These facts and hypotheses are very interesting and provocative; however additional research will be required to clarify the situation. All that can be said with confidence is that there is evidence that reassortant populations of BTV exist in nature in northern Colorado, and that more examples of similar reassortment events would probably be discovered if several or all of the RNA segments of viral isolates were molecularly characterized.

Phylogenetic Analyses of the S10 Segment of BTV Isolates from Northern Colorado

Since the full length of S10 was analyzed by Bonneau et al. (5), the rationale was to repeat their methods as closely as possible, to ensure that our results would be directly comparable to theirs in terms of the utility of the S10 segment for phylogenetic analysis.

S10 encodes NS3 and NS3a – non-structural proteins necessary for the egress of the virus from infected cells. As such, it represents a viral sequence that is removed from the selection pressures placed on VP2 and VP7 (encoded by L2 and S7, respectively). S10 segment sequences can also be the topotypic determinant of BTV(5). In addition, the sequence of the S10 segment is very similar between serotypes. For example, one of the Genbank BTV11 S10 sequences clusters with BTV1 (see Figure 2.7). Also, in the work of Pierce et al.(40), the topology of the phylogenetic tree based on the S10 sequence of several viral isolates from the western U.S. had little if any relationship to the serotype of the virus isolate. In the analysis of the Colorado isolates, I found a mixture of the expected and the unexpected: S10 segments of the 1980's isolates clusterrd with an S10 segment of BTV17 origin, suggesting a BTV17 origin of all of the viral segments. However, the 1990's isolates clustered with a BTV11 origin S10 segment, which would

be expected considering the serotype of the virus, but is in contrast to the BTV2-like origin of their S7 segment.

Overall, the analysis of the S10 segments support the original hypothesis: that BTV is maintained in a genetically stable state in endemic foci. The data do not support the annual reintroduction of BTV to endemic regions of the temperate world. In the case of either vertebrate or invertebrate annual reintroduction of BTV, different BTV topotypes would appear from year to year. This is not the case, regardless of how these data are viewed in terms of the reassortment of viral genome segments within a geographical region.

Summary

The analyses of all three genome segments support the hypothesis that the virus overwinters in endemic foci. The limited degree of genetic variability seen in a group of viral isolates from a single endemic focus is in direct contrast to the large degree of variation that would be expected in the analysis of isolates from several parts of the country or the world(5, 39). Taken together with the refutation of vertebrate overwintering by Tabachnick et al.(54), the overwintering of BTV in its invertebrate vector *Culicoides sonorensis* must be considered as the most likely mechanism of transeasonal maintenance of the virus.

In fact, it would be expected that in an analysis of a larger viral library of one serotype, collected over time from several endemic foci, the small differences leading to the groupings seen in this analysis would disappear, and samples would cluster simply based on location of isolation. These data would further support the hypothesis of genetic

identity within endemic foci, but would require a multi-state effort in the identification and isolation of virus from the field.

The possibility of a laboratory contamination must be addressed in both the S7 and S10 sequence analyses (Figures 2.6 and 2.7). These data would be consistent with that possibility, as there are not extraordinary segment sequences present in the analysis – sequences that could come only from genome segment trafficking in nature (i.e. segments from serotypes outside the United States, or at least segments not present in the laboratory). However, this possibility is still viewed as unlikely given the passage histories, origins, and isolation methods of the isolates.

These data demonstrate the necessity of sequencing multiple genome segments in any phylogenetic analysis of BTV. In the worst case scenario, multiple analyses would help in the identification of laboratory contamination. However, analysis of multiple segments from viral isolates could reveal potential trafficking of BTV genomes and genome segments in the natural world. This could be critical for molecular epidemiological studies of BTV: considering the "decoupling" of the evolution of S10 from L2 and S7 (i.e. its relative freedom from external evolutionary pressures), multiple analyses represent another source of information for the determination of topotypes. It is easy to imagine a scenario where the ability of the S10 segment to spread throughout a viral population after its introduction masks the true history of BTV isolates in an individual analysis of that segment. However, finding two viral isolates with very similar S10 segments, but different S7 segments, could further illuminate where the viruses came from, how and when they were introduced, and how best to stop their re-introduction in

the future. *In toto*, these analyses would provide invaluable information about the natural biology of BTV.

The limited number of viral isolates examined in these analyses limits the conclusions that can be drawn concerning the significance of reassortment events in nature to the overall epidemiology of BTV. There is the possibility that L2 could also be prone to reassortment – i.e. that a BTV17 "backbone" virus acquired a BTV11-like L2 segment in one case, while a BTV11 "backbone" virus acquired a BTV2-like S7 segment in another. This is somewhat unexpected, considering the importance that is attached to the L2 gene segment in regards to the life cycle. As can be seen, it is difficult to separate the concept (and language) of serotype from any discussion concerning the nature and origins of viral isolates (and/or their genome segments). However, the L2 segment may be the gene most prone to reassortment. As the main target of the vertebrate immune response, it is under the most extreme external selection pressures of all of the genome segments. Reassortment of L2 (and the "acquisition" of a new serotype) could provide a quantum leap in relative fitness. Indeed, there is a major precedent for this concept with the influenza viruses. The hemagglutinin (HA) and neuraminidase (NA) proteins, which are involved in virus binding and cell entry and egress, reassort on a regular basis over time, allowing for the spread of the virus around the world. Occasionally, with the introduction of a completely novel HA/NA combination, serious pandemics occur, as with the 1918 flu pandemic which killed more people than all of World War I(13, 38, 44), or at least major public health scares occur, as with the "Avian flu" incident of 1999(3, 27). Preliminary data concerning BTV in nature indicate a high frequency of reassortment events in nature, especially in the vector, but still significant in the

vertebrate hosts(2). Hence, the BTV L2 segment may be simply another nucleic acid sequence that can reassort to create a selective advantage for the "new" virus. Additionally, the L2 segment may be a focused target of reassortment in response to negative selection pressures, and must be viewed as such in any examination of the natural world.

In the end, it is clear that there is a significant need for more phylogenetic information concerning all segments of BTV to better understand the genetic composition, molecular epidemiology, and natural biology of the virus.

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CHAPTER 3 - MOLECULAR BASIS OF PERSISTENCE OF BTV IN *CULICOIDES SPP.*

INTRODUCTION

Molecular epidemiological studies (Chapter 2) suggested that BTV does overwinter in endemic foci. In order to increase sample sizes for additional phylogenetic studies, and to add a temporal dimension to the dataset, virus isolation (VI) was attempted in the 1996 transmission season. *C. sonorensis* larvae were collected from the BTV endemic premises sampled in the past, and VI was again attempted in *Aedes triseriatus* mosquitoes, embryonated chicken eggs, and BHK-21 cells as described in Chapter 2. No isolates were obtained from 7743 larvae that were reared to adults in the laboratory in the 1996 collection season (see Appendix G). This was consistent with studies in 1992 and 1993 in which only one isolate was obtained in 1993 and nine in 1994 (out of 901 light-trap collected flies, 49,521 larvae, and 11,221 larvae that were allowed to develop to adulthood – for a total of 61,643 total insects for 1992-1994)(2). Even in 1994, most of the isolates (5 of 9) came from a single larval collection, which was harvested many times over several weeks as the larvae developed and were collected as pupae.

Due to the difficulty with VI, considerable effort was also devoted to developing an RT-PCR approach for the detection of the L2 genome segment in insect vectors. RT-PCR was thought to be much more sensitive than the previously used blot hybridization technique(6) for detecting BTV L2 dsRNA in culicoids. Also, DNA from direct amplification of viral RNA in larvae would be a satisfactory alternative to virus isolation for phylogenetic analysis, and would thereby increase the sample size. However, amplification of L2 dsRNA from vectors also was unsuccessful. All samples from the 1996 transmission season listed in Appendix G were negative. In considering these

results, it seemed possible that the biology of the virus could be confounding the isolation and/or RT-PCR detection efforts. The precedent of viral genome segment down regulation in quantity or activity seen in wound tumor virus (WTV) and described in the Review of Literature (pg. 40) was used as a model. There, the long-term propagation of the virus in plant cells resulted in the "loss" of those genome segments necessary for insect cell infection(7). In the case of persistent infection of larval *C. sonorensis* by BTV, it was postulated that the L2 segment was in some way lost or modified such that it was undetectable by conventional molecular means. Thus, assays for BTV in larvae based on the detection of L2 dsRNA could yield falsely negative results.

Experiments were designed to test the hypothesis that persistent BTV infection in larvae was associated with down regulation of the L2 segment, and presumably the M5 segment as well. It was thought that since the subviral particle (VP's 2 and 5 removed) is more infectious for insect cells(5), long-term infection of vectors could occur without the two genome segments (and their gene products) that are necessary for the infection of vertebrate cells. Obviously, the actual loss of these segments would preclude replication of the virus in vertebrate animals. However, down regulation in the absolute number of these genome segments in larval stages would allow for the subsequent recovery of infectivity for vertebrates, possibly associated with the massive anatomical and physiological changes associated with metamorphosis from larva to pupa and then adult insect. These changes could most simply provide new and/or additional tissues for virus replication, and/or increase the absolute amount of the virus in the insect as a whole (increased body size), both of which could increase the chance of generating complete BT virions and thereby increase the chance of a positive isolation attempt. Alternatively, the

virus could have co-evolved with its vector, and thus somehow "recognize" the physiological cues and events associated with metamorphosis. Such signals could trigger the replication of the full viral genome complement in preparation for the emergence of the insect at the beginning of the transmission season. Both of these hypotheses are consistent with the observation that VI from insects requires that they be raised to the adult stage (as discussed in Chapter 2) instead of being able to isolate virus from field-collected larvae, despite the identification of BTV nucleic acid and antigen in those larvae(2, 6).

However, detection of a contaminant suspected to be BTV in cell cultures derived from colony larvae created a problem(10). The obvious experiment to test the hypothesis stated above would be to isolate virus from field collected larvae using *Culicoides* cell cultures, which would bypass the need for a functional VP2 and would enhance the rate of VI above that seen in vertebrate systems. In that experimental system, the insect cell receptor ligand (VP7) would bind to its target *Culicoides sonorensis* cells – presumably a highly efficient system for VI. However, one *Culicoides* cell line available was demonstrated to be persistently infected with a dsRNA-containing particle(10). Other cell lines, which had been derived from larvae collected from an endemic focus in Colorado instead of colony culicoids, were found to contain BTV segments L3 and S10 by RT-PCR (W. Wilson, personal communication). Without a cell culture system that was guaranteed to be BTV-free, VI from larval culicoids was viewed as problematic.

Thus, an RT-PCR approach was used to assay for L2, L3, S7, and S10 dsRNA in cell cultures and in larvae from endemic foci. Detection of L3, S7, and/or S10 dsRNA and not L2 dsRNA would further support the hypothesis of a persistent infection with a

BTV subviral particle in overwintering larvae, and would provide insight into a possible mechanism of viral persistence in the insect vector. Since the vertical transmission of BTV from infected adults to their overwintering progeny would necessitate persistent infection of the larvae during diapause, these experiments could further support the central hypothesis of this dissertation.

MATERIALS AND METHODS

Virus Isolation and Propagation

Virus isolation (VI) was attempted from field-collected material during 1996 transmission season as described in Chapter 2 (pg. 63). Briefly, mud samples were collected from endemic foci that had been monitored in the past by scientists from AIDL and ABADRL(2, 6). Larvae were collected daily, reared to adults, and VI was attempted by intravenous inoculation of embryonated chicken eggs, intrathoracic inoculation of *Aedes triseriatus* mosquitoes, and isolation in baby hamster kidney-clone 21 (BHK-21) cells. Three blind passages were performed as in Chapter 2.

Cell Cultures

C6/36 cells were cloned by Igarashi in 1978(4) from an original cell line first initiated by Singh and Paul in 1969(8) from *Aedes albopictus* larvae. C6/36 cells are commonly used at AIDL, and were obtained from frozen stocks there.

CuVaW3 cell cultures were obtained from USDA-ABADRL (initially provided by Dr. James Mecham). These cell cultures were derived from *C. sonorensis* larvae that had been collected at the Howard site in Northern Colorado, and cultured by traditional methods(9). They had previously been tested for the presence of BTV10 dsRNA by RT-PCR for segments 2, 7, and 10 by Dr. Bill Wilson (USDA-ABADRL). However, at the

Table 3.1
Primers used to amplify BTV sequences

Primer Name	Upper Primer Sequence (5'->3')	Upper Primer Position
L2Short F	CGGCGGTATTTCAGCACCAGAGTC	1770
L2Long F	CTTGCGGCAAGATTATGGTCGTAC	1183
L3 Forward	AGCCGTTAGATTTCCAGATTGGAA	1285
S7 Upper	ATGGACACTATCGCTGCAAGAGCG	18
S10 Upper	GTTAAAAAGTGTCGCTGCC	1
L2nest Forward	GCCAAAGGTGATGTGGTATGTCCC	1826
L3nest Forward	ACGGATGGGCTACAATTGATGTTG	1366
S7nest Forward	GCCCGACCTCGTTGGCACAGAGAA	169
S10nest Forward	CGGGCTGATCCAAAGGTTCGAGGA	28
Primer name	**Lower Primer Sequence (5'->3')**	**Lower Primer Position**
L2Short R	TGCGGTTCACCTAAACTCTGGACT	2104
L2Long R	GCGCGATCTTAGTGTTTGCGTAAT	2463
L3 Reverse	CGGCGTTCTGATGCGCCCCAGCAA	1687
S7 Lower	CCCGCTTGAGTTTGCTGTGAATTA	614
S10 Lower	GTAAGTGTGTAGCGCCGCA	804
L2nest Reverse	GCCGATGCTGGGGTGCGTATAAGT	1952
L3nest Reverse	CGCCGCCTCAGAATCTTTCCCTGC	1536
S7nest Reverse	GCGGCACGCATGAACCATCTCCCA	431
S10nest Reverse	CGCCACTCTACCTACTGATCTCAG	706

Primer Set Name	Optimal Annealing Temperature (^{O}C)	Product Length	Product G/C Content	Product Tm (^{O}C)
L2Short	56.1	358bp	45.30%	76.6
L2Long	56.6	1304bp	43.10%	77.1
L3	56	426bp	45.10%	76.8
S7	57.7	620bp	47.70%	78.4
S10	53.9	822bp	46.10%	78
L2nest	56.5	150bp	50.70%	76.2
L3nest	56	194bp	47.90%	76.1
S7nest	59.3	286bp	48.30%	77.3
S10nest	55.3	702bp	44.40%	77.2

time we received them, they had not been tested with primers designed for detection of BTV11 sequences, nor had they been tested for the presence of BTV antigen by immunofluorescence with polyclonal antiserum. BHK-21 cells were used as a model of vertebrate cell infection for these experiments. Cell cultures were propagated and maintained as described in Appendix H.

Positive controls were obtained by infecting subsets of all three cell cultures with approximately 10^3 PFU of BTV11 Station Strain (USDA-ABADRL) per $150cm^2$ of confluent cells (multiplicity of infection approximately equal to 0.01). Insect cell cultures were maintained on growth medium for 7 days to allow for establishment of the infection. BHK-21 cells were only infected for 3 days in an attempt to maximize the amount of viral RNA in intact cells (the normal lytic cycle being 5-7 days).

Assay of Cell Cultures for BTV Nucleic Acid

Isolation of total RNA was performed as described in Chapter 2 (pg. 67), with the PUREscript kit (Gentra Systems), and was the same for all pools of field-collected larvae. RT-PCR was performed by the same method as outlined in Chapter 2, using the primers delineated in Table 3.1, in an attempt to detect genome segments L2, L3, S7, S10. Nested PCR was performed on the cellular products to obtain enough DNA for automated sequencing reactions. A second ("nested") set of PCR reactions was performed according to the protocol outlined in Chapter 2, using 5µl of the first PCR reaction as the starting material for the second, and the appropriate set of nested primers (Table 3.1). Selected RT-PCR products were sequenced at the Davis Sequencing facility to confirm their identity as BTV sequences and not a spurious cellular product of similar size.

Assay for BTV Antigen in Cell Cultures

An indirect immunofluorescence assay was performed on uninfected CuVaW3 cells using the polyclonal guinea pig anti-BTV antiserum produced by el Hussein et al.(3). A commercial secondary goat anti-guinea pig antibody conjugated with fluorescein isothiocyanate (FITC) (Cappel, Inc.) as a reporter was used for detection. Confluent cells were scraped into the medium and dotted onto glass slides, fixed in 100% acetone for 10 minutes, and circled with an ink pen. Then, the primary antibody (diluted 1:200) was added to the fixed cells and incubated at 37^{O}C for 30 minutes. Cells were then washed 3 times in phosphate-buffered saline (PBS). The secondary antibody was then added, incubated, and washed following the same protocol. Coverslips were then adhered to the slide with a mixture of 50% glycerol/50% PBS, and cells were viewed with an Olympus BH2 fluorescence microscope with an appropriate UV light source and filter to see the FITC tag.

Assay of *C. sonorensis* Larvae from 1998 for BTV Nucleic Acid

In summer 1998, field-collected *C. sonorensis* larvae were assayed directly by RT-PCR for BTV analyte. Immunofluorescence and/or virus isolation were not performed on field-collected larvae to conserve starting materials for the RT-PCR. *C. sonorensis* larvae were collected from sites in Northern Colorado that have been the subject of longitudinal epidemiological studies by either researchers from AIDL and/or ABADRL, as noted previously. Larval culicoids were collected daily and grouped into pools of 1-50 in de-ionized water. These pools were stored at –70^{O}C until RNA extraction, which was performed as described in Chapter 2 (pg. 67). The larvae were thawed, triturated in 1.7ml microcentrifuge tubes, and total RNA was isolated using the

Table 3.2

***C. sonorensis* collections in 1998**

POOL NUMBER	DATE COLL.	SITE	# OF	STAGE	# OF	STAGE
A-98-1	7/24/98	Howard	8	Larva	1	Pupa
A-98-2	7/24/98	Howard	9	Larva	1	Pupa
A-98-3	7/24/98	Howard	4	Larva	1	Pupa
A-98-4	7/30/98	Fields	0	Larva	10	Pupa
A-98-5	7/30/98	Fields	5	Larva	0	Pupa
A-98-6	7/30/98	Fields	8	Larva	5	Pupa
A-98-7	7/30/98	Fields	2	Larva	3	Pupa
A-98-8	7/30/98	Fields	1	Larva	6	Pupa
A-98-9	7/30/98	Fields	1	Larva	1	Pupa
A-98-10	7/30/98	Fields	6	Larva	6	Pupa
A-98-11	7/30/98	Fields	0	Larva	1	Pupa
A-98-12	8/10/98	Howard	10	Larva	0	Pupa
A-98-13	8/10/98	Howard	10	Larva	0	Pupa
A-98-14	8/10/98	Howard	10	Larva	0	Pupa
A-98-15	8/10/98	Howard	10	Larva	0	Pupa
A-98-16	8/10/98	Howard	0	Larva	8	Pupa
A-98-17	8/10/98	Howard	10	Larva	0	Pupa
A-98-18	8/10/98	Howard	10	Larva	0	Pupa
A-98-19	8/10/98	Howard	10	Larva	0	Pupa
A-98-20	8/10/98	Howard	10	Larva	0	Pupa
A-98-21	8/10/98	Howard	10	Larva	0	Pupa
A-98-22	8/10/98	Howard	10	Larva	0	Pupa
A-98-23	8/10/98	Howard	10	Larva	0	Pupa
A-98-24	8/10/98	Howard	10	Larva	0	Pupa
A-98-25	8/10/98	Howard	10	Larva	0	Pupa
A-98-26	8/10/98	Howard	10	Larva	0	Pupa

Date Coll. = Date of mud collection from the site, NOT of the larval collection from the mud.

PUREscript kit (Gentra Systems). Total RNA was stored at $-70^{o}C$ until use. RT-PCR was performed as described in Chapter 2. Positive controls were obtained by infecting BHK-21 cells with BTV11 Station Strain as described above.

RESULTS

Virus Isolation and Detection of the L2 Genome Segment

As discussed in the introduction, all field-collected material from the 1996 transmission season was negative for either virus isolation and/or detection of the L2 dsRNA segment of BTV11 by RT-PCR. The methods used for virus isolation and nucleic acid detection are described in Chapter 2.

Detection of BTV Antigen and dsRNA in *C. sonorensis* Cell Cultures

Viral nucleic acid was detected in non-exogenously infected CuVaW3 cells (Figure 3.1) after 2 passages at AIDL. Genome segments L3, S7, and S10 were detected in the cell cultures (S7 data are not shown because clear pictures were never available). However, L2 dsRNA was notable in its absence (data not shown). These lines were approximately passage 30 before being received by our laboratory (L. McHolland, personal communication). Viral antigen was detected in CuVaW3 cells (Figure 3.2), but antigen detection was not performed on field-collected larvae to conserve starting materials for the RT-PCR.

It is obvious from the gel (Figure 3.1) that the amount of amplified DNA present is low relative to the controls, therefore sequencing was problematic. Sequence data were obtained for the S10 band from the CuVaW3 cell line using a nested PCR technique.

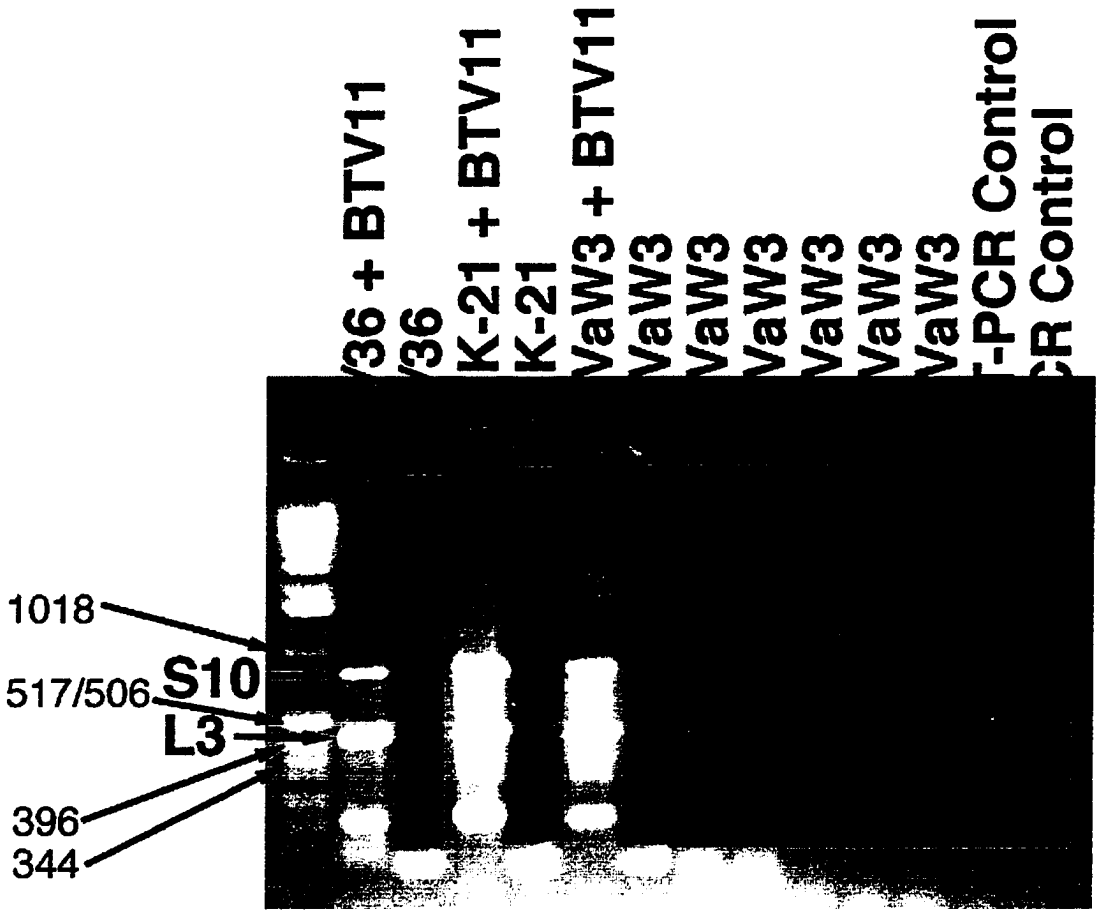


Figure 3.1: Agarose gel electrophoresis of RT-PCR products from CuVaW3 cell cultures, as described in the text. The CuVaW3 total RNA preps listed were from different passage numbers, isolated at different times. Bands were enhanced in Adobe Photoshop by increasing the contrast 69%, and decreasing the brightness 30% on all at the same time (a multiple selection was performed, as seen by the black boxes). The RT-PCR control was a reaction run with the others lacking added RNA. The PCR control was a reaction run with the others lacking any cDNA from the RT reaction, but similar to the others in all other respects. Labels were added with Adobe Photoshop. S10 = product obtained using primers for the S10 segment. L3 = product obtained using primers for the L3 segment. Numbers = Molecular size markers (number of nucleotides).

Table 3.3: Distance analyses of the BTV-like sequence found in CuVaW3 cells.

Pairwise distances between the 643bp S10 segment sequence from CuVaW3 cells and analogous, full length viral sequences from BTV's 10, 11, 13, and 17 from Genbank. The first chart is the total number of differences between the sequences, the second is the number of those which are transitions, and the third is the number of those distances that are transversions. The sequence from CuVaW3 cells has a maximum of 10 differences from the most dissimilar Genbank sequence.

Distance: Number of **different** nucleotides.
No. of Codons in Subset: 274 of 274
Codon Position(s) Used: 1 2 3
Gap Sites and Missing Information Data: All such sites were removed from the subset data

OTU Labels
1.. CuVaW3.S10N
2.. BTV17.S10GB
3.. BTV13.S10GB
4.. BTV10.S10GB
5.. BTV11.S10GB

Distances in the upper-right matrix
'*' indicates an invalid distance value

OTUs	1	2	3	4	5
1		1.0000	4.0000	5.0000	10.0000
2			3.0000	4.0000	9.0000
3				5.0000	10.0000
4					9.0000
5					

Distance: Number of different nucleotides (**transitions only**).
No. of Codons in Subset: 274 of 274
Codon Position(s) Used: 1 2 3
Gap Sites and Missing Information Data: All such sites were removed from the subset data

OTU Labels
1.. CuVaW3.S10N
2.. BTV17.S10GB
3.. BTV13.S10GB
4.. BTV10.S10GB
5.. BTV11.S10GB

Distances in the upper-right matrix
'*' indicates an invalid distance value

OTUs	1	2	3	4	5
1		1.0000	4.0000	3.0000	9.0000
2			3.0000	2.0000	8.0000
3				3.0000	9.0000
4					6.0000
5					

Distance: Number of different nucleotides (**transversions only**).
No. of Codons in Subset: 274 of 274
Codon Position(s) Used: 1 2 3
Gap Sites and Missing Information Data: All such sites were removed from the subset data

OTU Labels
1.. CuVaW3.S10N
2.. BTV17.S10GB
3.. BTV13.S10GB
4.. BTV10.S10GB
5.. BTV11.S10GB

Distances in the upper-right matrix
'*' indicates an invalid distance value

OTUs	1	2	3	4	5
1		0.0000	0.0000	2.0000	1.0000
2			0.0000	2.0000	1.0000
3				2.0000	1.0000
4					3.0000
5					

Table 3.4: Multiple alignment file for the comparison of the BTV-like sequence found by nested RT-PCR in CuVaW3 cells (CuVaW3.S10N), and several BTV sequences downloaded from Genbank (noted with the suffix "GB").

```
CLUSTAL W (1.74) multiple sequence alignment


CuVaW3.S10N       ------------------------------------------------------------T
BTV17.S10GB       GTTAAAAAGTGTCGCTGCCATGCTATCCGGGCTGATCCAAAGGTTCGAGGAAGAAAAAAT
BTV13.S10GB       GTTAAAAAGTGTCGCTGCCATGCTATCCGGGCTGATCCAAAGGTTCGAGGAAGAAAAAAT
BTV10.S10GB       GTTAAAAAGTGTCGCTGCCATGCTATCCGGGCTGATCCAAAGGTTCGAGGAAGAAAAAAT
BTV11.S10GB       GTTAAAAAGTGTCGCTGCCATGCTATCCGGGCTGATCCAAAGGTTCGAGGAAGAAAAAAT
                                                                            *

CuVaW3.S10N       GAAACATAATCAGGAACGGGTTGAAGAGTTGAGTTTGGTCCGTGTGGATGATACAATTTC
BTV17.S10GB       GAAACATAATCAGGAACGGGTTGAAGAGTTGAGTTTGGTCCGTGTGGATGATACAATTTC
BTV13.S10GB       GAAACATAATCAGGAACGGGTTGAAGAGTTGAGTTTGGTCCGTGTGGATGATACAATTTC
BTV10.S10GB       GAAACATAATCAGGAACGGGTTGAAGAGTTGAGTTTGGTCCGTGTGGATGATACAATTTA
BTV11.S10GB       GAAACATAATCAGGAACGGGTTGAAGAGTTGAGTTTGGTCCGTGTGGATGATACAATTTC
                  ************************************************************

CuVaW3.S10N       TCAGCCGCCAAGATATGCTCCGAGCGCGCCAATGCCATCATCTATGCCCACTGTTGCACT
BTV17.S10GB       TCAGCCGCCAAGATATGCTCCGAGCGCGCCAATGCCATCATCTATGCCCACTGTTGCACT
BTV13.S10GB       TCAGCCGCCAAGATATGCTCCGAGCGCGCCAATGCCATCATCTATGCCCACTGTTGCACT
BTV10.S10GB       TCAGCCGCCAAGATATGCTCCGAGCGCGCCAATGCCATCATCTATGCCTACTGTTGCACT
BTV11.S10GB       TCAGCCGCCAAGATATGCTCCGAGCGCGCCAATGCCATCGTCAATGCCTACTGTTGCACT
                  ************************************** ** ***** ***********

CuVaW3.S10N       TGAAATCTTGGACAAAGCGATGTCAAATACAACGGGTGCTACGCAAACACAAAAAGCGGA
BTV17.S10GB       TGAAATCTTGGACAAAGCGATGTCAAATACAACGGGTGCTACGCAAACACAAAAAGCGGA
BTV13.S10GB       TGAAATCTTGGACAAAGCGATGTCAAATACAACGGGTGCTACGCAAACACAAAAAGCGGA
BTV10.S10GB       TGAAATCTTGGACAAAGCGATGTCAAATACAACGGGTGCTACGCAAACACAAAAAGCGGA
BTV11.S10GB       TGAAATCTTGGACAAAGCGATGTCAAATACAACGGGTGCTACGCAAACACAAAAGGCGGA
                  ************************************************** *****

CuVaW3.S10N       GAAGGCTGCATTCGCATCGTACGCGGAAGCGTTTCGTGATGATGTGAGGTTAAGACAAAT
BTV17.S10GB       GAAGGCTGCATTCGCATCGTACGCGGAAGCGTTTCGTGATGATGTGAGGTTAAGACAAAT
BTV13.S10GB       GAAGGCTGCATTCGCATCGTACGCGGAAGCGTTTCGTGATGATGTGAGGTTAAGACAAAT
BTV10.S10GB       GAAGGCTGCATTCGCATCGTACGCGGAAGCGTTTCGTGATGATGTGAGGTTAAGACAAAT
BTV11.S10GB       AAAGGCTGCATTCGCATCGTACGCGGAAGCGTTTCGTGATGATGTGAGGTTAAGGCAAAT
                   ************************************************** *****

CuVaW3.S10N       TAAGCGACATGTGAATGRGCAAATTTTACCAAAGTTGAAAAGTGACCTAGGAGGCTTAAA
BTV17.S10GB       TAAGCGACATGTGAATGAGCAAATTTTACCAAAGTTGAAAAGTGACCTAGGAGGCTTAAA
BTV13.S10GB       TAAGCGACATGTGAATGAGCAAATTTTACCAAAGTTGAAAAGTGACCTAGGAGGCTTAAA
BTV10.S10GB       TAAGCGACATGTGAATGAGCAAATTTTACCAAAGTTGAAAAGTGACCTAGGAGGCTTAAA
BTV11.S10GB       TAAGCGACATGTGAATGAGCAAATTTTACCAAAGTTGAAAAGTGACCTAGGAGGCTTAAA
                  ***************** ******************************************

CuVaW3.S10N       GAAGAAAAGAGCTATTATACATATGACACTGCTGATAGCGGCAGTCGTTGCTCTGCTGAC
BTV17.S10GB       GAAGAAAAGAGCTATTATACATATGACACTGCTGATAGCGGCAGTCGTTGCTCTGCTGAC
BTV13.S10GB       GAAGAAAAGAGCTATTATACATATGACACTGCTGATAGCGGCAGTCGTTGCTCTGCTGAC
BTV10.S10GB       GAAGAAAAGAGCTATTATACATATGACACTGCTGATAGCTGCAGTCGTTGCTCTGCTGAC
BTV11.S10GB       GAAGAAAAGAGCTATTATACATATGACACTGCTGATAGCGGCAGTCGTTGCTCTGCTGAC
                  ****************************************** ******************

CuVaW3.S10N       ATCAGTTTGTACGTTGTCGAGCGATATGAGCGTGGCATTTAAATTAAATGGTACATCAGC
BTV17.S10GB       ATCAGTTTGTACGTTGTCGAGCGATATGAGCGTGGCATTTAAATTAAATGGTACATCAGC
BTV13.S10GB       ATCAGTTTGTACGTTGTCGAGCGATATGAGCGTGGCATTTAAATTAAATGGTACATCAGC
BTV10.S10GB       ATCAGTTTGTACGTTGTCGAGCGATATGAGCGTGGCATTTAAATTAAATGGTACATCAGC
BTV11.S10GB       ATCAGTTTGTACGTTGTCGAGCGATATGAGCGTGGCATTTAAATTAAATGGTACATCAGC
                  ************************************************************
```

```
CuVaW3.S10N     TGAAATACCTCAGTGGTTTAAGAGTTTGAATCCAATGTTGGGTGTGGTAAATCTGGGAGC
BTV17.S10GB     TGAAATACCTCAGTGGTTTAAGAGTTTGAATCCAATGTTGGGTGTGGTAAATCTGGGAGC
BTV13.S10GB     TGAAATACCTCAGTGGTTTAAGAGTTTGAATCCAATGTTGGGTGTGGTAAATCTGGGAGC
BTV10.S10GB     TGAAATACCTCAGTGGTTTAAGAGTTTGAATCCAATGTTGGGTGTGGTAAATCTGGGAGC
BTV11.S10GB     TGAAATACCTCAGTGGTTTAAGAGTTTGAATCCAATGTTGGGTGTGGTAAATCTGGGAGC
                ************************************************************

CuVaW3.S10N     TACCTTCCTGATGATGGTCTGTGCAAAAAGTGAAAGAAGCTTGAATCAGCAAATTGATAT
BTV17.S10GB     TACCTTCCTGATGATGGTCTGTGCAAAAAGTGAAAGAAGCTTGAATCAGCAAATTGATAT
BTV13.S10GB     TACCTTCCTGATGATGGTTTGTGCAAAAAGTGAAAGGAGCTTGAATCAGCAAATTGATAT
BTV10.S10GB     TACCTTCCTGATGATGGTTTGTGCAAAAAGTGAAAGAAGCTTGAATCAGCAAATTGATAT
BTV11.S10GB     TACCTTCCTGATGATGGTTTGTGCGAAAAGTGAAAGAAGCTTGAATCAGCAAATTGATAT
                ****************** ***** *********** ************************

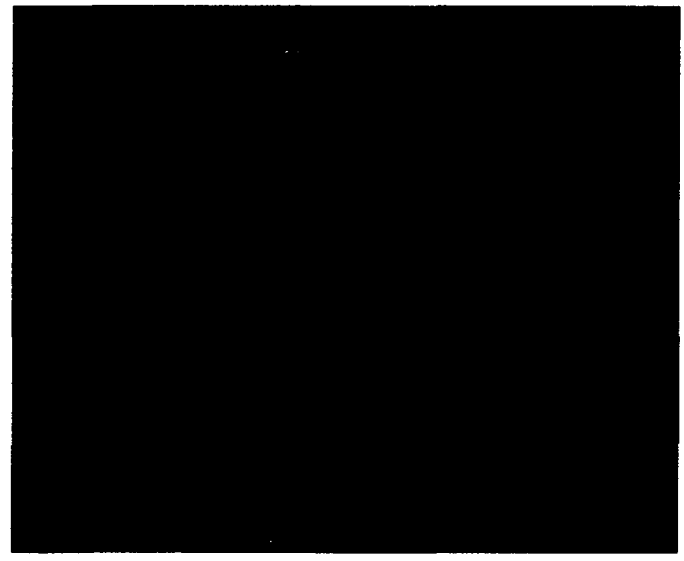
CuVaW3.S10N     GATTAAGAAGGAAGTGATGAAGAAGCAATCGTATAATGATGCGGTGAGGATGAGTTTTAC
BTV17.S10GB     GATTAAGAAGGAAGTGATGAAGAAGCAATCGTATAATGATGCGGTGAGGATGAGTTTTAC
BTV13.S10GB     GATTAAGAAGGAAGTGATGAAGAAGCAATCGTATAATGATGCGGTGAGGATGAGTTTTAC
BTV10.S10GB     GATTAAGAAGGAAGTGATGAAGAAGCAATCGTATAATGATGCGGTGAGGATGAGTTTTAC
BTV11.S10GB     GATTAAGAAGGAAGTGATGAAGAAGCAATCGTATAATGATGCGGTGAGGATGAGTTTTAC
                ************************************************************

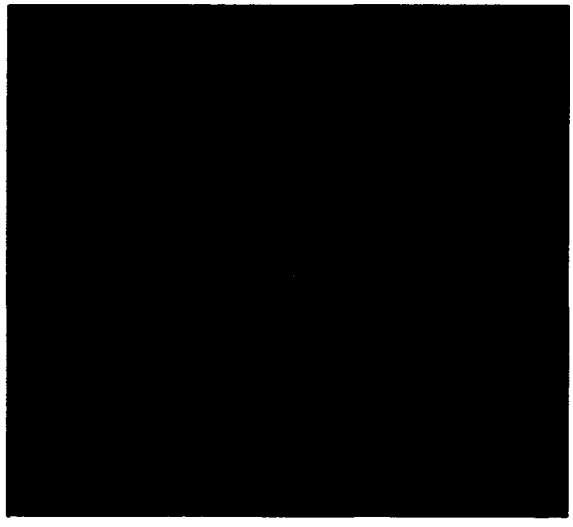
CuVaW3.S10N     GGAATTTTCATCAGTCCCGCTAGATGGTTTTGAAT-ACCATTA----------------
BTV17.S10GB     GGAATTTTCATCAGTCCCGCTAGATGGTTTTGAATTACCATTAACCTAAGGTCAGTAGGT
BTV13.S10GB     GGAATTTTCATCAGTCCCGCTAGATGGTTTTGAATTGCCATTAACCTAAGGTCAGTAGGT
BTV10.S10GB     GGAATTTTCATCAGTCCCGCTAGATGGTTTTGAATTACCATTAACCTAAGGTCGGTAGGT
BTV11.S10GB     AGAATTTTCATCAGTCCCGCTAGATGGTTTTGAATCACCATTAACCTAAGGTCAGTAGGT
                 ********************************** ******

CuVaW3.S10N     ------------------------------------------------------------
BTV17.S10GB     AGAGTGGCGCCCCAAGGTCCGTATCGTGTAGAGTGGTTGATCTCACGATGCGGACTCCTA
BTV13.S10GB     AGAGTGGCGCCCCAAGGTCCGTATCGTGTAGAGTGGTTGATCTCACGATGCGGACTCCTA
BTV10.S10GB     AGAGTGGCGCCCCAAGGTCCGTATCGTGTAGAGTGGTTGATCTCACGATGCGGACTCCTA
BTV11.S10GB     AGAGTGGCGCCCCAAGGTCCGTATCGTGTAGAGTGGTTGATCTCACGATGCGGACTCCTA

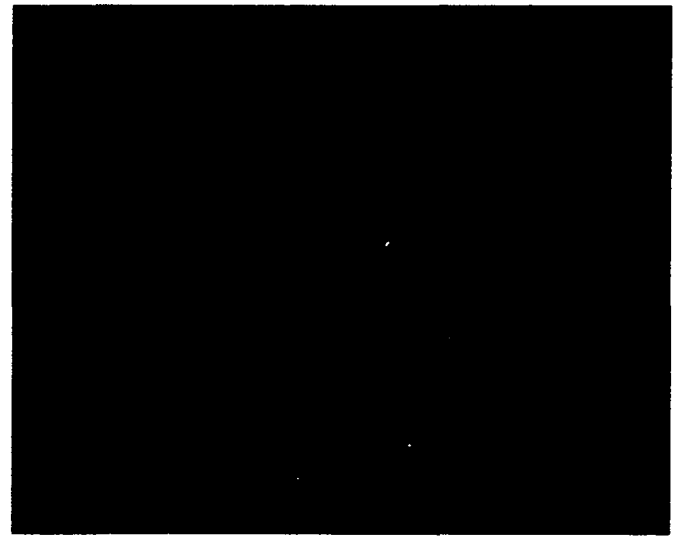
CuVaW3.S10N     ----------------------------------------
BTV17.S10GB     CTGCTGTCTAACGGGGGAGGGTACGCGGCGCTACACACTTAC
BTV13.S10GB     CTGCTGTCTAACGGGGGAGGGTACGCGGCGCTACACACTTAC
BTV10.S10GB     CTGCTGTCTAACGGGGGAGGGTACGCGGCGCTACACACTTAC
BTV11.S10GB     CTGCTGTCTAACGGGGGAGGGTACGCGGCGCTACACACTTAC
```

Antisera to BTV11 in CuVaW3 cells (20X).





Antisera to BTV11 in CuVaW3 cells (40X).



Antisera raised to BTV10 in CuVaW3 cells (20X)

Figure 3.2: Examples of indirect immunoflourescence with different polyclonal antisera for BTV10 and BTV11 in non-exogenously infected CuVaW3 cells. Antisera were prepared in guinea pigs by immunization with the virus noted as described in Materials and Methods (pg. 102). Secondary staining was accomplished with a commercially produced goat anti-guinea pig antibody.

The sequence of the DNA amplified from the CuVaW3 cell cultures was then compared with the analogous Genbank sequences from BTV11, BTV13, BTV17, and BTV10. The amplified sequence was most most similar to the S10 segment from BTV17. This is consistent with previous results from the amplification of BTV-like sequences from this cell line (W. Wilson, personal communication). As can be seen in Table 3.3, the number of differences between the all of sequences was not large (maximum of 10 differences, and a minimum of 1). The alignment of the sequences is shown in Table 3.4. The large gap at the beginning and end of the sequence from the CuVaW3 cells versus the viral sequences downloaded from Genbank is due to the fact that a nested PCR technique was required to obtain the sequence.

Detection of BTV Nucleic Acid in Field-Collected *C. sonorensis* Larvae

C. sonorensis larvae were collected in summer 1998 as described above (Table 3.2), and were used solely for the detection of BTV nucleic acid. BTV dsRNA was detected as shown in Figure 3.3. As can be seen in the figure, the absence of an RT-PCR product from the L2 segment is notable, especially considering the presence of RT-PCR products from the S7 segment in three of the nine pools shown (samples A-98-4, -5, and -8). Overall, RT-PCR products from the S7 segment were detected in 5 of 26 larval/pupal pools from 1998; the L2 segment was always absent. Samples were run side by side, with the same reaction mixtures, enzymes, etc. – the only difference in the reaction conditions was the primer set used.

Additionally, BTV S7 segment dsRNA was detected in 26 of 319 adult fly pools from the 1996 collection season (data not shown). These were pools that had been processed for viral isolation as described in Chapter 2. A portion of these samples had

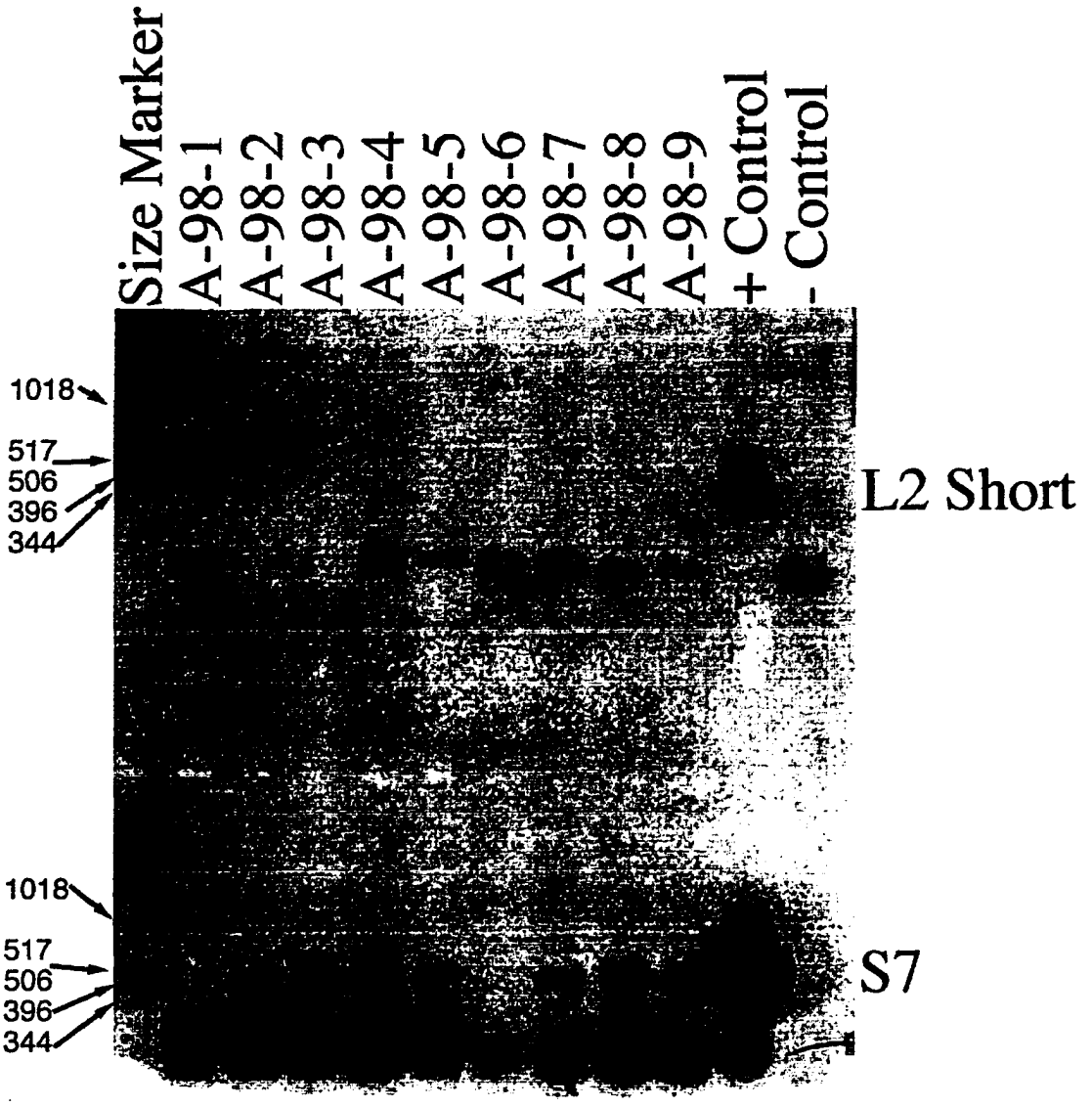


Figure 3.3: Agarose gel electrophoresis of RT-PCR products from field-collected *C. sonoresis* larvae. These samples represent pools 1-9 as shown in Table 3.2. Positive control dsRNA was obtained from BHK-21 cells infected with BTV11 Station Strain. Samples 4, 5, and 8 are positive for the presence of S7 dsRNA.

been stored at –70°C for possible use at a later date (Figure 2.2, pg. 65). It is expected that after processing and long-term storage, degradation of viral nucleic acid would lead to a lower rate of detection (8.15% positive for the 1996 season [processed adults] versus 19.23% positive for the 1998 season [larvae/pupae]).

DISCUSSION

These results support the hypothesis of a persistent BTV infection of overwintering *C. sonorensis*, consistent with previous data from AIDL showing detection of viral antigen in field-collected samples (larvae reared to adults in the lab) before the beginning of the transmission season (February(6) and April, see Appendix G). The lack of a detectable L2 segment suggests the reason for previous virus isolation failures from larval culicoids. In the persistently infected larvae, L2 is either not functional, not present, or not producing enough of its gene product VP2 (the vertebrate cell receptor ligand) for vertebrate cell infection. Hence, any isolation methods based on infection of vertebrate cells (embryonated chicken eggs, BHK-21 cells) are inefficient. We have observed that rearing field-collected larvae to adults increases the virus isolation rate(2). Indeed, all AIDL viral isolates are from field-collected larvae that were reared to adulthood(2). The process of metamorphosis may provide tissues or organs more permissive to BTV replication, including a productive cell infection involving all 10 dsRNA segments. Alternatively, the process may just increase the overall titer of live virus, resulting in an increase in the absolute number of intact virions and vertebrate cell infection.

In contrast to VI from arthropods, it is relatively easy to isolate virus from the blood or tissues of infected and viremic cattle. After experimental infection, virus can be

isolated nearly 100% of the time during the time of expected viremia(1), and virus can easily be isolated from sentinel animals placed in endemic foci(6). This difference provides indirect evidence in support of the hypothesis of BTV persistence in larval culicoids in a different form – the subviral particle – from that in infected vertebrates.

The sequence of segment S10 dsRNA derived from the CuVaW3 cell line was most similar to BTV17, which is consistent with previous data. This finding, however, is not consistent with the data presented in Chapter 2: since the larvae used to start the cell line were collected in 1995, it would be expected that the sequence would be most similar to the BTV11 S10 segment present in 1994, and not the BTV17 S10 segment that was seen during 1981-1983. This concern, while troubling, does not negate this work because it is possible that 1995 represented a year where a new S10 segment was introduced into the area, and spread through the viral population. Alternatively, 1995 could be the year where the previously existing S10 segment "reasserted control" of the area under study and won the fitness race with the invading sequence from 1993-4. This may seem untenable, but both of those hypotheses are consistent with a segment that determines topotype: by definition, any new genotype of that segment introduced into an area will either be extinguished or replace all previously existing versions so that it alone defines the topotype of that region. Any other situation would present a phylogeny of multiple groupings within a geographical region, and negate the ability of the virus to be placed geographically by that segment.

For example, imagine a scenario where topotype A (S10 segment sequence A) exists in a microgeographic region completely and entirely. Upon the introduction of another, competing sequence (topotype B), there are three possibilities: A could

extinguish B, B could replace A, or they could coexist stably in the long term. If we accept the premise that the S10 sequence can reliably and reproducibly locate BTV genotypes geographically, we must reject the possibility that the two sequences could coexist over time, as the presence of a new, "invading" sequence B would disrupt the geographical groupings of any phylogeny based on a sampling period after its introduction. However, if we again accept the premise that S10 is a topotypic determinant, we can imagine a scenario where the invading (B) and previously existing (A) sequences do coexist for a short time, during which they compete until the most fit "wins" and replaces the other sequence. In this case, we would see multiple sequences within an area representing the 2 types. A large enough dataset would allow us to define where the invader came from, and longitudinal data would allow us to see which was "winning" by the change in the relative gene frequencies upon repeated sampling.

This latter situation is lent support by the data in Chapter 2 in their entirety: if "A" in the above scenario describes the genotype present from 1981-1983 (BTV11-L2, BTV17-S7, BTV17-S10) and "B" describes the genotype present in 1994 (BTV11-L2, BTV2-S7, BTV11-S10), then isolate 270 from 1993 represents an intermediate genotype (BTV11-L2, BTV2-S7, BTV17-S10). 1993 could have been a year where we were witnessing a Darwinian struggle between 2 BTV genotypes – the single grouping seen in the S10 segment phylogeny (Figure 2.7) an artificial result of the limited sample size and the inefficient virus isolation process. The recommendation for analysis of multiple segments of BTV in molecular epidemiological studies is again emphasized by these seemingly contradictory results.

However, regardless of the source of the persistent infection, the real value of this experiment is the demonstration of a persistent infection of vector cells. While previous work had hinted at the possibility, it has never been demonstrated with confidence before now.

Despite the mentioned problems, the importance of this demonstration of BTV persistence in vector cells should not be underrated – either in the context of the basic biology of the virus, or in the larger sense of the epidemiology of bluetongue as a whole. When considering arboviruses as biological entities, it is evident that their success depends upon the success of their invertebrate vectors. An arbovirus exists in close association with its vector, thereby maximizing its opportunities for dispersal and transmission. For these and other reasons, it has become a paradigm in the field of arbovirology that viruses persistently infect their invertebrate vectors. This demonstration of a persistent infection of vector cell lines, taken together with the demonstration of BTV-like nucleic acid in field-collected larvae, completes the bluetongue virus life cycle in a compelling and consistent fashion. These results suggest a model for BTV long-term survival in nature, which could have profound implications for the control and/or eradication of the virus and disease from endemic areas of the world. This model will be discussed more fully in the summary of this dissertation.

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SUMMARY

When taken as a whole, the body of work presented in this dissertation lends significant support to the hypothesis that bluetongue virus overwinters by vertical transmission in its invertebrate vector, *Culicoides sonorensis*. First, an examination of the literature surrounding the three main hypotheses of the transeasonal maintenance mechanism leads one to the conclusion that overwintering in the invertebrate vector is most consistent with the body of knowledge concerning BTV. While it has been impossible to entirely refute either of the other hypotheses, their reliance on events which occur only rarely in the natural world must argue against their importance in the long-term, year to year maintenance of the virus in endemic foci.

Second, the demonstration of the genetic identity of BTV in northern Colorado over time is inconsistent with the annual reintroduction of the virus to endemic foci from continuous transmission cycles. It is clear that the topology of a phylogenetic tree representing a continual immigration of BTV's from other areas of the world would include different serotypes and topotypes – which was not seen here. While the phylogenetic analyses do not refute the maintenance of the virus in a vertebrate host, they do provide the basis of a conclusion refuting the constant influx of viruses from external sources.

Last, this work culminates with the demonstration of a persistent infection of a *Culicoides* cell line with BTV, in addition to the demonstration of BTV sequences in *Culicoides* larvae. While this finding by no means proffers a sweeping refutation of all other possible transeasonal maintenance mechanisms, it does complete the life cycle of BTV in a way that is consistent with previous studies as well as current thought in the

field. There are some obvious weaknesses in this work: the larvae were collected at the end of the transmission season, there is only a small amount of RT-PCR product, giving rise to concerns of contamination, and the sequence is not what would be expected from previous phylogenetic analyses. These problems will hopefully be corrected through repetition and the analysis of multiple gene segments from the *Culicoides* cell lines, as well as an actual examination of overwintering larvae.

Most convincingly, however, these data give rise to a consistent and cohesive model of the transeasonal maintenance mechanism of BTV in temperate regions of the world. We can now imagine a scenario where the virus overwinters as a persistent infection of *Culicoides* larvae, those larvae having been vertically infected by one or both of their parents. These larvae mature and emerge in the spring as infected adults, who then have the possibility of transmitting the virus to naïve vertebrates that they encounter. This gives rise to the presence of the disease within defined geographical regions of the country on an annual basis, as well as amplifying the virus to infect any naïve culicoids, which can then pass the virus on to their progeny. Studies designed to investigate the nature of the persistent infection of *Culicoides* larvae could include (but are not limited to): gene expression analyses (capture of poly-A mRNA's with poly-T substrates, *in vitro* translation of isolated transcripts), electron microscopic examination of sectioned larvae for subviral particles and/or intracellular localization of active transcription units (cores) with anti-VP3 antibodies (either gold-labelled for electron microscopy, or flourescently tagged for confocal microscopy), and/or co-cultivation of demonstrably uninfected *Culicoides* cells with non-exogenously infected cell lines (to demonstrate the presence or

absence to transmission of the persistently infecting virus to other cells within the infected larvae).

Perhaps the most interesting part of this model is the state of the virus while persistently infecting the invertebrate vector. Here, the data presented lead one to the conclusion that the virus exists in an altered state in the insect vector – most probably by downregulating the activity or absolute number of those viral segments which encode gene products necessary for vertebrate cell infection (L2 and M5). The nature of this change is unclear, but could be tied to the life cycle of the insect and the immense morphologic and physiologic changes that it goes through in the course of its development. In any case, this change makes sense in terms of the basic biology of the virus and its relationship with its vector: by downregulating any genome segment activity which is unnecessary for its survival in the invertebrate, the virus is sparing cellular resources which are then available to the insect in its attempt to survive the winter in a quiescent state. While the savings may seem small, it should be obvious that every microjoule of stored energy and microgram of physical and chemical resources are precious to the insect, and that by enabling the survival of its host, the virus is ensuring its own survival.

This change would also explain previous failures at virus isolation from field-collected insects. Traditional methods were all based on the presence and function of L2, first being antibody based and then nucleic acid methods designed to detect the "most important" of the segments – the antigenic determinant and the determinant of serotype. By demonstrating the lack of a functional L2 in persistently infected larvae, and by analogy postulating its lack of abundance relative to the other segments in the adult

insect, the failures of the past take on the role of supporting the model by increasing our confidence in the lack of a detectable L2 in field-collected adult insects and larvae.

In the strictest sense, this work does nothing to change the debate about the overwintering mechanism of BTV. There is no conclusive proof presented here that demonstrates BTV overwinters in the juvenile stages of its invertebrate vector. However, the development of a working model for a BTV overwintering mechanism, and the acquisition of a substantial amount of circumstantial evidence in support of that model cannot be ignored. This model can now serve as the basis for more stringent experimental design; and it will eventually be discarded or, hopefully, borne out by a larger, more conclusive body of evidence.

APPENDICES

L2

APPENDIX A – Distance analyses for the 1304bp fragment of L2

Distance: Number of different nucleotides.
No. of Codons in Subset: 417 of 417
Codon Position(s) Used: 1 2 3
Gap Sites and Missing Information Data: All such sites were removed from the subset data

OTU Labels
1.. E94393
2.. M94430
3.. A9464
4.. M94368
5.. M9442
6.. A945
7.. M94389
8.. A94389
9.. 270
10.. BTV11SS
11.. 203794
12.. 203853
13.. 203843
14.. 201767
15.. 201773
16.. 202020
17.. 203826
18.. 203734
19.. 203733
20.. 202894
21.. 203732
22.. 201400
23.. BTV11
24.. BTV10
25.. BTV17
26.. BTV23
27.. BTV2

Distances in the upper-right matrix
'*' indicates an invalid distance value

OTUs	1	2	3	4	5	6	7
1		0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
2			0.0000	0.0000	0.0000	0.0000	0.0000
3				0.0000	0.0000	0.0000	0.0000
4					0.0000	0.0000	0.0000
5						0.0000	0.0000
6							0.0000
7							

OTUs	8	9	10	11	12	13	14
1	0.0000	1.0000	1.0000	17.0000	14.0000	14.0000	14.0000
2	0.0000	1.0000	1.0000	17.0000	14.0000	14.0000	14.0000
3	0.0000	1.0000	1.0000	17.0000	14.0000	14.0000	14.0000
4	0.0000	1.0000	1.0000	17.0000	14.0000	14.0000	14.0000
5	0.0000	1.0000	1.0000	17.0000	14.0000	14.0000	14.0000
6	0.0000	1.0000	1.0000	17.0000	14.0000	14.0000	14.0000
7	0.0000	1.0000	1.0000	17.0000	14.0000	14.0000	14.0000

8		1.0000	1.0000	17.0000	14.0000	14.0000	14.0000
9			0.0000	16.0000	13.0000	13.0000	13.0000
10				16.0000	13.0000	13.0000	13.0000
11					21.0000	21.0000	21.0000
12						0.0000	0.0000
13							0.0000
14							

OTUs	15	16	17	18	19	20	21
1	14.0000	14.0000	14.0000	14.0000	14.0000	14.0000	14.0000
2	14.0000	14.0000	14.0000	14.0000	14.0000	14.0000	14.0000
3	14.0000	14.0000	14.0000	14.0000	14.0000	14.0000	14.0000
4	14.0000	14.0000	14.0000	14.0000	14.0000	14.0000	14.0000
5	14.0000	14.0000	14.0000	14.0000	14.0000	14.0000	14.0000
6	14.0000	14.0000	14.0000	14.0000	14.0000	14.0000	14.0000
7	14.0000	14.0000	14.0000	14.0000	14.0000	14.0000	14.0000
8	14.0000	14.0000	14.0000	14.0000	14.0000	14.0000	14.0000
9	13.0000	13.0000	13.0000	13.0000	13.0000	13.0000	13.0000
10	13.0000	13.0000	13.0000	13.0000	13.0000	13.0000	13.0000
11	21.0000	21.0000	21.0000	21.0000	21.0000	21.0000	21.0000
12	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
13	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
14	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
15		0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
16			0.0000	0.0000	0.0000	0.0000	0.0000
17				0.0000	0.0000	0.0000	0.0000
18					0.0000	0.0000	0.0000
19						0.0000	0.0000
20							0.0000
21							

OTUs	22	23	24	25	26	27
1	14.0000	16.0000	403.0000	376.0000	601.0000	598.0000
2	14.0000	16.0000	403.0000	376.0000	601.0000	598.0000
3	14.0000	16.0000	403.0000	376.0000	601.0000	598.0000
4	14.0000	16.0000	403.0000	376.0000	601.0000	598.0000
5	14.0000	16.0000	403.0000	376.0000	601.0000	598.0000
6	14.0000	16.0000	403.0000	376.0000	601.0000	598.0000
7	14.0000	16.0000	403.0000	376.0000	601.0000	598.0000
8	14.0000	16.0000	403.0000	376.0000	601.0000	598.0000
9	13.0000	15.0000	402.0000	377.0000	600.0000	597.0000
10	13.0000	15.0000	402.0000	377.0000	600.0000	597.0000
11	21.0000	27.0000	407.0000	374.0000	606.0000	599.0000
12	0.0000	24.0000	402.0000	371.0000	605.0000	597.0000
13	0.0000	24.0000	402.0000	371.0000	605.0000	597.0000
14	0.0000	24.0000	402.0000	371.0000	605.0000	597.0000
15	0.0000	24.0000	402.0000	371.0000	605.0000	597.0000
16	0.0000	24.0000	402.0000	371.0000	605.0000	597.0000
17	0.0000	24.0000	402.0000	371.0000	605.0000	597.0000
18	0.0000	24.0000	402.0000	371.0000	605.0000	597.0000
19	0.0000	24.0000	402.0000	371.0000	605.0000	597.0000
20	0.0000	24.0000	402.0000	371.0000	605.0000	597.0000
21	0.0000	24.0000	402.0000	371.0000	605.0000	597.0000
22		24.0000	402.0000	371.0000	605.0000	597.0000
23			402.0000	380.0000	607.0000	601.0000
24				394.0000	608.0000	599.0000
25					599.0000	583.0000
26						497.0000
27						

Distance: Number of different nucleotides (transversions only).
No. of Codons in Subset: 417 of 417
Codon Position(s) Used: 1 2 3
Gap Sites and Missing Information Data: All such sites were removed from the subset data

OTU Labels
1.. E94393
2.. M94430
3.. A9464
4.. M94368
5.. M9442
6.. A945
7.. M94389
8.. A94389
9.. 270
10.. BTV11SS
11.. 203794
12.. 203853
13.. 203843
14.. 201767
15.. 201773
16.. 202020
17.. 203826
18.. 203734
19.. 203733
20.. 202894
21.. 203732
22.. 201400
23.. BTV11
24.. BTV10
25.. BTV17
26.. BTV23
27.. BTV2

Distances in the upper-right matrix
'*' indicates an invalid distance value

OTUs	1	2	3	4	5	6	7
1		0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
2			0.0000	0.0000	0.0000	0.0000	0.0000
3				0.0000	0.0000	0.0000	0.0000
4					0.0000	0.0000	0.0000
5						0.0000	0.0000
6							0.0000
7							

OTUs	8	9	10	11	12	13	14
1	0.0000	0.0000	0.0000	1.0000	0.0000	0.0000	0.0000
2	0.0000	0.0000	0.0000	1.0000	0.0000	0.0000	0.0000
3	0.0000	0.0000	0.0000	1.0000	0.0000	0.0000	0.0000
4	0.0000	0.0000	0.0000	1.0000	0.0000	0.0000	0.0000
5	0.0000	0.0000	0.0000	1.0000	0.0000	0.0000	0.0000
6	0.0000	0.0000	0.0000	1.0000	0.0000	0.0000	0.0000
7	0.0000	0.0000	0.0000	1.0000	0.0000	0.0000	0.0000
8		0.0000	0.0000	1.0000	0.0000	0.0000	0.0000
9			0.0000	1.0000	0.0000	0.0000	0.0000
10				1.0000	0.0000	0.0000	0.0000
11					1.0000	1.0000	1.0000
12						0.0000	0.0000
13							0.0000
14							

OTUs	15	16	17	18	19	20	21
1	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
2	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
3	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
4	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
5	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
6	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
7	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
8	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
9	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
10	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
11	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
12	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
13	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
14	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
15		0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
16			0.0000	0.0000	0.0000	0.0000	0.0000
17				0.0000	0.0000	0.0000	0.0000
18					0.0000	0.0000	0.0000
19						0.0000	0.0000
20							0.0000
21							

OTUs	22	23	24	25	26	27
1	0.0000	3.0000	165.0000	155.0000	353.0000	371.0000
2	0.0000	3.0000	165.0000	155.0000	353.0000	371.0000
3	0.0000	3.0000	165.0000	155.0000	353.0000	371.0000
4	0.0000	3.0000	165.0000	155.0000	353.0000	371.0000
5	0.0000	3.0000	165.0000	155.0000	353.0000	371.0000
6	0.0000	3.0000	165.0000	155.0000	353.0000	371.0000
7	0.0000	3.0000	165.0000	155.0000	353.0000	371.0000
8	0.0000	3.0000	165.0000	155.0000	353.0000	371.0000
9	0.0000	3.0000	165.0000	155.0000	353.0000	371.0000
10	0.0000	3.0000	165.0000	155.0000	353.0000	371.0000
11	1.0000	4.0000	166.0000	154.0000	352.0000	370.0000
12	0.0000	3.0000	165.0000	155.0000	353.0000	371.0000
13	0.0000	3.0000	165.0000	155.0000	353.0000	371.0000
14	0.0000	3.0000	165.0000	155.0000	353.0000	371.0000
15	0.0000	3.0000	165.0000	155.0000	353.0000	371.0000
16	0.0000	3.0000	165.0000	155.0000	353.0000	371.0000
17	0.0000	3.0000	165.0000	155.0000	353.0000	371.0000
18	0.0000	3.0000	165.0000	155.0000	353.0000	371.0000
19	0.0000	3.0000	165.0000	155.0000	353.0000	371.0000
20	0.0000	3.0000	165.0000	155.0000	353.0000	371.0000
21	0.0000	3.0000	165.0000	155.0000	353.0000	371.0000
22		3.0000	165.0000	155.0000	353.0000	371.0000
23			166.0000	156.0000	352.0000	372.0000
24				158.0000	358.0000	364.0000
25					340.0000	354.0000
26						272.0000
27						

Distance: Number of different nucleotides (transitions only).
No. of Codons in Subset: 417 of 417
Codon Position(s) Used: 1 2 3
Gap Sites and Missing Information Data: All such sites were removed from the subset data

OTU Labels

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4.. M94368
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7.. M94389
8.. A94389
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14.. 201767
15.. 201773
16.. 202020
17.. 203826
18.. 203734
19.. 203733
20.. 202894
21.. 203732
22.. 201400
23.. BTV11
24.. BTV10
25.. BTV17
26.. BTV23
27.. BTV2

Distances in the upper-right matrix
'*' indicates an invalid distance value

OTUs	1	2	3	4	5	6	7
1		0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
2			0.0000	0.0000	0.0000	0.0000	0.0000
3				0.0000	0.0000	0.0000	0.0000
4					0.0000	0.0000	0.0000
5						0.0000	0.0000
6							0.0000
7							

OTUs	8	9	10	11	12	13	14
1	0.0000	1.0000	1.0000	16.0000	14.0000	14.0000	14.0000
2	0.0000	1.0000	1.0000	16.0000	14.0000	14.0000	14.0000
3	0.0000	1.0000	1.0000	16.0000	14.0000	14.0000	14.0000
4	0.0000	1.0000	1.0000	16.0000	14.0000	14.0000	14.0000
5	0.0000	1.0000	1.0000	16.0000	14.0000	14.0000	14.0000
6	0.0000	1.0000	1.0000	16.0000	14.0000	14.0000	14.0000
7	0.0000	1.0000	1.0000	16.0000	14.0000	14.0000	14.0000
8		1.0000	1.0000	16.0000	14.0000	14.0000	14.0000
9			0.0000	15.0000	13.0000	13.0000	13.0000
10				15.0000	13.0000	13.0000	13.0000
11					20.0000	20.0000	20.0000
12						0.0000	0.0000
13							0.0000
14							

OTUs	15	16	17	18	19	20	21
1	14.0000	14.0000	14.0000	14.0000	14.0000	14.0000	14.0000
2	14.0000	14.0000	14.0000	14.0000	14.0000	14.0000	14.0000
3	14.0000	14.0000	14.0000	14.0000	14.0000	14.0000	14.0000
4	14.0000	14.0000	14.0000	14.0000	14.0000	14.0000	14.0000
5	14.0000	14.0000	14.0000	14.0000	14.0000	14.0000	14.0000
6	14.0000	14.0000	14.0000	14.0000	14.0000	14.0000	14.0000

7	14.0000	14.0000	14.0000	14.0000	14.0000	14.0000	14.0000
8	14.0000	14.0000	14.0000	14.0000	14.0000	14.0000	14.0000
9	13.0000	13.0000	13.0000	13.0000	13.0000	13.0000	13.0000
10	13.0000	13.0000	13.0000	13.0000	13.0000	13.0000	13.0000
11	20.0000	20.0000	20.0000	20.0000	20.0000	20.0000	20.0000
12	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
13	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
14	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
15		0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
16			0.0000	0.0000	0.0000	0.0000	0.0000
17				0.0000	0.0000	0.0000	0.0000
18					0.0000	0.0000	0.0000
19						0.0000	0.0000
20							0.0000
21							

OTUs	22	23	24	25	26	27
1	14.0000	13.0000	238.0000	221.0000	248.0000	227.0000
2	14.0000	13.0000	238.0000	221.0000	248.0000	227.0000
3	14.0000	13.0000	238.0000	221.0000	248.0000	227.0000
4	14.0000	13.0000	238.0000	221.0000	248.0000	227.0000
5	14.0000	13.0000	238.0000	221.0000	248.0000	227.0000
6	14.0000	13.0000	238.0000	221.0000	248.0000	227.0000
7	14.0000	13.0000	238.0000	221.0000	248.0000	227.0000
8	14.0000	13.0000	238.0000	221.0000	248.0000	227.0000
9	13.0000	12.0000	237.0000	222.0000	247.0000	226.0000
10	13.0000	12.0000	237.0000	222.0000	247.0000	226.0000
11	20.0000	23.0000	241.0000	220.0000	254.0000	229.0000
12	0.0000	21.0000	237.0000	216.0000	252.0000	226.0000
13	0.0000	21.0000	237.0000	216.0000	252.0000	226.0000
14	0.0000	21.0000	237.0000	216.0000	252.0000	226.0000
15	0.0000	21.0000	237.0000	216.0000	252.0000	226.0000
16	0.0000	21.0000	237.0000	216.0000	252.0000	226.0000
17	0.0000	21.0000	237.0000	216.0000	252.0000	226.0000
18	0.0000	21.0000	237.0000	216.0000	252.0000	226.0000
19	0.0000	21.0000	237.0000	216.0000	252.0000	226.0000
20	0.0000	21.0000	237.0000	216.0000	252.0000	226.0000
21	0.0000	21.0000	237.0000	216.0000	252.0000	226.0000
22		21.0000	237.0000	216.0000	252.0000	226.0000
23			236.0000	224.0000	255.0000	229.0000
24				236.0000	250.0000	235.0000
25					259.0000	229.0000
26						225.0000
27						

APPENDIX B Multiple alignment file for the 1304bp fragment of L2

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M94389.L2     -ACG-GAGGTTCCGCTAGAGAACAACAAATATGTGTTTAGTCGGATTAATTTGTTTGATT
BTV10.L2GB    --CGTGAGAGGCCGTTAGAAGACAATAAATATGTGTTCGCTCGTCTTAATCTATTTGATA
A94389.L2     -ACG-GAGGTTCCGCTAGAGAACAACAAATATGTGTTTAGTCGGATTAATTTGTTTGATT
BTV11.L2GB    -ACG-GAGGTTCCGCTAGAGAACAACAAATATGTGTTTAGTCGGATTAATCTGTTTGATT
202894.L2     -ACG-GAGGTTCCGCTAGAGAACAACAAATATGTGTTTAGTCGGATTAATCTGTTTGATT
203733.L2     -ACG-GAGGTTCCGCTAGAGAACAACAAATATGTGTTTAGTCGGATTAATCTGTTTGATT
203794.L2     -ACG-GAGGTTCCGCTAGAGAACAACAAATATGTGTTCAGTCGGATTAATCTGTTTGATT
203826.L2     -ACG-GAGGTTCCGCTAGAGAACAACAAATATGTGTTTAGTCGGATTAATCTGTTTGATT
270.L2        -ACG-GAGGTTCCGCTAGAGAACAACAAATATGTGTTTAGTCGGATTAATCTGTTTGATT
M9442.L2      -ACG-GAGGTTCCGCTAGAGAACAACAAATATGTGTTTAGTCGGATTAATTTGTTTGATT
A945.L2       -ACG-GAGGTTCCGCTAGAGAACAACAAATATGTGTTTAGTCGGATTAATTTGTTTGATT
M94368.L2     -ACG-GAGGTTCCGCTAGAGAACAACAAATATGTGTTTAGTCGGATTAATTTGTTTGATT
A9464.L2      -ACG-GAGGTTCCGCTAGAGAACAACAAATATGTGTTTAGTCGGATTAATTTGTTTGATT
E94393.L2     -ACG-GAGGTTCCGCTAGAGAACAACAAATATGTGTTTAGTCGGATTAATTTGTTTGATT
M94430.L2     -ACG-GAGGTTCCGCTAGAGAACAACAAATATGTGTTTAGTCGGATTAATTTGTTTGATT
BTV17.L2GB    -ACG--AGCGACAGTTAGAGAACAATAAATATGTCTTCAATCGTATAAATTTATTTGATT
BTV11.L2      -ACG-GAGGTTCCGCTAGAGAACAACAAATATGTGTTTAGTCGGATTAATCTGTTTGATT
201400.L2     -ACG-GAGGTTCCGCTAGAGAACAACAAATATGTGTTTAGTCGGATTAATCTGTTTGATT
203734.L2     -ACG-GAGGTTCCGCTAGAGAACAACAAATATGTGTTTAGTCGGATTAATCTGTTTGATT
201767.L2     -ACG-GAGGTTCCGCTAGAGAACAACAAATATGTGTTTAGTCGGATTAATCTGTTTGATT
201773.L2     -ACG-GAGGTTCCGCTAGAGAACAACAAATATGTGTTTAGTCGGATTAATCTGTTTGATT
202020.L2     -ACG-GAGGTTCCGCTAGAGAACAACAAATATGTGTTTAGTCGGATTAATCTGTTTGATT
203853.L2     -ACG-GAGGTTCCGCTAGAGAACAACAAATATGTGTTTAGTCGGATTAATCTGTTTGATT
203732.L2     -ACG-GAGGTTCCGCTAGAGAACAACAAATATGTGTTTAGTCGGATTAATCTGTTTGATT
203843.L2     -ACG-GAGGTTCCGCTAGAGAACAACAAATATGTGTTTAGTCGGATTAATCTGTTTGATT
BTV2.L2GB     --CGTGAAAGGGAGCGGGAAACGGAGAAGTATATATTTAGTAAGATAAACCTATTTGATT
BTV23.L2GB    TACGATAGGAGCAG--AGAGAGCGACAAATACGTATATAGCCGAGTGAATCTGTTTGATT
                ** *      *   **     * ** **  * *         * **  * ******

M94389.L2     -CAAACCTGGAAGTCGGTGATCAAG-TAGTGCATTGGAAGTACGAAATTGATGGCCCAGC
BTV10.L2GB    -CTAACTTAGCCGTTGGGGATGAAA-TCATTCACTGGCGTTATGAGGTTTATCAGCCTAA
A94389.L2     -CAAACCTGGAAGTCGGTGATCAAG-TAGTGCATTGGAAGTACGAAATTGATGGCCCAGC
BTV11.L2GB    -CAAACCTGGAAGTCGGTGATCAAG-TAGTGCATTGGAAGTACGAAATTGATGGCCCAGC
202894.L2     -CAAACCTGGAAGTCGGTGATCAAG-TAGTGCATTGGAAGTACGAAATTGATGGCCCAGC
203733.L2     -CAAACCTGGAAGTCGGTGATCAAG-TAGTGCATTGGAAGTACGAAATTGATGGCCCAGC
203794.L2     -CAAACCTGGAAGTCGGTGATCAAG-TAGTGCATTGGAAGTACGAAATTGATGGCCCAGC
203826.L2     -CAAACCTGGAAGTCGGTGATCAAG-TAGTGCATTGGAAGTACGAAATTGATGGCCCAGC
270.L2        -CAAACCTGGAAGTCGGTGATCAAG-TAGTGCATTGGAAGTACGAAATTGATGGCCCAGC
M9442.L2      -CAAACCTGGAAGTCGGTGATCAAG-TAGTGCATTGGAAGTACGAAATTGATGGCCCAGC
A945.L2       -CAAACCTGGAAGTCGGTGATCAAG-TAGTGCATTGGAAGTACGAAATTGATGGCCCAGC
M94368.L2     -CAAACCTGGAAGTCGGTGATCAAG-TAGTGCATTGGAAGTACGAAATTGATGGCCCAGC
A9464.L2      -CAAACCTGGAAGTCGGTGATCAAG-TAGTGCATTGGAAGTACGAAATTGATGGCCCAGC
E94393.L2     -CAAACCTGGAAGTCGGTGATCAAG-TAGTGCATTGGAAGTACGAAATTGATGGCCCAGC
M94430.L2     -CAAACCTGGAAGTCGGTGATCAAG-TAGTGCATTGGAAGTACGAAATTGATGGCCCAGC
BTV17.L2GB    -CAAACTTAGCGGTCGGCGACCAGA-TAATTCATTGGCGTTATGAGGTTAAAGCATCGGC
BTV11.L2      -CAAACCTGGAAGTCGGTGATCAAG-TAGTGCATTGGAAGTACGAAATTGATGGCCCAGC
201400.L2     -CAAACCTGGAAGTCGGTGATCAAG-TAGTGCATTGGAAGTACGAAATTGATGGCCCAGC
203734.L2     -CAAACCTGGAAGTCGGTGATCAAG-TAGTGCATTGGAAGTACGAAATTGATGGCCCAGC
201767.L2     -CAAACCTGGAAGTCGGTGATCAAG-TAGTGCATTGGAAGTACGAAATTGATGGCCCAGC
201773.L2     -CAAACCTGGAAGTCGGTGATCAAG-TAGTGCATTGGAAGTACGAAATTGATGGCCCAGC
202020.L2     -CAAACCTGGAAGTCGGTGATCAAG-TAGTGCATTGGAAGTACGAAATTGATGGCCCAGC
203853.L2     -CAAACCTGGAAGTCGGTGATCAAG-TAGTGCATTGGAAGTACGAAATTGATGGCCCAGC
203732.L2     -CAAACCTGGAAGTCGGTGATCAAG-TAGTGCATTGGAAGTACGAAATTGATGGCCCAGC
203843.L2     -CAAACCTGGAAGTCGGTGATCAAG-TAGTGCATTGGAAGTACGAAATTGATGGCCCAGC
BTV2.L2GB     ACGAAGCTGGT--CCATCGAGCAAGGTTATACATTGGGAGTATCAATTATATAAACGAGA
BTV23.L2GB    -CAACGCTTAGACCTGGCGATAAGA-TTATACATTGGGAGTAT-AAATTGCTCAACGAAG
               * *   *          **  *   *  * ** ***   **  *  *
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M94389.L2     TGAGACA---ACGTATGA-CAATGGCTATATATGCAAGA------CTGAACGAGAAGATG
BTV10.L2GB    AGAAACA---ACTCATGA-TGATGGATATATCTGTGTGT------CACAGAAAGGTGATG
A94389.L2     TGAGACA---ACGTATGA-CAATGGCTATATATGCAAGA------CTGAACGAGAAGATG
BTV11.L2GB    TGAGACA---ACGTATGA-CAATGGCTATATATGCAAGA------CTGAACGAGAAGATG
202894.L2     TGAGACA---ACGTATGA-CAATGGCTATATGTGCAAGA------CTGAACGAGAAGATG
203733.L2     TGAGACA---ACGTATGA-CAATGGCTATATGTGCAAGA------CTGAACGAGAAGATG
203794.L2     TGAGACA---ACGTATGA-CAATGGCTATATATGCAAGA------CTGAACGAGAAGATG
203826.L2     TGAGACA---ACGTATGA-CAATGGCTATATGTGCAAGA------CTGAACGAGAAGATG
270.L2        TGAGACA---ACGTATGA-CAATGGCTATATATGCAAGA------CTGAACGAGAAGATG
M9442.L2      TGAGACA---ACGTATGA-CAATGGCTATATATGCAAGA------CTGAACGAGAAGATG
A945.L2       TGAGACA---ACGTATGA-CAATGGCTATATATGCAAGA------CTGAACGAGAAGATG
M94368.L2     TGAGACA---ACGTATGA-CAATGGCTATATATGCAAGA------CTGAACGAGAAGATG
A9464.L2      TGAGACA---ACGTATGA-CAATGGCTATATATGCAAGA------CTGAACGAGAAGATG
E94393.L2     TGAGACA---ACGTATGA-CAATGGCTATATATGCAAGA------CTGAACGAGAAGATG
M94430.L2     TGAGACA---ACGTATGA-CAATGGCTATATATGCAAGA------CTGAACGAGAAGATG
BTV17.L2GB    GGAGACG---ACTTATGA-CAGCGGATACATGTGTCGGC------ATGAAGCTGAGGAGG
BTV11.L2      TGAGACA---ACGTATGA-CAATGGCTATATATGCAAGA------CTGAACGAGAAGATG
201400.L2     TGAGACA---ACGTATGA-CAATGGCTATATGTGCAAGA------CTGAACGAGAAGATG
203734.L2     TGAGACA---ACGTATGA-CAATGGCTATATGTGCAAGA------CTGAACGAGAAGATG
201767.L2     TGAGACA---ACGTATGA-CAATGGCTATATGTGCAAGA------CTGAACGAGAAGATG
201773.L2     TGAGACA---ACGTATGA-CAATGGCTATATGTGCAAGA------CTGAACGAGAAGATG
202020.L2     TGAGACA---ACGTATGA-CAATGGCTATATGTGCAAGA------CTGAACGAGAAGATG
203853.L2     TGAGACA---ACGTATGA-CAATGGCTATATGTGCAAGA------CTGAACGAGAAGATG
203732.L2     TGAGACA---ACGTATGA-CAATGGCTATATGTGCAAGA------CTGAACGAGAAGATG
203843.L2     TGAGACA---ACGTATGA-CAATGGCTATATGTGCAAGA------CTGAACGAGAAGATG
BTV2.L2GB     GCGGGTGGTTACCCTAGA-GAGAGGCAATCCCTGTGACTTGTATCCTGATGAAGATGATG
BTV23.L2GB    TACGAGAGGTGTGCATAAAGGATGGTAATGAATGTGATC------TATTCCCTGAAGATG
                            *     **  *  .  **                    *  **  *

M94389.L2     GAGAATTGGTGTGCAAGATTAGCGAAGAAAAATATAAGACAATGCTTGATAGAATGATCC
BTV10.L2GB    ATGAATTATTATGTGAGGTTGATGAAGATAGGTATAAGGAGATGTTTGATCGTATGATTC
A94389.L2     GAGAATTGGTGTGCAAGATTAGCGAAGAAAAATATAAGACAATGCTTGATAGAATGATCC
BTV11.L2GB    GAGAATTGGTGTGCAAGATTAGCGAAGAAAAATATAAGACAATGCTTGATAGAATGATCC
202894.L2     GAGAATTGGTGTGCAAGATTAGCGAAGAAAAATATAAGACAATGCTTGATAGAATGATCC
203733.L2     GAGAATTGGTGTGCAAGATTAGCGAAGAAAAATATAAGACAATGCTTGATAGAATGATCC
203794.L2     GAGAATTGGTGTGCAAGATTAGCGAAGAAAAATATAAGACAATGCTTGATAGAATGATCC
203826.L2     GAGAATTGGTGTGCAAGATTAGCGAAGAAAAATATAAGACAATGCTTGATAGAATGATCC
270.L2        GAGAATTGGTGTGCAAGATTAGCGAAGAAAAATATAAGACAATGCTTGATAGAATGATCC
M9442.L2      GAGAATTGGTGTGCAAGATTAGCGAAGAAAAATATAAGACAATGCTTGATAGAATGATCC
A945.L2       GAGAATTGGTGTGCAAGATTAGCGAAGAAAAATATAAGACAATGCTTGATAGAATGATCC
M94368.L2     GAGAATTGGTGTGCAAGATTAGCGAAGAAAAATATAAGACAATGCTTGATAGAATGATCC
A9464.L2      GAGAATTGGTGTGCAAGATTAGCGAAGAAAAATATAAGACAATGCTTGATAGAATGATCC
E94393.L2     GAGAATTGGTGTGCAAGATTAGCGAAGAAAAATATAAGACAATGCTTGATAGAATGATCC
M94430.L2     GAGAATTGGTGTGCAAGATTAGCGAAGAAAAATATAAGACAATGCTTGATAGAATGATCC
BTV17.L2GB    ATGAACTACTATGTAAAATCAATGAGGACAAATATAAAGACATGCTGGACAGAATGATTC
BTV11.L2      GAGAATTGGTGTGCAAGATTAGCGAAGAAAAATATAAGACAATGCTTGATAGAATGATCC
201400.L2     GAGAATTGGTGTGCAAGATTAGCGAAGAAAAATATAAGACAATGCTTGATAGAATGATCC
203734.L2     GAGAATTGGTGTGCAAGATTAGCGAAGAAAAATATAAGACAATGCTTGATAGAATGATCC
201767.L2     GAGAATTGGTGTGCAAGATTAGCGAAGAAAAATATAAGACAATGCTTGATAGAATGATCC
201773.L2     GAGAATTGGTGTGCAAGATTAGCGAAGAAAAATATAAGACAATGCTTGATAGAATGATCC
202020.L2     GAGAATTGGTGTGCAAGATTAGCGAAGAAAAATATAAGACAATGCTTGATAGAATGATCC
203853.L2     GAGAATTGGTGTGCAAGATTAGCGAAGAAAAATATAAGACAATGCTTGATAGAATGATCC
203732.L2     GAGAATTGGTGTGCAAGATTAGCGAAGAAAAATATAAGACAATGCTTGATAGAATGATCC
203843.L2     GAGAATTGGTGTGCAAGATTAGCGAAGAAAAATATAAGACAATGCTTGATAGAATGATCC
BTV2.L2GB     AAGTCATTATCACGAAATTTGATGACGCTAAATACTCTGAGATGGTCGGGGAAATTATAG
BTV23.L2GB    AAGAGATGACCACCAAATTTAATGAGGGAAAATATACAGAAATGAAGGGCCAGATCATTC
                *   *       *  *    ** *  *  **       ***   *     ** **
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M94389.L2     AAGGTGGTTGGGATCAGGAAAGATTCAAACTTTATAGCGTATT---GACAGATCCAAATT
BTV10.L2GB    AAGGCGGATGGGATCAGGAACGGTTCAAATTACACAACATATT---GACTGAACCAAACC
A94389.L2     AAGGTGGTTGGGATCAGGAAAGATTCAAACTTTATAGCGTATT---GACAGATCCAAATT
BTV11.L2GB    AAGGTGGTTGGGATCAGGAAAGATTCAAACTTTATAGCGTATT---GACAGATCCAAATT
202894.L2     AAGGTGGTTGGGATCAGGAAAGATTCAAACTTTATAGCGTATT---GACAGATCCAAATT
203733.L2     AAGGTGGTTGGGATCAGGAAAGATTCAAACTTTATAGCGTATT---GACAGATCCAAATT
203794.L2     AAGGTGGTTGGGATCAGGAAAGATTCAAACTTTATAGCGTACT---GACAGATCCAAATT
203826.L2     AAGGTGGTTGGGATCAGGAAAGATTCAAACTTTATAGCGTATT---GACAGATCCAAATT
270.L2        AAGGTGGTTGGGATCAGGAAAGATTCAAACTTTATAGCGTATT---GACAGATCCAAATT
M9442.L2      AAGGTGGTTGGGATCAGGAAAGATTCAAACTTTATAGCGTATT---GACAGATCCAAATT
A945.L2       AAGGTGGTTGGGATCAGGAAAGATTCAAACTTTATAGCGTATT---GACAGATCCAAATT
M94368.L2     AAGGTGGTTGGGATCAGGAAAGATTCAAACTTTATAGCGTATT---GACAGATCCAAATT
A9464.L2      AAGGTGGTTGGGATCAGGAAAGATTCAAACTTTATAGCGTATT---GACAGATCCAAATT
E94393.L2     AAGGTGGTTGGGATCAGGAAAGATTCAAACTTTATAGCGTATT---GACAGATCCAAATT
M94430.L2     AAGGTGGTTGGGATCAGGAAAGATTCAAACTTTATAGCGTATT---GACAGATCCAAATT
BTV17.L2GB    AGGGTGGGTGGGATCAGGAAAGATTTAAACTTCATAACATACT---GACGGACCCTAACT
BTV11.L2      AAGGTGGTTGGGATCAGGAAAGATTCAAACTTTATAGCGTATT---GACAGATCCAAATT
201400.L2     AAGGTGGTTGGGATCAGGAAAGATTCAAACTTTATAGCGTATT---GACAGATCCAAATT
203734.L2     AAGGTGGTTGGGATCAGGAAAGATTCAAACTTTATAGCGTATT---GACAGATCCAAATT
201767.L2     AAGGTGGTTGGGATCAGGAAAGATTCAAACTTTATAGCGTATT---GACAGATCCAAATT
201773.L2     AAGGTGGTTGGGATCAGGAAAGATTCAAACTTTATAGCGTATT---GACAGATCCAAATT
202020.L2     AAGGTGGTTGGGATCAGGAAAGATTCAAACTTTATAGCGTATT---GACAGATCCAAATT
203853.L2     AAGGTGGTTGGGATCAGGAAAGATTCAAACTTTATAGCGTATT---GACAGATCCAAATT
203732.L2     AAGGTGGTTGGGATCAGGAAAGATTCAAACTTTATAGCGTATT---GACAGATCCAAATT
203843.L2     AAGGTGGTTGGGATCAGGAAAGATTCAAACTTTATAGCGTATT---GACAGATCCAAATT
BTV2.L2GB     ATGGTGGATGGAATGATGAGGAATTTAAAATGTATAAGTTACTTCAGGAAAAAGGTAATG
BTV23.L2GB    AGGATGGATGGAACCATCGAGATTTTAAAATGCATAAAATACTGCAAGATGGTGCTAACG
              * *  ** *** *  *       ** *** *  * *   ** *            **

M94389.L2     TGCTTACCATAGATTTTGAGAAGGATGCGTACTTGAATATTAGATCGGAGTTTGTGCTCC
BTV10.L2GB    TACTTACCATCGATTTCGAGAAGGATGCTTACCTTGGCGCGCGTTCTGAATTAGTATTCC
A94389.L2     TGCTTACCATAGATTTTGAGAAGGATGCGTACTTGAATATTAGATCGGAGTTTGTGCTCC
BTV11.L2GB    TGCTAACCATAGATTTTGAGAAGGATGCGTACTTGAATATTAGATCGGAGTTTGTGCTCC
202894.L2     TGCTTACCATAGATTTTGAGAAGGATGCGTACTTGAATATTAGATCGGAGTTTGTGCTCC
203733.L2     TGCTTACCATAGATTTTGAGAAGGATGCGTACTTGAATATTAGATCGGAGTTTGTGCTCC
203794.L2     TGCTTACCATAGATTTTGAGAAGGATGCGTACTTGAATATTAAATCGGAGTTTGTGCTCC
203826.L2     TGCTTACCATAGATTTTGAGAAGGATGCGTACTTGAATATTAGATCGGAGTTTGTGCTCC
270.L2        TGCTTACCATAGATTTTGAGAAGGATGCGTACTTGAATATTAGATCGGAGTTTGTGCTCC
M9442.L2      TGCTTACCATAGATTTTGAGAAGGATGCGTACTTGAATATTAGATCGGAGTTTGTGCTCC
A945.L2       TGCTTACCATAGATTTTGAGAAGGATGCGTACTTGAATATTAGATCGGAGTTTGTGCTCC
M94368.L2     TGCTTACCATAGATTTTGAGAAGGATGCGTACTTGAATATTAGATCGGAGTTTGTGCTCC
A9464.L2      TGCTTACCATAGATTTTGAGAAGGATGCGTACTTGAATATTAGATCGGAGTTTGTGCTCC
E94393.L2     TGCTTACCATAGATTTTGAGAAGGATGCGTACTTGAATATTAGATCGGAGTTTGTGCTCC
M94430.L2     TGCTTACCATAGATTTTGAGAAGGATGCGTACTTGAATATTAGATCGGAGTTTGTGCTCC
BTV17.L2GB    TATTGACGATTGATTTTGAAAAAGATGCGTATCTGAATTTACGGTCCGAGTTAGTTTTGC
BTV11.L2      TGCTTACCATAGATTTTGAGAAGGATGCGTACTTGAATATTAGATCGGAGTTTGTGCTCC
201400.L2     TGCTTACCATAGATTTTGAGAAGGATGCGTACTTGAATATTAGATCGGAGTTTGTGCTCC
203734.L2     TGCTTACCATAGATTTTGAGAAGGATGCGTACTTGAATATTAGATCGGAGTTTGTGCTCC
201767.L2     TGCTTACCATAGATTTTGAGAAGGATGCGTACTTGAATATTAGATCGGAGTTTGTGCTCC
201773.L2     TGCTTACCATAGATTTTGAGAAGGATGCGTACTTGAATATTAGATCGGAGTTTGTGCTCC
202020.L2     TGCTTACCATAGATTTTGAGAAGGATGCGTACTTGAATATTAGATCGGAGTTTGTGCTCC
203853.L2     TGCTTACCATAGATTTTGAGAAGGATGCGTACTTGAATATTAGATCGGAGTTTGTGCTCC
203732.L2     TGCTTACCATAGATTTTGAGAAGGATGCGTACTTGAATATTAGATCGGAGTTTGTGCTCC
203843.L2     TGCTTACCATAGATTTTGAGAAGGATGCGTACTTGAATATTAGATCGGAGTTTGTGCTCC
BTV2.L2GB     TTCTCACAATTGACTTCGAAAAGGATACGAAGCTGTATAATACTTCAGAGGTGGTTTTAC
BTV23.L2GB    TTTTGACAATTGATTTTGAAAAAGACGCACGTATAGGAAATGGATCAGTTTTGACGTTAC
              *  * ** ** ** ** ** ** ** **  *     *          ** *   *     * *
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M94389.L2     CAAGTTATTTTGATCAATGGATCTACTCGCCAATGTTTAATGCGCGACTGCGAATAACTC
BTV10.L2GB    CGCCCTATTATGACAAATGGATCAATTCACCGATGTTTAATGCCCGGCTTAAAATTGCGC
A94389.L2     CAAGTTATTTTGATCAATGGATCTACTCGCCAATGTTTAATGCGCGACTGCGAATAACTC
BTV11.L2GB    CAAGTTATTTCGATCAATGGATCTACTCGCCGATGTTTAATGCGCGACTGCGAATAACTC
202894.L2     CAAGTTATTTCGATCAATGGATCTACTCGCCGATGTTTAACGCGCGACTGCGAATAACTC
203733.L2     CAAGTTATTTCGATCAATGGATCTACTCGCCGATGTTTAACGCGCGACTGCGAATAACTC
203794.L2     CAAGTTATTTCGATCAATGGATCTACTCGCCGATGTTTAATGCGCGACTGCGAATAACTC
203826.L2     CAAGTTATTTCGATCAATGGATCTACTCGCCGATGTTTAACGCGCGACTGCGAATAACTC
270.L2        CAAGTTATTTTGATCAATGGATCTACTCGCCAATGTTTAATGCGCGACTGCGAATAACTC
M9442.L2      CAAGTTATTTTGATCAATGGATCTACTCGCCAATGTTTAATGCGCGACTGCGAATAACTC
A945.L2       CAAGTTATTTTGATCAATGGATCTACTCGCCAATGTTTAATGCGCGACTGCGAATAACTC
M94368.L2     CAAGTTATTTTGATCAATGGATCTACTCGCCAATGTTTAATGCGCGACTGCGAATAACTC
A9464.L2      CAAGTTATTTTGATCAATGGATCTACTCGCCAATGTTTAATGCGCGACTGCGAATAACTC
E94393.L2     CAAGTTATTTTGATCAATGGATCTACTCGCCAATGTTTAATGCGCGACTGCGAATAACTC
M94430.L2     CAAGTTATTTTGATCAATGGATCTACTCGCCAATGTTTAATGCGCGACTGCGAATAACTC
BTV17.L2GB    CGGATTATTTCGATAAATGGATCAGTTCACCAATGTTTAACGCGCGCTTAAGAATTACTA
BTV11.L2      CAAGTTATTTTGATCAATGGATCTACTCGCCAATGTTTAATGCGCGACTGCGAATAACTC
201400.L2     CAAGTTATTTCGATCAATGGATCTACTCGCCGATGTTTAACGCGCGACTGCGAATAACTC
203734.L2     CAAGTTATTTCGATCAATGGATCTACTCGCCGATGTTTAACGCGCGACTGCGAATAACTC
201767.L2     CAAGTTATTTCGATCAATGGATCTACTCGCCGATGTTTAACGCGCGACTGCGAATAACTC
201773.L2     CAAGTTATTTCGATCAATGGATCTACTCGCCGATGTTTAACGCGCGACTGCGAATAACTC
202020.L2     CAAGTTATTTCGATCAATGGATCTACTCGCCGATGTTTAACGCGCGACTGCGAATAACTC
203853.L2     CAAGTTATTTCGATCAATGGATCTACTCGCCGATGTTTAACGCGCGACTGCGAATAACTC
203732.L2     CAAGTTATTTCGATCAATGGATCTACTCGCCGATGTTTAACGCGCGACTGCGAATAACTC
203843.L2     CAAGTTATTTCGATCAATGGATCTACTCGCCGATGTTTAACGCGCGACTGCGAATAACTC
BTV2.L2GB     CAGACTATTATGGTAAGTGGATAGTTGCGCCGATGTTCAACTCTAAAATGAGAATTATTG
BTV23.L2GB    CCGAATATTATAACAAATGGATAGTTGCCCCAATGTTCAATGCGAAATTACGCATCTCTG
              *    ****       * *****     * ** ***** **  *     *    **

M94389.L2     ATGGAGAGATCGGGACCAGGAAGTCGGCGGATCCGTGGAATAAGCGCGT-CGTATTCGGA
BTV10.L2GB    GTGGCGAGATTGCAACCTGGAAGGCGGATGATCCTTGGAGTAATCGGGC-GGTTCATGGG
A94389.L2     ATGGAGAGATCGGGACCAGGAAGTCGGCGGATCCGTGGAATAAGCGCGT-CGTATTCGGA
BTV11.L2GB    ATGGAGAGATCGGGACCAGGAAGTCGGCGGATCCGTGGAATAAGCGCGT-CGTATTCGGA
202894.L2     ATGGAGAGATCGGGACCAGGAAGTCGGCGGATCCGTGGAATAAGCGCGT-CGTATTCGGA
203733.L2     ATGGAGAGATCGGGACCAGGAAGTCGGCGGATCCGTGGAATAAGCGCGT-CGTATTCGGA
203794.L2     ATGGAGAGATCGGGACCAGGAAGTCGGCGGATCCGTGGAATAAGCGCGT-CGTATTCGGA
203826.L2     ATGGAGAGATCGGGACCAGGAAGTCGGCGGATCCGTGGAATAAGCGCGT-CGTATTCGGA
270.L2        ATGGAGAGATCGGGACCAGGAAGTCGGCGGATCCGTGGAATAAGCGCGT-CGTATTCGGA
M9442.L2      ATGGAGAGATCGGGACCAGGAAGTCGGCGGATCCGTGGAATAAGCGCGT-CGTATTCGGA
A945.L2       ATGGAGAGATCGGGACCAGGAAGTCGGCGGATCCGTGGAATAAGCGCGT-CGTATTCGGA
M94368.L2     ATGGAGAGATCGGGACCAGGAAGTCGGCGGATCCGTGGAATAAGCGCGT-CGTATTCGGA
A9464.L2      ATGGAGAGATCGGGACCAGGAAGTCGGCGGATCCGTGGAATAAGCGCGT-CGTATTCGGA
E94393.L2     ATGGAGAGATCGGGACCAGGAAGTCGGCGGATCCGTGGAATAAGCGCGT-CGTATTCGGA
M94430.L2     ATGGAGAGATCGGGACCAGGAAGTCGGCGGATCCGTGGAATAAGCGCGT-CGTATTCGGA
BTV17.L2GB    AAGGGGAGATCGGAACATCGAAAAAGGATGATCCATGGAACAACCGCGC-AGTACGTGGA
BTV11.L2      ATGGAGAGATCGGGACCAGGAAGTCGGCGGATCCGTGGAATAAGCGCGT-CGTATTCGGA
201400.L2     ATGGAGAGATCGGGACCAGGAAGTCGGCGGATCCGTGGAATAAGCGCGT-CGTATTCGGA
203734.L2     ATGGAGAGATCGGGACCAGGAAGTCGGCGGATCCGTGGAATAAGCGCGT-CGTATTCGGA
201767.L2     ATGGAGAGATCGGGACCAGGAAGTCGGCGGATCCGTGGAATAAGCGCGT-CGTATTCGGA
201773.L2     ATGGAGAGATCGGGACCAGGAAGTCGGCGGATCCGTGGAATAAGCGCGT-CGTATTCGGA
202020.L2     ATGGAGAGATCGGGACCAGGAAGTCGGCGGATCCGTGGAATAAGCGCGT-CGTATTCGGA
203853.L2     ATGGAGAGATCGGGACCAGGAAGTCGGCGGATCCGTGGAATAAGCGCGT-CGTATTCGGA
203732.L2     ATGGAGAGATCGGGACCAGGAAGTCGGCGGATCCGTGGAATAAGCGCGT-CGTATTCGGA
203843.L2     ATGGAGAGATCGGGACCAGGAAGTCGGCGGATCCGTGGAATAAGCGCGT-CGTATTCGGA
BTV2.L2GB     AAACGGAGATTGCAACCAACAAAAGTGATGATCCCATGATTAAAAGAACGCTCAAACCGA
BTV23.L2GB    AGACTGTAATCGCGAAAGCACACTCAGATGACCC-TGCCGTAGCTAGGACAGCTAAATCG
                   *  ** *  *       *    *  ** **        *
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M94389.L2     TATGTTAAAGCGTCAACGGAAAGCCCTGAATACGCTCTAGGACAATATTTCGACACGCGT
BTV10.L2GB    TATATAAAGACGTCAGCGGAATCTTTAGAGTATGCGTTAGGGCCATATTATGACCTACGT
A94389.L2     TATGTTAAAGCGTCAACGGAAAGCCCTGAATACGCTCTAGGACAATATTTCGACACGCGT
BTV11.L2GB    TATGTTAAAGCGTCAACGGAAAGCCCTGAATACGCTCTAGGACAATATTTCGACACGCGT
202894.L2     TATGTTAAAGCGTCAACGGAAAGCCCTGAATACGCTCTAGGACAATATTTCGACACGCGT
203733.L2     TATGTTAAAGCGTCAACGGAAAGCCCTGAATACGCTCTAGGACAATATTTCGACACGCGT
203794.L2     TATATTAAAGCGTCAACGGAAAGCCCTGAATACGCTCTAGGACAATATTTCGACACGCGT
203826.L2     TATGTTAAAGCGTCAACGGAAAGCCCTGAATACGCTCTAGGACAATATTTCGACACGCGT
270.L2        TATGTTAAAGCGTCAACGGAAAGCCCTGAATACGCTCTAGGACAATATTTCGACACGCGT
M9442.L2      TATGTTAAAGCGTCAACGGAAAGCCCTGAATACGCTCTAGGACAATATTTCGACACGCGT
A945.L2       TATGTTAAAGCGTCAACGGAAAGCCCTGAATACGCTCTAGGACAATATTTCGACACGCGT
M94368.L2     TATGTTAAAGCGTCAACGGAAAGCCCTGAATACGCTCTAGGACAATATTTCGACACGCGT
A9464.L2      TATGTTAAAGCGTCAACGGAAAGCCCTGAATACGCTCTAGGACAATATTTCGACACGCGT
E94393.L2     TATGTTAAAGCGTCAACGGAAAGCCCTGAATACGCTCTAGGACAATATTTCGACACGCGT
M94430.L2     TATGTTAAAGCGTCAACGGAAAGCCCTGAATACGCTCTAGGACAATATTTCGACACGCGT
BTV17.L2GB    TACATTAAGTCCCTTGCGGAGTCGTTGGATTTTGTTCTCGGGCCTTACTACGATCTGCGG
BTV11.L2      TATGTTAAAGCGTCAACGGAAAGCCCTGAATACGCTCTAGGACAATATTTCGACACGCGT
201400.L2     TATGTTAAAGCGTCAACGGAAAGCCCTGAATACGCTCTAGGACAATATTTCGACACGCGT
203734.L2     TATGTTAAAGCGTCAACGGAAAGCCCTGAATACGCTCTAGGACAATATTTCGACACGCGT
201767.L2     TATGTTAAAGCGTCAACGGAAAGCCCTGAATACGCTCTAGGACAATATTTCGACACGCGT
201773.L2     TATGTTAAAGCGTCAACGGAAAGCCCTGAATACGCTCTAGGACAATATTTCGACACGCGT
202020.L2     TATGTTAAAGCGTCAACGGAAAGCCCTGAATACGCTCTAGGACAATATTTCGACACGCGT
203853.L2     TATGTTAAAGCGTCAACGGAAAGCCCTGAATACGCTCTAGGACAATATTTCGACACGCGT
203732.L2     TATGTTAAAGCGTCAACGGAAAGCCCTGAATACGCTCTAGGACAATATTTCGACACGCGT
203843.L2     TATGTTAAAGCGTCAACGGAAAGCCCTGAATACGCTCTAGGACAATATTTCGACACGCGT
BTV2.L2GB     TG-ACTGATGATCCCGTCGAGCTACAGAGATATACTTTAGCCAGATACTATGATATCCGT
BTV23.L2GB    TTTGATCATGACCCCTTCAACTTACAACGGTACTGTTTAGCGAGATATTATGATGTACGA
                    *       *             *             *       * *      ** *  **     **

M94389.L2     ATACAACTTTATGGGGATGCGTTGAGTTTGAAACAGAGCCCAGTCGGC----GGTATTTC
BTV10.L2GB    CTGCAGTTATTCGGCGACACCCTCAGTCTGGGACAAAGAC-AGTCAGC----TGTATTCG
A94389.L2     ATACAACTTTATGGGGATGCGTTGAGTTTGAAACAGAGCC-AGTCGGC----GGTATTTC
BTV11.L2GB    ATACAACTTTATGGGGATGCGTTGAGTTTGAAACAGAGCC-AGTCGGC----GGTATTTC
202894.L2     ATACAATTTTATGGGGATGCGTTGAGTTTGAAACAGAGCC-AGTCGGC----GGTATTTC
203733.L2     ATACAATTTTATGGGGATGCGTTGAGTTTGAAACAGAGCC-AGTCGGC----GGTATTTC
203794.L2     ATACAACTTTATGGGGATGCGTTGAGTTTGAAACAGAGCC-AGTCGGC----GGTATTTC
203826.L2     ATACAATTTTATGGGGATGCGTTGAGTTTGAAACAGAGCC-AGTCGGC----GGTATTTC
270.L2        ATACAACTTTATGGGGATGCGTTGAGTTTGAAACAGAGCC-AGTCGGC----GGTATTTC
M9442.L2      ATACAACTTTATGGGGATGCGTTGAGTTTGAAACAGAGCC-AGTCGGC----GGTATTTC
A945.L2       ATACAACTTTATGGGGATGCGTTGAGTTTGAAACAGAGCC-AGTCGGC----GGTATTTC
M94368.L2     ATACAACTTTATGGGGATGCGTTGAGTTTGAAACAGAGCC-AGTCGGC----GGTATTTC
A9464.L2      ATACAACTTTATGGGGATGCGTTGAGTTTGAAACAGAGCC-AGTCGGC----GGTATTTC
E94393.L2     ATACAACTTTATGGGGATGCGTTGAGTTTGAAACAGAGCC-AGTCGGC----GGTATTTC
M94430.L2     ATACAACTTTATGGGGATGCGTTGAGTTTGAAACAGAGCC-AGTCGGC----GGTATTTC
BTV17.L2GB    CTACTATTTTTTGGCGAGACGTTGAGCTTAAAACAGGAAC-AATCCGC----GGTTTTTC
BTV11.L2      ATACAACTTTATGGGGATGCGTTGAGTTTGAAACAGAGCC-AGTCGGC----GGTATTTC
201400.L2     ATACAATTTTATGGGGATGCGTTGAGTTTGAAACAGAGCC-AGTCGGC----GGTATTTC
203734.L2     ATACAATTTTATGGGGATGCGTTGAGTTTGAAACAGAGCC-AGTCGGC----GGTATTTC
201767.L2     ATACAATTTTATGGGGATGCGTTGAGTTTGAAACAGAGCC-AGTCGGC----GGTATTTC
201773.L2     ATACAATTTTATGGGGATGCGTTGAGTTTGAAACAGAGCC-AGTCGGC----GGTATTTC
202020.L2     ATACAATTTTATGGGGATGCGTTGAGTTTGAAACAGAGCC-AGTCGGC----GGTATTTC
203853.L2     ATACAATTTTATGGGGATGCGTTGAGTTTGAAACAGAGCC-AGTCGGC----GGTATTTC
203732.L2     ATACAATTTTATGGGGATGCGTTGAGTTTGAAACAGAGCC-AGTCGGC----GGTATTTC
203843.L2     ATACAATTTTATGGGGATGCGTTGAGTTTGAAACAGAGCC-AGTCGGC----GGTATTTC
BTV2.L2GB     CCTGGACTAATGGGTCGTTCGCTAAATAGGACACAGACTC-AATCAACATTTGACGCGAA
BTV23.L2GB    CCAGGATTGATGGGACGAGCATTATCCAAAGAGCAGGAAG-GGTCAGCCATAGCGAACAA
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M94389.L2     AGCACCAGAGTCA-------ACAAGAGGATTTTCCTGTGCTAACG-AGTTATGCCAAAGG
BTV10.L2GB    AACATATGGCTCA-------ACAAGATGATTTTTCTACGCTAACG-GATTATACGAAAGG
A94389.L2     AGCACCAGAGTCA-------ACAAGA-GATTTTCCTGTGCTAACG-AGTTATGCCAAAG-
BTV11.L2GB    AGCACCAGAGTCA-------ACAAGAGGATTTTCCTGTGCTAACG-AGTTATGCCAAAGG
202894.L2     AGCACCAGAGTCA-------ACAAGAGGATTT-CCTGTGCTAACG-AGTTATGCCAAAG-
203733.L2     AGCACCAGAGTCA-------ACAAGAGGATTT-CCTGTGCTAACG-AGTTATGCCAAAG-
203794.L2     AGCACCAGAGTCA-------ACAAGAGGATTTTCCTGTGCTAACG-AGTTATGCCAAAGG
203826.L2     AGCACCAGAGTCA-------ACAAGAGGATTTTCCTGTGCTAACG-AGTTATGCCAAAG-
270.L2        AGCACCAGAGTCA-------ACAAGAGGATTTTCCTGTGCTAACG-AGTTATGCCAAAGG
M9442.L2      AGCACCAGAGTCA-------ACAAGAGGATTTTCCTGTGCTAACG-AGTTATGCCAAAGG
A945.L2       AGCACCAGAGTCA-------ACAAGAGGATTTTCCTGTGCTAACG-AGTTATGCCAAAGG
M94368.L2     AGCACCAGAGTCA-------ACAAGAGGATTTTCCTGTGCTAACG-AGTTATGCCAAAGG
A9464.L2      AGCACCAGAGTCA-------ACAAGAGGATTTTCCTGTGCTAACG-AGTTATGCCAAAGG
E94393.L2     AGCACCAGAGTCA-------ACAAGAGGATTTTCCTGTGCTAACG-AGTTATGCCAAAGG
M94430.L2     AGCACCAGAGTCA-------ACAAGAGGATTTTCCTGTGCTAACG-AGTTATGCCAAAGG
BTV17.L2GB    AATATTTGAGTCA-------GCTCGATGATTTTCCCGCGCTTACGCAGCTA----AGAGG
BTV11.L2      AGCACCAGAGTCA-------ACAAGAGGATTTTCCTGTGCTAACG-AGTTATGCCAAAGG
201400.L2     AGCACCAGAGTCA-------ACAAGAGGATTTTCCTGTGCTAACG-AGTTATGCCAAAG-
203734.L2     AGCACCAGAGTCA-------ACAAGAGGATTT-CCTGTGCTAACG-AGTTATGCCAAAG-
201767.L2     AGCACCAGAGTCA-------ACAAGAGGATTTTCCTGTGCTAACG-AGTTATGCCAAAGG
201773.L2     AGCACCAGAGTCA-------ACAAGAGGATTTTCCTGTGCTAACG-AGTTATGCCAAAGG
202020.L2     AGCACCAGAGTCA-------ACAAGAGGATTTTCCTGTGCTAACG-AGTTATGCCAAAGG
203853.L2     AGCACCAGAGTCA-------ACAAGAGGATTTTCCTGTGCTAACG-AGTTATGCCAAAGG
203732.L2     AGCACCAGAGTCA-------ACAAGAGGATTT-CCTGTGCTAACG-AGTTATGCCAAAGG
203843.L2     AGCACCAGAGTCA-------ACAAGAGGATTTTCCTGTGCTAACG-AGTTATGCCAAAGG
BTV2.L2GB     GGTGTCAGAGTTGCCCGACTATGAAAAGGTGGTGTCACGGTTTGG-TGTAATCAAGAAAC
BTV23.L2GB    AGTGGCTGCGCTTGAGGATTATCGGGACGTTATATCAAGGCGTTTAGGGTATGTTGAACG
                     *                    *       *         *       *

M94389.L2     TGATGTGGTATGTCC-CATTCGGGTGGTGCGTTGTATACTTTTCGGAAGGTGGCTCTGAT
BTV10.L2GB    AAGAACAGTGTGCCCTCACTCAGGCGGGACTTTTTACACTTTCCGCAAGGTAGCGTTGAT
A94389.L2     TGATGTGGTATGTCC-CATTCGGGTGGTGCGTTGTATACTTTTCGGAAGGTGGCTCTGAT
BTV11.L2GB    TGATGTGGTATGTCCCCATTCGGGTGGTGCGTTGTATACTTTTCGGAAGGTGGCTCTGAT
202894.L2     TGATGTGGTATGTCCC-ATTCGGGTGGTGCGTTGTATACTTTTCGGAAGGTGGCTCTGAT
203733.L2     TGATGTGGTATGTCCC-ATTCGGGTGGTGCGTTGTATACTTTTCGGAAGGTGGCTCTGAT
203794.L2     TGATGTGGTATGTCCC-ATTCGGGTGGTGCGTTGTATACTTTTAGGAAGGTGGCTCTGAT
203826.L2     TGATGTGGTATGTCCCCATTCGGGTGGTGCGTTGTATACTTTTCGGAAGGTGGCTCTGAT
270.L2        TGATGTGGTATGTCCCCATTCGGGTGGTGCGTTGTATACTTTTCGGAAGGTGGCTCTGAT
M9442.L2      TGATGTGGTATGTCCC-ATTCGGGTGGTGCGTTGTATACTTTTCGGAAGGTGGCTCTGAT
A945.L2       TGATGTGGTATGTCCC-ATTCGGGTGGTGCGTTGTATACTTTTCGGAAGGTGGCTCTGAT
M94368.L2     TGATGTGGTATGTCCCCATTCGGGTGGTGCGTTGTATACTTTTCGGAAGGTGGCTCTGAT
A9464.L2      TGATGTGGTATGTCCCCATTCGGGTGGTGCGTTGTATACTTTTCGGAAGGTGGCTCTGAT
E94393.L2     TGATGTGGTATGTCCCCATTCGGGTGGTGCGTTGTATACTTTTCGGAAGGTGGCTCTGAT
M94430.L2     TGATGTGGTATGTCCCCATTCGGGTGGTGCGTTGTATACTTTTCGGAAGGTGGCTCTGAT
BTV17.L2GB    AGATGCCGTATGCCCACATTCAGGCGGAGCGCTATATACGTTTAGGAAAGTCGCGCTATT
BTV11.L2      TGATGTGGTATGTCCCCATTCGGGTGGTGCGTTGTATACTTTTCGGAAGGTGGCTCTGAT
201400.L2     TGATGTGGTATGTCCCATTCGGG-TGGTGCGTTGTATACTTTTCGGAAGGTGGCTCTGAT
203734.L2     TGATGTGGTATGTCCCCATTCGG-TGGTGCGTTGTATACTTTTCGGAAGGTGGCTCTGAT
201767.L2     TGATGTGGTATGTCCCCATTCGGGTGGTGCGTTGTATACTTTTCGGAAGGTGGCTCTGAT
201773.L2     TGATGTGGTATGTCCCCATTCGGGTGGTGCGTTGTATACTTTTCGGAAGGTGGCTCTGAT
202020.L2     TGATGTGGTATGTCCCCATTCGGGTGGTGCGTTGTATACTTTTCGGAAGGTGGCTCTGAT
203853.L2     TGATGTGGTATGTCCCCATTCGGGTGGTGCGTTGTATACTTTTCGGAAGGTGGCTCTGAT
203732.L2     TGATGTGGTATGTCCC-ATTCGGGTGGTGCGTTGTATACTTTTCGGAAGGTGGCTCTGAT
203843.L2     TGATGTGGTATGTCCCCATTCGGGTGGTGCGTTGTATACTTTTCGGAAGGTGGCTCTGAT
BTV2.L2GB     CGACAAGACCTTGCGTAACTTTAACCGGGCG--ATACATTCTGGAGAAATATTCTTTACT
BTV23.L2GB    TGAAAAACGATGTTT-AACTGAAACGGCGCA--GTATATATTGGAGAAAACATCATTGTA
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M94389.L2     GTTGATGGCGAATTACGAAAGGTTGAGCCCAGATT-GCATGAGGGTATGGAAGATCATAC
BTV10.L2GB    TATTTTATCGAATTACGAACGATTGGACCCAAGTTTACATGAGGGGAGGGAACATGAGAC
A94389.L2     GTTGATGGCGAATTACGAAAGGTTGAGCCCAGATCTGCATGAGGGTATGGAAGATCATAC
BTV11.L2GB    GTTGATGGCGAATTACGAAAGGTTGAGCCCAGATCTGCATGAGGGTATGGAAGATCATAC
202894.L2     GTTGATGGCGAATTATGAAAAGTTGAGCCCAGATCTGCATGAGGGTATGGAAGATCATAC
203733.L2     GTTGATGGCGAATTATGAAAAGTTGAGCCCAGATCTGCATGAGGGTATGGAAGATCATAC
203794.L2     GTTGATGGCGAATTACGAAAAGTTGAGCCCAGATCTGCATGAAGGTATGGAAGATCATAC
203826.L2     GTTGATGGCGAATTATGAAAAGTTGAGCCCAGATCTGCATGAGGGTATGGAAGATCATAC
270.L2        GTTGATGGCGAATTACGAAAGGTTGAGCCCAGATCTGCATGAGGGTATGGAAGATCATAC
M9442.L2      GTTGATGGCGAATTACGAAAGGTTGAGCCCAGATCTGCATGAGGGTATGGAAGATCATAC
A945.L2       GTTGATGGCGAATTACGAAAGGTTGAGCCCAGATCTGCATGAGGGTATGGAAGATCATAC
M94368.L2     GTTGATGGCGAATTACGAAAGGTTGAGCCCAGATCTGCATGAGGGTATGGAAGATCATAC
A9464.L2      GTTGATGGCGAATTACGAAAGGTTGAGCCCAGATCTGCATGAGGGTATGGAAGATCATAC
E94393.L2     GTTGATGGCGAATTACGAAAGGTTGAGCCCAGATCTGCATGAGGGTATGGAAGATCATAC
M94430.L2     GTTGATGGCGAATTACGAAAGGTTGAGCCCAGATCTGCATGAGGGTATGGAAGATCATAC
BTV17.L2GB    TTTAATCGGGAATTATGAAAAGTTAAGTCCGGATCTACATGAAGGTATGGAACATCAAGC
BTV11.L2      GTTGATGGCGAATTACGAAAGGTTGAGCCCAGATCTGCATGAGGGTATGGAAGATCATAC
201400.L2     GTTGATGGCGAATTATGAAAAGTTGAGCCCAGATCTGCATGAGGGTATGGAAGATCATAC
203734.L2     GTTGATGGCGAATTATGAAAAGTTGAGCCCAGATCTGCATGAGGGTATGGAAGATCATAC
201767.L2     GTTGATGGCGAATTATGAAAAGTTGAGCCCAGATCTGCATGAGGGTATGGAAGATCATAC
201773.L2     GTTGATGGCGAATTATGAAAAGTTGAGCCCAGATCTGCATGAGGGTATGGAAGATCATAC
202020.L2     GTTGATGGCGAATTATGAAAAGTTGAGCCCAGATCTGCATGAGGGTATGGAAGATCATAC
203853.L2     GTTGATGGCGAATTATGAAAAGTTGAGCCCAGATCTGCATGAGGGTATGGAAGATCATAC
203732.L2     GTTGATGGCGAATTATGAAAAGTTGAGCCCAGATCTGCATGAGGGTATGGAAGATCATAC
203843.L2     GTTGATGGCGAATTATGAAAAGTTGAGCCCAGATCTGCATGAGGGTATGGAAGATCATAC
BTV2.L2GB     TCTTATAG-ATATATTGAAGTATCATACTGAGGTT----GAAGGGAATCCACAAGAAGAA
BTV23.L2GB    TCTGTTAA--ACGTACTGAGTATGCACGTTAAACCGAC-TATTGATATGGAT-ATTAAAT
                * *           *   *                   *  *   *  *  *

M94389.L2     TTATACGCACCCCAGCATCGGCGGCGCCTATCAAAAACGCATCCTTGAGATGAGAGATTT
BTV10.L2GB    ATACATGCACCCGGCAGTTAATGATGTCTTTAGACGACACGTTTTAGAAATGAAAGATTT
A94389.L2     TTATACGCACCCCAGCATCGGCGGCGCCTATCAAAAACGCATCCTTGAGATGAGAGATTT
BTV11.L2GB    TTATACGCACCCCAGCATCGGCGGCGCCAATCAAAAACGCATCCTTGAGATGAGAGATTT
202894.L2     TTATACGCACCCCAGCATCGGCGGCGCCTATCAAAAACGCATCCTTGAGATGAGAGATTT
203733.L2     TTATACGCACCCCAGCATCGGCGGCGCCTATCAAAAACGCATCCTTGAGATGAGAGATTT
203794.L2     CTATACGCACCCCAGCATCGACGGCGCCTATCAGAAACGCATCCTTGAGATGAGAGATTT
203826.L2     TTATACGCACCCCAGCATCGGCGGCGCCTATCAAAAACGCATCCTTGAGATGAGAGATTT
270.L2        TTATACGCACCCCAGCATCGGCGGCGCCTATCAAAAACGCATCCTTGAGATGAGAGATTT
M9442.L2      TTATACGCACCCCAGCATCGGCGGCGCCTATCAAAAACGCATCCTTGAGATGAGAGATTT
A945.L2       TTATACGCACCCCAGCATCGGCGGCGCCTATCAAAAACGCATCCTTGAGATGAGAGATTT
M94368.L2     TTATACGCACCCCAGCATCGGCGGCGCCTATCAAAAACGCATCCTTGAGATGAGAGATTT
A9464.L2      TTATACGCACCCCAGCATCGGCGGCGCCTATCAAAAACGCATCCTTGAGATGAGAGATTT
E94393.L2     TTATACGCACCCCAGCATCGGCGGCGCCTATCAAAAACGCATCCTTGAGATGAGAGATTT
M94430.L2     TTATACGCACCCCAGCATCGGCGGCGCCTATCAAAAACGCATCCTTGAGATGAGAGATTT
BTV17.L2GB    ATATGTGCATCCGTCGACTGGTGGGACGTATCAAAAATGCGTGCTAGAGATGAAGGACCC
BTV11.L2      TTATACGCACCCCAGCATCGGCGGCGCCTATCAAAAACGCATCCTTGAGATGAGAGATTT
201400.L2     TTATACGCACCCCAGCATCGGCGGCGCCTATCAAAAACGCATCCTTGAGATGAGAGATTT
203734.L2     TTATACGCACCCCAGCATCGGCGGCGCCTATCAAAAACGCATCCTTGAGATGAGAGATTT
201767.L2     TTATACGCACCCCAGCATCGGCGGCGCCTATCAAAAACGCATCCTTGAGATGAGAGATTT
201773.L2     TTATACGCACCCCAGCATCGGCGGCGCCTATCAAAAACGCATCCTTGAGATGAGAGATTT
202020.L2     TTATACGCACCCCAGCATCGGCGGCGCCTATCAAAAACGCATCCTTGAGATGAGAGATTT
203853.L2     TTATACGCACCCCAGCATCGGCGGCGCCTATCAAAAACGCATCCTTGAGATGAGAGATTT
203732.L2     TTATACGCACCCCAGCATCGGCGGCGCCTATCAAAAACGCATCCTTGAGATGAGAGATTT
203843.L2     TTATACGCACCCCAGCATCGGCGGCGCCTATCAAAAACGCATCCTTGAGATGAGAGATTT
BTV2.L2GB     TT-TACCCATCCGAGGATTGATCCACAGTTTAAATTCAACGGTAATACATTATCTGATCT
BTV23.L2GB    TT--AAGCACCCATCGATTGATACACACCTTGATATTGATACCTGGAAGATCGCGGATAT
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M94389.L2     CTCGCAATTAATATGCTTTATAATCGATTATATATTTGAGAGGCATGATCAATTGCGCGA
BTV10.L2GB    TTCCCAGCTGATTTGTTTTGTGTTCGACTACATTTTTGAGAAACATGTACAGCTACGTAA
A94389.L2     CTCGCAATTAATATGCTTTATAATCGATTATATATTTGAGAGGCATGATCAATTGCGCGA
BTV11.L2GB    CTCGCAATTAATATGCTTTATAATCGATTATATATTTGAGAGGCATGATCAATTGCGCGA
202894.L2     CTCGCAATTAATATGCTTTATAATCGATTATATATTTGAGAGGCATGATCAATTGCGCGA
203733.L2     CTCGCAATTAATATGCTTTATAATCGATTATATATTTGAGAGGCATGATCAATTGCGCGA
203794.L2     CTCGCAATTAATATGCTTTATAATCGATTATATATTTGAGAGGCATGATCAATTGCGCGA
203826.L2     CTCGCAATTAATATGCTTTATAATCGATTATATATTTGAGAGGCATGATCAATTGCGCGA
270.L2        CTCGCAATTAATATGCTTTATAATCGATTATATATTTGAGAGGCATGATCAATTGCGCGA
M9442.L2      CTCGCAATTAATATGCTTTATAATCGATTATATATTTGAGAGGCATGATCAATTGCGCGA
A945.L2       CTCGCAATTAATATGCTTTATAATCGATTATATATTTGAGAGGCATGATCAATTGCGCGA
M94368.L2     CTCGCAATTAATATGCTTTATAATCGATTATATATTTGAGAGGCATGATCAATTGCGCGA
A9464.L2      CTCGCAATTAATATGCTTTATAATCGATTATATATTTGAGAGGCATGATCAATTGCGCGA
E94393.L2     CTCGCAATTAATATGCTTTATAATCGATTATATATTTGAGAGGCATGATCAATTGCGCGA
M94430.L2     CTCGCAATTAATATGCTTTATAATCGATTATATATTTGAGAGGCATGATCAATTGCGCGA
BTV17.L2GB    TTGTCAACTCATGTGCTTTGTGATTGATTACATCTTTGAAAAACGTGAACAGCTACGTGA
BTV11.L2      CTCGCAATTAATATGCTTTATAATCGATTATATATTTGAGAGGCATGATCAATTGCGCGA
201400.L2     CTCGCAATTAATATGCTTTATAATCGATTATATATTTGAGAGGCATGATCAATTGCGCGA
203734.L2     CTCGCAATTAATATGCTTTATAATCGATTATATATTTGAGAGGCATGATCAATTGCGCGA
201767.L2     CTCGCAATTAATATGCTTTATAATCGATTATATATTTGAGAGGCATGATCAATTGCGCGA
201773.L2     CTCGCAATTAATATGCTTTATAATCGATTATATATTTGAGAGGCATGATCAATTGCGCGA
202020.L2     CTCGCAATTAATATGCTTTATAATCGATTATATATTTGAGAGGCATGATCAATTGCGCGA
203853.L2     CTCGCAATTAATATGCTTTATAATCGATTATATATTTGAGAGGCATGATCAATTGCGCGA
203732.L2     CTCGCAATTAATATGCTTTATAATCGATTATATATTTGAGAGGCATGATCAATTGCGCGA
203843.L2     CTCGCAATTAATATGCTTTATAATCGATTATATATTTGAGAGGCATGATCAATTGCGCGA
BTV2.L2GB     GAATCAAACGGTCGTTTTTATAGTGGATTATTTGCATGAAAAGCGTAACTATGTACGATC
BTV23.L2GB    TTCTCAACTTATAGTATTACTATTTGATTATCTGTTTGAAAGTCGTAAGGAGATTAGAAG
                 **     *    **  *  * ** **  *   *** *  * *    *  *  *

M94389.L2     TATGAGAGAGGCGAGGAGGATTTTATATTTGATCCAGAGTTTAGATGAACCGCAGCGATT
BTV10.L2GB    TGCCAAAGAGGCGAGAAGGATTATATATTTAATACAGAATACGTCCGGTGCGTATCGATT
A94389.L2     TATGAGAGAGGCGAGGAGGATTTTATATTTGATCCAGAGTTTAGATGAACCGCAGCGATT
BTV11.L2GB    TATGAGAGAGGCGAGGAGGATTTTATATTTAGTCCAGAGTTTAGGTGAACCGCAGCGATT
202894.L2     TGTGAGAGAGGCGAGGAGGATTTTATATTTGATCCAGAGTTTAGATGAACCGCAGCGATT
203733.L2     TGTGAGAGAGGCGAGGAGGATTTTATATTTGATCCAGAGTTTAGATGAACCGCAGCGATT
203794.L2     TATGAGAGAGGCGAGGAGAATTCTATATTTGATCCAGAGTTTAGATGAACCACAGCGATT
203826.L2     TGTGAGAGAGGCGAGGAGGATTTTATATTTGATCCAGAGTTTAGATGAACCGCAGCGATT
270.L2        TATGAGAGAGGCGAGGAGGATTTTATATTTGATCCAGAGTTTAGATGAACCGCAGCGATT
M9442.L2      TATGAGAGAGGCGAGGAGGATTTTATATTTGATCCAGAGTTTAGATGAACCGCAGCGATT
A945.L2       TATGAGAGAGGCGAGGAGGATTTTATATTTGATCCAGAGTTTAGATGAACCGCAGCGATT
M94368.L2     TATGAGAGAGGCGAGGAGGATTTTATATTTGATCCAGAGTTTAGATGAACCGCAGCGATT
A9464.L2      TATGAGAGAGGCGAGGAGGATTTTATATTTGATCCAGAGTTTAGATGAACCGCAGCGATT
E94393.L2     TATGAGAGAGGCGAGGAGGATTTTATATTTGATCCAGAGTTTAGATGAACCGCAGCGATT
M94430.L2     TATGAGAGAGGCGAGGAGGATTTTATATTTGATCCAGAGTTTAGATGAACCGCAGCGATT
BTV17.L2GB    TACCAAAGAGGCGAGGTACATCGTGTATCTAATTCAAAGTCTCACTGGGACACAACGGCT
BTV11.L2      TATGAGAGAGGCGAGGAGGATTTTATATTTGATCCAGAGTTTAGATGAACCGCAGCGATT
201400.L2     TGTGAGAGAGGCGAGGAGGATTTTATATTTGATCCAGAGTTTAGATGAACCGCAGCGATT
203734.L2     TGTGAGAGAGGCGAGGAGGATTTTATATTTGATCCAGAGTTTAGATGAACCGCAGCGATT
201767.L2     TGTGAGAGAGGCGAGGAGGATTTTATATTTGATCCAGAGTTTAGATGAACCGCAGCGATT
201773.L2     TGTGAGAGAGGCGAGGAGGATTTTATATTTGATCCAGAGTTTAGATGAACCGCAGCGATT
202020.L2     TGTGAGAGAGGCGAGGAGGATTTTATATTTGATCCAGAGTTTAGATGAACCGCAGCGATT
203853.L2     TGTGAGAGAGGCGAGGAGGATTTTATATTTGATCCAGAGTTTAGATGAACCGCAGCGATT
203732.L2     TGTGAGAGAGGCGAGGAGGATTTTATATTTGATCCAGAGTTTAGATGAACCGCAGCGATT
203843.L2     TGTGAGAGAGGCGAGGAGGATTTTATATTTGATCCAGAGTTTAGATGAACCGCAGCGATT
BTV2.L2GB     GATATACGAGGCGAGATACATTATATCTAGGATAAGGTCGTCTACCGGTGCGGCGCGTAT
BTV23.L2GB    CGTGACTGAAGCGAGGTGGATTTTGTTTAAGGTCAGATCCGTTTCGGGACAAGAAAGACT
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M94389.L2     GGATAT-GTTAAATGTTACGTTTCCTAACTTTT-TTCGCTATTTTTTGAAATTAAAGGAC
BTV10.L2GB    AGATGT-TTTACGCGAAGCTTTCCCCAACTTTT-TAAAGCACGTTATGAATTTACGTGAT
A94389.L2     GGATAT-GTTAAATGTTACGTTTCCTAACTTTT-TTCGCTATTTTTTGAAATTAAAGGAC
BTV11.L2GB    GGATGT-GTTAAGTGTCGCGTCCCCTAACTTTT-CGCGCTATTTTTTGAAATTAAAGGAC
202894.L2     GGATAT-GTTAAATGTTACGTTTCCTAACTTTT-TTCGCTATTTTTTGAAATTGAAGGAC
203733.L2     GGATAT-GTTAAATGTTACGTTTCCTAACTTTT-TTCGCTATTTTTTGAAATTGAAGGAC
203794.L2     GGATAT-GTTAAATGTTACGTTTCCTAACTTTT-TTCGCTATTTTTCTGAAATTAAAGGAC
203826.L2     GGATAT-GTTAAATGTTACGTTTCCTAACTTTT-TTCGCTATTTTTTGAAATTGAAGGAC
270.L2        GGATAT-GTTAAATGTTACGTTTCCTAACTTTT-TTCGCTATTTTTTGAAATTAAAGGAC
M9442.L2      GGATAT-GTTAAATGTTACGTTTCCTAACTTTT-TTCGCTATTTTTTGAAATTAAAGGAC
A945.L2       GGATAT-GTTAAATGTTACGTTTCCTAACTTTT-TTCGCTATTTTTTGAAATTAAAGGAC
M94368.L2     GGATAT-GTTAAATGTTACGTTTCCTAACTTTT-TTCGCTATTTTTTGAAATTAAAGGAC
A9464.L2      GGATAT-GTTAAATGTTACGTTTCCTAACTTTT-TTCGCTATTTTTTGAAATTAAAGGAC
E94393.L2     GGATAT-GTTAAATGTTACGTTTCCTAACTTTT-TTCGCTATTTTTTGAAATTAAAGGAC
M94430.L2     GGATAT-GTTAAATGTTACGTTTCCTAACTTTT-TTCGCTATTTTTTGAAATTAAAGGAC
BTV17.L2GB    GGATGT-TCTGAAATCGACGTTCCCGAATTTTT-TCCAACGATTATTAATGCTGAAAGAG
BTV11.L2      GGATAT-GTTAAATGTTACGTTTCCTAACTTTT-TTCGCTATTTTTTGAAATTAAAGGAC
201400.L2     GGATAT-GTTAAATGTTACGTTTCCTAACTTTT-TTCGCTATTTTTTGAAATTGAAGGAC
203734.L2     GGATAT-GTTAAATGTTACGTTTCCTAACTTTT-TTCGCTATTTTTTGAAATTGAAGGAC
201767.L2     GGATAT-GTTAAATGTTACGTTTCCTAACTTTT-TTCGCTATTTTTTGAAATTGAAGGAC
201773.L2     GGATAT-GTTAAATGTTACGTTTCCTAACTTTT-TTCGCTATTTTTTGAAATTGAAGGAC
202020.L2     GGATAT-GTTAAATGTTACGTTTCCTAACTTTT-TTCGCTATTTTTTGAAATTGAAGGAC
203853.L2     GGATAT-GTTAAATGTTACGTTTCCTAACTTTT-TTCGCTATTTTTTGAAATTGAAGGAC
203732.L2     GGATAT-GTTAAATGTTACGTTTCCTAACTTTT-TTCGCTATTTTTTGAAATTGAAGGAC
203843.L2     GGATAT-GTTAAATGTTACGTTTCCTAACTTTT-TTCGCTATTTTTTGAAATTGAAGGAC
BTV2.L2GB     GAGTATCATCGAATTTTA--TTTCCCAACATTTGCGCGCTTGATTTCGAACGCGCGGGAA
BTV23.L2GB    GAATATGATTCAATCTTTC-TTTCCACGGTTTGGACGAGTCGTTAGAGACATCGCTAAAG
                 * *              *  **     **          *    *        *

M94389.L2     GTTCAGCGAATCAGTGATTTAAACGTAATAAACTTTTTACCGCTGCTGTTCTTGATACAG
BTV10.L2GB    GTGAAACGTATATGCGATCTGAATGTGATTAATTTCTTTCCATTGTTATTTTTGGTTCAA
A94389.L2     GTTCAGCGAATCAGTGATTTAAACGTAATAAACTTTTTACCGCTGCTGTTCTTGATACAG
BTV11.L2GB    GTTCAGCGAATCAGTGATTTAAACGTAATAAACTTTTTACCGCTGCTGTTCTTGATACAG
202894.L2     GTTCAGCGAATCAGTGATTTAAACGTAATAAACTTTTTACCGCTGTTGTTCTTGGTACAG
203733.L2     GTTCAGCGAATCAGTGATTTAAACGTAATAAACTTTTTACCGCTGTTGTTCTTGGTACAG
203794.L2     GTTCAGCGAATCAGTGATTTAAACGTAATAAACTTTTTACCGCTGCTGTTCTTGATACAG
203826.L2     GTTCAGCGAATCAGTGATTTAAACGTAATAAACTTTTTACCGCTGTTGTTCTTGGTACAG
270.L2        GTTCAGCGAATCAGTGATTTAAACGTAATAAACTTTTTACCGCTGCTGTTCTTGATACAG
M9442.L2      GTTCAGCGAATCAGTGATTTAAACGTAATAAACTTTTTACCGCTGCTGTTCTTGATACAG
A945.L2       GTTCAGCGAATCAGTGATTTAAACGTAATAAACTTTTTACCGCTGCTGTTCTTGATACAG
M94368.L2     GTTCAGCGAATCAGTGATTTAAACGTAATAAACTTTTTACCGCTGCTGTTCTTGATACAG
A9464.L2      GTTCAGCGAATCAGTGATTTAAACGTAATAAACTTTTTACCGCTGCTGTTCTTGATACAG
E94393.L2     GTTCAGCGAATCAGTGATTTAAACGTAATAAACTTTTTACCGCTGCTGTTCTTGATACAG
M94430.L2     GTTCAGCGAATCAGTGATTTAAACGTAATAAACTTTTTACCGCTGCTGTTCTTGATACAG
BTV17.L2GB    ATCAAATTTGTGCGTGATCTGAATGTGATCAATTTCCTCCCTCTGATGTTCCTTGTTCAT
BTV11.L2      GTTCAGCGAATCAGTGATTTAAACGTAATAAACTTTTTACCGCTGCTGTTCTTGATACAG
201400.L2     GTTCAGCGAATCAGTGATTTAAACGTAATAAACTTTTTACCGCTGTTGTTCTTGGTACAG
203734.L2     GTTCAGCGAATCAGTGATTTAAACGTAATAAACTTTTTACCGCTGTTGTTCTTGGTACAG
201767.L2     GTTCAGCGAATCAGTGATTTAAACGTAATAAACTTTTTACCGCTGTTGTTCTTGGTACAG
201773.L2     GTTCAGCGAATCAGTGATTTAAACGTAATAAACTTTTTACCGCTGTTGTTCTTGGTACAG
202020.L2     GTTCAGCGAATCAGTGATTTAAACGTAATAAACTTTTTACCGCTGTTGTTCTTGGTACAG
203853.L2     GTTCAGCGAATCAGTGATTTAAACGTAATAAACTTTTTACCGCTGTTGTTCTTGGTACAG
203732.L2     GTTCAGCGAATCAGTGATTTAAACGTAATAAACTTTTTACCGCTGTTGTTCTTGGTACAG
203843.L2     GTTCAGCGAATCAGTGATTTAAACGTAATAAACTTTTTACCGCTGTTGTTCTTGGTACAG
BTV2.L2GB     CCGACTTATGTGAAAGATCTGATGGCGTTGAACTTCTTGCCGTTACTATTCATAGTAGGT
BTV23.L2GB    CGAAAATCACTCAGGATGTTGCGCAT-CTTAACTTCTTTCCCTTATTTTTTATCATCGGC
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M94389.L2     GATAACATATCCTATTGGCATAGGCAATGGGCAGTCCCGATGATACTATACGATGACACA
BTV10.L2GB    GATAATATTTCATATTGGCATAGACAGTGGTCGATCCCAATGATTTTATTTGATCAAGTT
A94389.L2     GATAACATATCCTATTGGCATAGGCAATGGGCAGTCCCGATGATACTATACGATGACACA
BTV11.L2GB    GATAACATATCCTATTGGCATAGGCAATGGGCAGTCCCGATGATACTATACGATGACACA
202894.L2     GATAACATATCCTATTGGCATAGGCAATGGGCAGTCCCGATGATACTATACGATGACACA
203733.L2     GATAACATATCCTATTGGCATAGGCAATGGGCAGTCCCGATGATACTATACGATGACACA
203794.L2     GATAACATATCCTATTGGCATAGGCAATGGGCAGTCCCGATGATACTATACGATGACACA
203826.L2     GATAACATATCCTATTGGCATAGGCAATGGGCAGTCCCGATGATACTATACGATGACACA
270.L2        GATAACATATCCTATTGGCATAGGCAATGGGCAGTCCCGATGATACTATACGATGACACA
M9442.L2      GATAACATATCCTATTGGCATAGGCAATGGGCAGTCCCGATGATACTATACGATGACACA
A945.L2       GATAACATATCCTATTGGCATAGGCAATGGGCAGTCCCGATGATACTATACGATGACACA
M94368.L2     GATAACATATCCTATTGGCATAGGCAATGGGCAGTCCCGATGATACTATACGATGACACA
A9464.L2      GATAACATATCCTATTGGCATAGGCAATGGGCAGTCCCGATGATACTATACGATGACACA
E94393.L2     GATAACATATCCTATTGGCATAGGCAATGGGCAGTCCCGATGATACTATACGATGACACA
M94430.L2     GATAACATATCCTATTGGCATAGGCAATGGGCAGTCCCGATGATACTATACGATGACACA
BTV17.L2GB    GATAACATCTCGTATTGGCATAGACAGTGGTCAATTCCAATGGTGCTGTTTGACGATACG
BTV11.L2      GATAACATATCCTATTGGCATAGGCAATGGGCAGTCCCGATGATACTATACGATGACACA
201400.L2     GATAACATATCCTATTGGCATAGGCAATGGGCAGTCCCGATGATACTATACGATGACACA
203734.L2     GATAACATATCCTATTGGCATAGGCAATGGGCAGTCCCGATGATACTATACGATGACACA
201767.L2     GATAACATATCCTATTGGCATAGGCAATGGGCAGTCCCGATGATACTATACGATGACACA
201773.L2     GATAACATATCCTATTGGCATAGGCAATGGGCAGTCCCGATGATACTATACGATGACACA
202020.L2     GATAACATATCCTATTGGCATAGGCAATGGGCAGTCCCGATGATACTATACGATGACACA
203853.L2     GATAACATATCCTATTGGCATAGGCAATGGGCAGTCCCGATGATACTATACGATGACACA
203732.L2     GATAACATATCCTATTGGCATAGGCAATGGGCAGTCCCGATGATACTATACGATGACACA
203843.L2     GATAACATATCCTATTGGCATAGGCAATGGGCAGTCCCGATGATACTATACGATGACACA
BTV2.L2GB     GATAATATGATATATAAACATAGGCAGTGGTCGATACCATTATTGCTGTATACAGACCGA
BTV23.L2GB    GACAACATGGCTTACGCTCATAGACAGTGGAGCATACCTGTCCTATTATACGCTCATGAC
              ** ** **    **    ***** ** ***    * **  *  *  * *      *

M94389.L2     ATTAAATTGATTCCTGTGGAGGTTGGGGCATACGCGAATCGATTTGGGATAAAGAGCTTT
BTV10.L2GB    ATCAGATTAATTCCCGTCGAAGTCGGGGCATACGCAAATAGATTTGGACTTAAGAGCTTC
A94389.L2     ATTAAATTGATTCCTGTGGAGGTTGGGGCATACGCGAATCGATTTGGGATAAAGAGCTTT
BTV11.L2GB    ATTAAATTGATTCCTGTGGAGGTTGGGGCATACGCGAATCGATTTGGGATAAAGAGCTTT
202894.L2     ATTAAATTGATTCCCGTGGAGGTTGGGGCATACGCGAATCGATTTGGGATAAAGAGCTTT
203733.L2     ATTAAATTGATTCCCGTGGAGGTTGGGGCATACGCGAATCGATTTGGGATAAAGAGCTTT
203794.L2     ATTAAATTGATTCCTGTGGAGGTTGGGGCATACGCGAATCGATTTGGGATAAAGAGCTTT
203826.L2     ATTAAATTGATTCCCGTGGAGGTTGGGGCATACGCGAATCGATTTGGGATAAAGAGCTTT
270.L2        ATTAAATTGATTCCTGTGGAGGTTGGGGCATACGCGAATCGATTTGGGATAAAGAGCTTT
M9442.L2      ATTAAATTGATTCCTGTGGAGGTTGGGGCATACGCGAATCGATTTGGGATAAAGAGCTTT
A945.L2       ATTAAATTGATTCCTGTGGAGGTTGGGGCATACGCGAATCGATTTGGGATAAAGAGCTTT
M94368.L2     ATTAAATTGATTCCTGTGGAGGTTGGGGCATACGCGAATCGATTTGGGATAAAGAGCTTT
A9464.L2      ATTAAATTGATTCCTGTGGAGGTTGGGGCATACGCGAATCGATTTGGGATAAAGAGCTTT
E94393.L2     ATTAAATTGATTCCTGTGGAGGTTGGGGCATACGCGAATCGATTTGGGATAAAGAGCTTT
M94430.L2     ATTAAATTGATTCCTGTGGAGGTTGGGGCATACGCGAATCGATTTGGGATAAAGAGCTTT
BTV17.L2GB    ATCAAGTTAATACCCGTAGAGGTTGGTGCATATGCGAATAGATTTGGGTTCAAAAGTTTT
BTV11.L2      ATTAAATTGATTCCTGTGGAGGTTGGGGCATACGCGAATCGATTTGGGATAAAGAGCTTT
201400.L2     ATTAAATTGATTCCCGTGGAGGTTGGGGCATACGCGAATCGATTTGGGATAAAGAGCTTT
203734.L2     ATTAAATTGATTCCCGTGGAGGTTGGGGCATACGCGAATCGATTTGGGATAAAGAGCTTT
201767.L2     ATTAAATTGATTCCCGTGGAGGTTGGGGCATACGCGAATCGATTTGGGATAAAGAGCTTT
201773.L2     ATTAAATTGATTCCCGTGGAGGTTGGGGCATACGCGAATCGATTTGGGATAAAGAGCTTT
202020.L2     ATTAAATTGATTCCCGTGGAGGTTGGGGCATACGCGAATCGATTTGGGATAAAGAGCTTT
203853.L2     ATTAAATTGATTCCCGTGGAGGTTGGGGCATACGCGAATCGATTTGGGATAAAGAGCTTT
203732.L2     ATTAAATTGATTCCCGTGGAGGTTGGGGCATACGCGAATCGATTTGGGATAAAGAGCTTT
203843.L2     ATTAAATTGATTCCCGTGGAGGTTGGGGCATACGCGAATCGATTTGGGATAAAGAGCTTT
BTV2.L2GB     GTGAAGGTGATTCCATTGGAGGTGGGCTCATCGAACAATAGACAGGGATTCGTATCTTAT
BTV23.L2GB    ATAAGAATAATACCATTAGAGATTGGTGCATATAACAACCGATTTGGCTTTACATCCTAC
               * *   * ** **  * **  * **  ***     **  **   **  *       *
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M94389.L2     TTCAATTTTACGAGGTTCCACCC-TGGTGATGCGAAAAAACGACAGAAGGCGG----
BTV10.L2GB    TTCAATTTCATTAGGTTTCACCC-CGGCGATTCAAAGAAGAGACAGGATGCGG----
A94389.L2     TTCAATTTTACGAGGTTCCACCC-TGGTGATGCGAAAAAACGACAGAAGGCGG----
BTV11.L2GB    TTCAATTTTACGAGGTTCCACCC-TGGTGATGCGAAAAAACGACAGAAGGCGG----
202894.L2     TTCAATTTTACGAGATTCCACCC-TGGTGATGCGAAAAAACGACAGAAGGCGG----
203733.L2     TTCAATTTTACGAGATTCCACCC-TGGTGATGCGAAAAAACGACAGAAGGCGG----
203794.L2     TTCAATTTTACGAGATTCCACCC-TGGTGATGCGAAAAAACGACAGAAAGCGG----
203826.L2     TTCAATTTTACGAGATTCCACCC-TGGTGATGCGAAAAAACGACAGAAGGCGG----
270.L2        TTCAATTTTACGAGGTTCCACCC-TGGTGATGCGAAAAAACGACAGAAGGCGG----
M9442.L2      TTCAATTTTACGAGGTTCCACCC-TGGTGATGCGAAAAAACGACAGAAGGCGG----
A945.L2       TTCAATTTTACGAGGTTCCACCC-TGGTGATGCGAAAAAACGACAGAAGGCGG----
M94368.L2     TTCAATTTTACGAGGTTCCACCC-TGGTGATGCGAAAAAACGACAGAAGGCGG----
A9464.L2      TTCAATTTTACGAGGTTCCACCC-TGGTGATGCGAAAAAACGACAGAAGGCGG----
E94393.L2     TTCAATTTTACGAGGTTCCACCC-TGGTGATGCGAAAAAACGACAGAAGGCGG----
M94430.L2     TTCAATTTTACGAGGTTCCACCC-TGGTGATGCGAAAAAACGACAGAAGGCGG----
BTV17.L2GB    ATGAACTTTACACGGTTTCACCC-TGGTGAGTCAAAGAAAAAACAGATTGCCGAGGA
BTV11.L2      TTCAATTTTACGAGGTTCCACCC-TGGTGATGCGAAAAAACGACAGAAGGCGG----
201400.L2     TTCAATTTTACGAGATTCCACCC-TGGTGATGCGAAAAAACGACAGAAGGCGG----
203734.L2     TTCAATTTTACGAGATTCCACCC-TGGTGATGCGAAAAAACGACAGAAGGCGG----
201767.L2     TTCAATTTTACGAGATTCCACCC-TGGTGATGCGAAAAAACGACAGAAGGCGG----
201773.L2     TTCAATTTTACGAGATTCCACCC-TGGTGATGCGAAAAAACGACAGAAGGCGG----
202020.L2     TTCAATTTTACGAGATTCCACCC-TGGTGATGCGAAAAAACGACAGAAGGCGG----
203853.L2     TTCAATTTTACGAGATTCCACCC-TGGTGATGCGAAAAAACGACAGAAGGCGG----
203732.L2     TTCAATTTTACGAGATTCCACCC-TGGTGATGCGAAAAAACGACAGAAGGCGG----
203843.L2     TTCAATTTTACGAGATTCCACCC-TGGTGATGCGAAAAAACGACAGAAGGCGG----
BTV2.L2GB     CTAGAGTATATGTTCTTTTTTCCATCGTTAGCTGATC--------------------
BTV23.L2GB    TTGGAGTATATGACGTTCTTTCC---GTCACACG-CGACACGAGAGG----------
               *  *  *  *     **    **   *  *
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APPENDIX C – Distance analyses for the 620bp fragment of S7

Distance: Number of different nucleotides.
No. of Codons in Subset: 208 of 208
Codon Position(s) Used: 1 2 3
Gap Sites and Missing Information Data: All such sites were removed from the subset data

OTU Labels
1.. BTV11gb.VP7
2.. BTV11.VP7-SS
3.. 201773.VP7
4.. 201400.VP7
5.. 203794.VP7
6.. 203733.VP7
7.. 203843.VP7
8.. 202894.VP7
9.. BTV17.VP7
10.. 203732.VP7
11.. 270.VP7
12.. 203826.VP7
13.. 203734.VP7
14.. 203853.VP7
15.. 202020.VP7
16.. 201767.VP7
17.. M94430.VP7
18.. M94389.VP7
19.. E94393.VP7
20.. M9442.VP7
21.. M94368.VP7
22.. A945.VP7
23.. A94389.VP7
24.. A9464.VP7
25.. BTV2.VP7
26.. BTV13.VP7

Distances in the upper-right matrix
'*' indicates an invalid distance value

OTUs	1	2	3	4	5	6	7
1		0.0000	29.0000	29.0000	29.0000	29.0000	29.0000
2			29.0000	29.0000	29.0000	29.0000	29.0000
3				0.0000	0.0000	0.0000	0.0000
4					0.0000	0.0000	0.0000
5						0.0000	0.0000
6							0.0000
7							

OTUs	8	9	10	11	12	13	14
1	30.0000	31.0000	29.0000	28.0000	30.0000	30.0000	37.0000
2	30.0000	31.0000	29.0000	28.0000	30.0000	30.0000	37.0000
3	1.0000	2.0000	0.0000	1.0000	1.0000	1.0000	17.0000
4	1.0000	2.0000	0.0000	1.0000	1.0000	1.0000	17.0000
5	1.0000	2.0000	0.0000	1.0000	1.0000	1.0000	17.0000
6	1.0000	2.0000	0.0000	1.0000	1.0000	1.0000	17.0000
7	1.0000	2.0000	0.0000	1.0000	1.0000	1.0000	17.0000
8		3.0000	1.0000	2.0000	2.0000	2.0000	18.0000
9			2.0000	3.0000	3.0000	3.0000	19.0000
10				1.0000	1.0000	1.0000	17.0000
11					2.0000	2.0000	18.0000

12						2.0000	16.0000
13							16.0000
14							

OTUs	15	16	17	18	19	20	21
1	36.0000	29.0000	45.0000	45.0000	45.0000	45.0000	45.0000
2	36.0000	29.0000	45.0000	45.0000	45.0000	45.0000	45.0000
3	18.0000	8.0000	38.0000	38.0000	38.0000	38.0000	38.0000
4	18.0000	8.0000	38.0000	38.0000	38.0000	38.0000	38.0000
5	18.0000	8.0000	38.0000	38.0000	38.0000	38.0000	38.0000
6	18.0000	8.0000	38.0000	38.0000	38.0000	38.0000	38.0000
7	18.0000	8.0000	38.0000	38.0000	38.0000	38.0000	38.0000
8	19.0000	9.0000	39.0000	39.0000	39.0000	39.0000	39.0000
9	20.0000	10.0000	40.0000	40.0000	40.0000	40.0000	40.0000
10	18.0000	8.0000	38.0000	38.0000	38.0000	38.0000	38.0000
11	19.0000	9.0000	39.0000	39.0000	39.0000	39.0000	39.0000
12	17.0000	9.0000	37.0000	37.0000	37.0000	37.0000	37.0000
13	17.0000	7.0000	39.0000	39.0000	39.0000	39.0000	39.0000
14	1.0000	11.0000	43.0000	43.0000	43.0000	43.0000	43.0000
15		10.0000	42.0000	42.0000	42.0000	42.0000	42.0000
16			39.0000	39.0000	39.0000	39.0000	39.0000
17				0.0000	0.0000	0.0000	0.0000
18					0.0000	0.0000	0.0000
19						0.0000	0.0000
20							0.0000
21							

OTUs	22	23	24	25	26
1	45.0000	45.0000	45.0000	47.0000	123.0000
2	45.0000	45.0000	45.0000	47.0000	123.0000
3	38.0000	38.0000	38.0000	40.0000	116.0000
4	38.0000	38.0000	38.0000	40.0000	116.0000
5	38.0000	38.0000	38.0000	40.0000	116.0000
6	38.0000	38.0000	38.0000	40.0000	116.0000
7	38.0000	38.0000	38.0000	40.0000	116.0000
8	39.0000	39.0000	39.0000	41.0000	116.0000
9	40.0000	40.0000	40.0000	40.0000	117.0000
10	38.0000	38.0000	38.0000	40.0000	116.0000
11	39.0000	39.0000	39.0000	41.0000	115.0000
12	37.0000	37.0000	37.0000	39.0000	115.0000
13	39.0000	39.0000	39.0000	41.0000	117.0000
14	43.0000	43.0000	43.0000	45.0000	122.0000
15	42.0000	42.0000	42.0000	44.0000	121.0000
16	39.0000	39.0000	39.0000	41.0000	118.0000
17	0.0000	0.0000	0.0000	4.0000	110.0000
18	0.0000	0.0000	0.0000	4.0000	110.0000
19	0.0000	0.0000	0.0000	4.0000	110.0000
20	0.0000	0.0000	0.0000	4.0000	110.0000
21	0.0000	0.0000	0.0000	4.0000	110.0000
22		0.0000	0.0000	4.0000	110.0000
23			0.0000	4.0000	110.0000
24				4.0000	110.0000
25					111.0000
26					

Distance: Number of different nucleotides (transversions only).
No. of Codons in Subset: 208 of 208
Codon Position(s) Used: 1 2 3
Gap Sites and Missing Information Data: All such sites were removed from the subset data

OTU Labels

1.. BTV11gb.VP7
2.. BTV11.VP7-SS
3.. 201773.VP7
4.. 201400.VP7
5.. 203794.VP7
6.. 203733.VP7
7.. 203843.VP7
8.. 202894.VP7
9.. BTV17.VP7
10.. 203732.VP7
11.. 270.VP7
12.. 203826.VP7
13.. 203734.VP7
14.. 203853.VP7
15.. 202020.VP7
16.. 201767.VP7
17.. M94430.VP7
18.. M94389.VP7
19.. E94393.VP7
20.. M9442.VP7
21.. M94368.VP7
22.. A945.VP7
23.. A94389.VP7
24.. A9464.VP7
25.. BTV2.VP7
26.. BTV13.VP7

Distances in the upper-right matrix
'*' indicates an invalid distance value

OTUs	1	2	3	4	5	6	7	8
1		0.0000	4.0000	4.0000	4.0000	4.0000	4.0000	5.0000
2			4.0000	4.0000	4.0000	4.0000	4.0000	5.0000
3				0.0000	0.0000	0.0000	0.0000	1.0000
4					0.0000	0.0000	0.0000	1.0000
5						0.0000	0.0000	1.0000
6							0.0000	1.0000
7								1.0000
8								

OTUs	9	10	11	12	13	14	15	16
1	4.0000	4.0000	4.0000	4.0000	4.0000	5.0000	4.0000	3.0000
2	4.0000	4.0000	4.0000	4.0000	4.0000	5.0000	4.0000	3.0000
3	0.0000	0.0000	0.0000	0.0000	0.0000	1.0000	2.0000	1.0000
4	0.0000	0.0000	0.0000	0.0000	0.0000	1.0000	2.0000	1.0000
5	0.0000	0.0000	0.0000	0.0000	0.0000	1.0000	2.0000	1.0000
6	0.0000	0.0000	0.0000	0.0000	0.0000	1.0000	2.0000	1.0000
7	0.0000	0.0000	0.0000	0.0000	0.0000	1.0000	2.0000	1.0000
8	1.0000	1.0000	1.0000	1.0000	1.0000	2.0000	3.0000	2.0000
9		0.0000	0.0000	0.0000	0.0000	1.0000	2.0000	1.0000
10			0.0000	0.0000	0.0000	1.0000	2.0000	1.0000
11				0.0000	0.0000	1.0000	2.0000	1.0000
12					0.0000	1.0000	2.0000	1.0000
13						1.0000	2.0000	1.0000
14							1.0000	2.0000
15								1.0000
16								

OTUs	17	18	19	20	21	22	23	24
1	8.0000	8.0000	8.0000	8.0000	8.0000	8.0000	8.0000	8.0000
2	8.0000	8.0000	8.0000	8.0000	8.0000	8.0000	8.0000	8.0000

3	10.0000	10.0000	10.0000	10.0000	10.0000	10.0000	10.0000	10.0000
4	10.0000	10.0000	10.0000	10.0000	10.0000	10.0000	10.0000	10.0000
5	10.0000	10.0000	10.0000	10.0000	10.0000	10.0000	10.0000	10.0000
6	10.0000	10.0000	10.0000	10.0000	10.0000	10.0000	10.0000	10.0000
7	10.0000	10.0000	10.0000	10.0000	10.0000	10.0000	10.0000	10.0000
8	11.0000	11.0000	11.0000	11.0000	11.0000	11.0000	11.0000	11.0000
9	10.0000	10.0000	10.0000	10.0000	10.0000	10.0000	10.0000	10.0000
10	10.0000	10.0000	10.0000	10.0000	10.0000	10:0000	10.0000	10.0000
11	10.0000	10.0000	10.0000	10.0000	10.0000	10.0000	10.0000	10.0000
12	10.0000	10.0000	10.0000	10.0000	10.0000	10.0000	10.0000	10.0000
13	10.0000	10.0000	10.0000	10.0000	10.0000	10.0000	10.0000	10.0000
14	11.0000	11.0000	11.0000	11.0000	11.0000	11.0000	11.0000	11.0000
15	10.0000	10.0000	10.0000	10.0000	10.0000	10.0000	10.0000	10.0000
16	9.0000	9.0000	9.0000	9.0000	9.0000	9.0000	9.0000	9.0000
17		0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
18			0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
19				0.0000	0.0000	0.0000	0.0000	0.0000
20					0.0000	0.0000	0.0000	0.0000
21						0.0000	0.0000	0.0000
22							0.0000	0.0000
23								0.0000
24								

OTUs	25	26
1	8.0000	57.0000
2	8.0000	57.0000
3	10.0000	57.0000
4	10.0000	57.0000
5	10.0000	57.0000
6	10.0000	57.0000
7	10.0000	57.0000
8	11.0000	56.0000
9	10.0000	57.0000
10	10.0000	57.0000
11	10.0000	57.0000
12	10.0000	57.0000
13	10.0000	57.0000
14	11.0000	58.0000
15	10.0000	57.0000
16	9.0000	56.0000
17	0.0000	55.0000
18	0.0000	55.0000
19	0.0000	55.0000
20	0.0000	55.0000
21	0.0000	55.0000
22	0.0000	55.0000
23	0.0000	55.0000
24	0.0000	55.0000
25		55.0000
26		

Distance: Number of different nucleotides (transitions only).
No. of Codons in Subset: 208 of 208
Codon Position(s) Used: 1 2 3
Gap Sites and Missing Information Data: All such sites were removed from the subset data

OTU Labels
1.. BTV11gb.VP7
2.. BTV11.VP7-SS

3.. 201773.VP7
4.. 201400.VP7
5.. 203794.VP7
6.. 203733.VP7
7.. 203843.VP7
8.. 202894.VP7
9.. BTV17.VP7
10.. 203732.VP7
11.. 270.VP7
12.. 203826.VP7
13.. 203734.VP7
14.. 203853.VP7
15.. 202020.VP7
16.. 201767.VP7
17.. M94430.VP7
18.. M94389.VP7
19.. E94393.VP7
20.. M9442.VP7
21.. M94368.VP7
22.. A945.VP7
23.. A94389.VP7
24.. A9464.VP7
25.. BTV2.VP7
26.. BTV13.VP7

Distances in the upper-right matrix
'*' indicates an invalid distance value

OTUs	1	2	3	4	5	6	7	8
1		0.0000	25.0000	25.0000	25.0000	25.0000	25.0000	25.0000
2			25.0000	25.0000	25.0000	25.0000	25.0000	25.0000
3				0.0000	0.0000	0.0000	0.0000	0.0000
4					0.0000	0.0000	0.0000	0.0000
5						0.0000	0.0000	0.0000
6							0.0000	0.0000
7								0.0000
8								

OTUs	9	10	11	12	13	14	15	16
1	27.0000	25.0000	24.0000	26.0000	26.0000	32.0000	32.0000	26.0000
2	27.0000	25.0000	24.0000	26.0000	26.0000	32.0000	32.0000	26.0000
3	2.0000	0.0000	1.0000	1.0000	1.0000	16.0000	16.0000	7.0000
4	2.0000	0.0000	1.0000	1.0000	1.0000	16.0000	16.0000	7.0000
5	2.0000	0.0000	1.0000	1.0000	1.0000	16.0000	16.0000	7.0000
6	2.0000	0.0000	1.0000	1.0000	1.0000	16.0000	16.0000	7.0000
7	2.0000	0.0000	1.0000	1.0000	1.0000	16.0000	16.0000	7.0000
8	2.0000	0.0000	1.0000	1.0000	1.0000	16.0000	16.0000	7.0000
9		2.0000	3.0000	3.0000	3.0000	18.0000	18.0000	9.0000
10			1.0000	1.0000	1.0000	16.0000	16.0000	7.0000
11				2.0000	2.0000	17.0000	17.0000	8.0000
12					2.0000	15.0000	15.0000	8.0000
13						15.0000	15.0000	6.0000
14							0.0000	9.0000
15								9.0000
16								

OTUs	17	18	19	20	21	22	23	24
1	37.0000	37.0000	37.0000	37.0000	37.0000	37.0000	37.0000	37.0000
2	37.0000	37.0000	37.0000	37.0000	37.0000	37.0000	37.0000	37.0000
3	28.0000	28.0000	28.0000	28.0000	28.0000	28.0000	28.0000	28.0000
4	28.0000	28.0000	28.0000	28.0000	28.0000	28.0000	28.0000	28.0000
5	28.0000	28.0000	28.0000	28.0000	28.0000	28.0000	28.0000	28.0000

6	28.0000	28.0000	28.0000	28.0000	28.0000	28.0000	28.0000	28.0000
7	28.0000	28.0000	28.0000	28.0000	28.0000	28.0000	28.0000	28.0000
8	28.0000	28.0000	28.0000	28.0000	28.0000	28.0000	28.0000	28.0000
9	30.0000	30.0000	30.0000	30.0000	30.0000	30.0000	30.0000	30.0000
10	28.0000	28.0000	28.0000	28.0000	28.0000	28.0000	28.0000	28.0000
11	29.0000	29.0000	29.0000	29.0000	29.0000	29.0000	29.0000	29.0000
12	27.0000	27.0000	27.0000	27.0000	27.0000	27.0000	27.0000	27.0000
13	29.0000	29.0000	29.0000	29.0000	29.0000	29.0000	29.0000	29.0000
14	32.0000	32.0000	32.0000	32.0000	32.0000	32.0000	32.0000	32.0000
15	32.0000	32.0000	32.0000	32.0000	32.0000	32.0000	32.0000	32.0000
16	30.0000	30.0000	30.0000	30.0000	30.0000	30.0000	30.0000	30.0000
17		0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
18			0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
19				0.0000	0.0000	0.0000	0.0000	0.0000
20					0.0000	0.0000	0.0000	0.0000
21						0.0000	0.0000	0.0000
22							0.0000	0.0000
23								0.0000
24								

OTUs	25	26
1	39.0000	66.0000
2	39.0000	66.0000
3	30.0000	59.0000
4	30.0000	59.0000
5	30.0000	59.0000
6	30.0000	59.0000
7	30.0000	59.0000
8	30.0000	60.0000
9	30.0000	60.0000
10	30.0000	59.0000
11	31.0000	58.0000
12	29.0000	58.0000
13	31.0000	60.0000
14	34.0000	64.0000
15	34.0000	64.0000
16	32.0000	62.0000
17	4.0000	55.0000
18	4.0000	55.0000
19	4.0000	55.0000
20	4.0000	55.0000
21	4.0000	55.0000
22	4.0000	55.0000
23	4.0000	55.0000
24	4.0000	55.0000
25		56.0000
26		

APPENDIX D – Multiple Alignment file for the 620bp fragment of S7

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BTV17.S7GB  ATGGACACT-ATCGCTGCAAGAGCACTCACTGTGATGCGAGCATGTGCTACGCTTCAAGA
203853.S7   ATGGACACT-ATCGCTGCAAGAGCGCTCACTGTGATGCGAGCATGTGCTACGCTTCAAGA
A945.S7     ATGGACACT-ATCGCTGCAAGAGCGCTCACTGTGATGCGAGCATGTGCTACGCTTCAAGA
BTV11.S7    ATGGACACT-ATCGCTGCAAGAGCGCTCACTGTGATGCGAGCATGCGCTACGCTTCAAGA
BTV13.S7GB  ATGGACACT-ATCGCAGCAAGAGCTCTCACTGTGATGCGGGTATGTGCTACCTTACAAGA
203732.S7   ATGGACACT-ATCGCTGCAAGAGCGCTCACTGTGATGCGAGCATGTGCTACGCTTCAAGA
201773.S7   ATGGACACT-ATCGCTGCAAGAGCGCTCACTGTGATGCGAGCATGTGCTACGCTTCAAGA
201400.S7   ATGGACACT-ATCGCTGCAAGAGCGCTCACTGTGATGCGAGCATGTGCTACGCTTCAAGA
203843.S7   ATGGACACT-ATCGCTGCAAGAGCGCTCACTGTGATGCGAGCATGTGCTACGCTTCAAGA
270.S7      ATGGACACT-ATCGCTGCAAGAGCGCTCACTGTGATGCGAGCATGTGCTACGCTTCAAGA
201767.S7   ATGGACACT-ATCGCTGCAAGAGCGCTCACTGTGATGCGAGCATGTGCTACGCTTCAAGA
203794.S7   ATGGACACT-ATCGCTGCAAGAGCGCTCACTGTGATGCGAGCATGTGCTACGCTTCAAGA
203733.S7   ATGGACACT-ATCGCTGCAAGAGCGCTCACTGTGATGCGAGCATGTGCTACGCTTCAAGA
M94389.S7   ATGGACACTTATCGCTGCAAGAGCGCTCACTGTGATGCGAGCATGTGCTACGCTTCAAGA
203734.S7   ATGGACACT-ATCGCTGCAAGAGCGCTCACTGTGATGCGAGCATGTGCTACGCTTCAAGA
202894.S7   ATGGACACT-ATCGCTGCAAGAGCGCTCACTGTGATGCGAGCATGTGCTACGCTTCAAGA
203826.S7   ATGGACACT-ATCGCTGCAAGAGCGCTCACTGTGATGCGAGCATGTGCTACGCTTCAAGA
A94389.S7   ATGGACACT-ATCGCTGCAAGAGCGCTCACTGTGATGCGAGCATGTGCTACGCTTCAAGA
202020.S7   ATGGACACT-ATCGCTGCAAGAGCGCTCACTGTGATGCGAGCATGTGCTACGCTTCAAGA
M9442.S7    ATGGACACT-ATCGCTGCAAGAGCGCTCACTGTGATGCGAGCATGTGCTACGCTTCAAGA
E94393.S7   ATGGACACT-ATCGCTGCAAGAGCGCTCACTGTGATGCGAGCATGTGCTACGCTTCAAGA
BTV11.S7GB  ATGGACACT-ATCGCTGCAAGAGCGCTCACTGTGATGCGAGCATGCGCTACGCTTCAAGA
A9464.S7    ATGGACACT-ATCGCTGCAAGAGCGCTCACTGTGATGCGAGCATGTGCTACGCTTCAAGA
BTV2.S7GB   ATGGACACT-ATCGCTGCAAGAGCACTCACTGTGATGCGAGCATGTGCTACGCTTCAAGA
M94368.S7   ATGGACACT-ATCGCTGCAAGAGCGCTCACTGTGATGCGAGCATGTGCTACGCTTCAAGA
M94430.S7   ATGGACACT-ATCGCTGCAAGAGCGCTCACTGTGATGCGAGCATGTGCTACGCTTCAAGA
            ********* ***** ******** ************** * *** *****  * *****

BTV17.S7GB  GGCAAGAATTGTGTTGGAAGCCAATGTGATGGAAATTCT-GGGGATTGCTATCAATAGGT
203853.S7   GGCAAGAATTGTGTTGGAAGCCAATGTGATGGAAATTTT-GGGGATTGCTATCAATAGGT
A945.S7     AGCAAGAATTGTGTTGGAAGCTAATGTGATGGAAATACT-GGGGATAGCTATCAACAGGT
BTV11.S7    AGCAAGAATTGTGTTGGAAGCCAATGTGATGGAAATTCT-AGGGATAGCTATCAACAGGT
BTV13.S7GB  GGCCCGAATTGTCCTAGAACCCAATGTAATGGAGATACT-GGGAATAGCAATCAATAGAT
203732.S7   GGCAAGAATTGTGTTGGAAGCCAATGTGATGGAAATTCTTGGGGATTGCTATCAATAGGT
201773.S7   GGCAAGAATTGTGTTGGAAGCCAATGTGATGGAAATTCT-GGGGATTGCTATCAATAGGT
201400.S7   GGCAAGAATTGTGTTGGAAGCCAATGTGATGGAAATTCT-GGGGATTGCTATCAATAGGT
203843.S7   GGCAAGAATTGTGTTGGAAGCCAATGTGATGGAAATTCT-GGGGATTGCTATCAATAGGT
270.S7      GGCAAGAATTGTGTTGGAAGCCAATGTGATGGAAATTCT-GGGGATTGCTATCAATAGGT
201767.S7   GGCAAGAATTGTGTTGGAAGCCAATGTGATGGAAATTTT-GGGGATAGCTATCAATAGGT
203794.S7   GGCAAGAATTGTGTTGGAAGCCAATGTGATGGAAATTCT-GGGGATTGCTATCAATAGGT
203733.S7   GGCAAGAATTGTGTTGGAAGCCAATGTGATGGAAATTCT-GGGGATTGCTATCAATAGGT
M94389.S7   AGCAAGAATTGTGTTGGAAGCTAATGTGATGGAAATACT-GGGGATAGCTATCAACAGGT
203734.S7   GGCAAGAATTGTGTTGGAAGCCAATGTGATGGAAATTCT-GGGGATTGCTATCAATAGGT
202894.S7   GGCAAGAATTGTGTTGGAAGCCAATGTGATGGAAATTCT-GGGGATTGCTATCAATAGGT
203826.S7   GGCAAGAATTGTGTTGGAAGCCAATGTGATGGAAATTCT-GGGGATTGCTATCAATAGGT
A94389.S7   AGCAAGAATTGTGTTGGAAGCTAATGTGATGGAAATACT-GGGGATAGCTATCAACAGGT
202020.S7   GGCAAGAATTGTGTTGGAAGCCAATGTGATGGAAATTTT-GGGGATAGCTATCAATAGGT
M9442.S7    AGCAAGAATTGTGTTGGAAGCTAATGTGATGGAAATACT-GGGGATAGCTATCAACAGGT
E94393.S7   AGCAAGAATTGTGTTGGAAGCTAATGTGATGGAAATACT-GGGGATAGCTATCAACAGGT
BTV11.S7GB  AGCAAGAATTGTGTTGGAAGCCAATGTGATGGAAATTCT-AGGGATAGCTATCAACAGGT
A9464.S7    AGCAAGAATTGTGTTGGAAGCTAATGTGATGGAAATACT-GGGGATAGCTATCAACAGGT
BTV2.S7GB   AGCAAGAATTGTGTTGGAAGCTAATGTGATGGAAATACT-GGGGATAGCTATCAACAGGT
M94368.S7   AGCAAGAATTGTGTTGGAAGCTAATGTGATGGAAATACT-GGGGATAGCTATCAACAGGT
M94430.S7   AGCAAGAATTGTGTTGGAAGCTAATGTGATGGAAATACT-GGGGATAGCTATCAACAGGT
             **  *******  * *** * ***** ***** **  *  ** ** ** ***** ** *
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BTV17.S7GB   ACAATGGACTTACTCTACGAGGAGTGACGATGCGCCCAACCTCGTTAGCACAAAGAAATG
203853.S7    ATAATGGACTCACTTTACGAGGAGTGACGATGCGCCCGACCTCGTTAGCACAAAGAAATG
A945.S7      ACAATGGACTAACTCTACGTGGGGTAACGATGCGCCCGACCTCGTTAGCGCAAAGAAATG
BTV11.S7     ACAATGGGCTCACTCTACGAGGAGTGACGATGCGCCCGACCTCGTTGGCACAGAGAAATG
BTV13.S7GB   ATAATGGATTAACTCTACGTGGAGTTACGATGAGACCCACTTCATTAGCTCAAAGAAATG
203732.S7    ACAATGGACTTACTCTACGAGGAGTGACGATGCGCCCAACCTCGTTAGCACAAAGAAATG
201773.S7    ACAATGGACTTACTCTACGAGGAGTGACGATGCGCCCAACCTCGTTAGCACAAAGAAATG
201400.S7    ACAATGGACTTACTCTACGAGGAGTGACGATGCGCCCAACCTCGTTAGCACAAAGAAATG
203843.S7    ACAATGGACTTACTCTACGAGGAGTGACGATGCGCCCAACCTCGTTAGCACAAAGAAATG
270.S7       ACAATGGACTTACTCTACGAGGAGTGACGATGCGCCCAACCTCGTTAGCACAAAGAAATG
201767.S7    ATAATGGACTCACTTTACGAGGAGTGACGATGCGCCCGACCTCGTTAGCACAAAGAAATG
203794.S7    ACAATGGACTTACTCTACGAGGAGTGACGATGCGCCCAACCTCGTTAGCACAAAGAAATG
203733.S7    ACAATGGACTTACTCTACGAGGAGTGACGATGCGCCCAACCTCGTTAGCACAAAGAAATG
M94389.S7    ACAATGGACTAACTCTACGTGGGGTAACGATGCGCCCGACCTCGTTAGCGCAAAGAAATG
203734.S7    ACAATGGACTTACTCTACGAGGAGTGACGATGCGCCCAACCTCGTTAGCACAAAGAAATG
202894.S7    ACAATGGACTTACTCTACGAGGAGTGACGATGCGCCCAACCTCGTTAGCACAAAGAAATG
203826.S7    ACAATGGACTTACTCTACGAGGAGTGACGATGCGCCCAACCTCGTTAGCACAAAGAAATG
A94389.S7    ACAATGGACTAACTCTACGTGGGGTAACGATGCGCCCGACCTCGTTAGCGCAAAGAAATG
202020.S7    ATAATGGACTCACTTTACGAGGAGTGACGATGCGCCCGACCTCGTTAGCACAAAGAAATG
M9442.S7     ACAATGGACTAACTCTACGTGGGGTAACGATGCGCCCGACCTCGTTAGCGCAAAGAAATG
E94393.S7    ACAATGGACTAACTCTACGTGGGGTAACGATGCGCCCGACCTCGTTAGCGCAAAGAAATG
BTV11.S7GB   ACAATGGGCTCACTCTACGAGGAGTGACGATGCGCCCGACCTCGTTGGCACAGAGAAATG
A9464.S7     ACAATGGACTAACTCTACGTGGGGTAACGATGCGCCCGACCTCGTTAGCGCAAAGAAATG
BTV2.S7GB    ACAATGGACTAACTCTACGTGGGGTAACGATGCGCCCGACCTCGTTAGCGCAAAGAAATG
M94368.S7    ACAATGGACTAACTCTACGTGGGGTAACGATGCGCCCGACCTCGTTAGCGCAAAGAAATG
M94430.S7    ACAATGGACTAACTCTACGTGGGGTAACGATGCGCCCGACCTCGTTAGCGCAAAGAAATG
             * ******  * *** **** ** ** ****** * ** ** ** ** ** ** *******

BTV17.S7GB   AGATGTTTTTTATGTGTTTGGATATGATGCT-GTCTG--CTACTGGGATAAATGTCGGAC
203853.S7    AGATGTTCTTTATGTGTTTGGATATGATGCT-GTCTG--CTGCTGGGATAAATGTTGGAC
A945.S7      AGATGTTTTTTATGTGTCTTGATATGATGTT-GTCTTGCTTGCGGGAATAAATGTTGGAC
BTV11.S7     AGATGTTTTTTATGTGTTTGGATATGATGCTTGTCTG--CTGCTGGGATAAATGTTGGAC
BTV13.S7GB   AAATGTTTTTCATGTGTTTAGATATGATGGT-GTCAG--CAGCGGGAATAAATGTCGGAC
203732.S7    AGATGTTTTTTATGTGTTTGGATATGATGCT-GTCTG--CTACTGGGATAAATGTCGGAC
201773.S7    AGATGTTTTTTATGTGTTTGGATATGATGCT-GTCTG--CTACTGGGATAAATGTCGGAC
201400.S7    AGATGTTTTTTATGTGTTTGGATATGATGCT-GTCTG--CTACTGGGATAAATGTCGGAC
203843.S7    AGATGTTTTTTATGTGTTTGGATATGATGCT-GTCTG--CTACTGGGATAAATGTCGGAC
270.S7       AGATGTTTTTTATGTGTTTGGATATGATGCT-GTCTG--CTACTGGGATAAATGTCGGAC
201767.S7    AGATGTTCTTTATGTGTTTGGATATGATGCT-GTCTG--CTACTGGGATAAATGTTGGAC
203794.S7    AGATGTTTTTTATGTGTTTGGATATGATGCT-GTCTG--CTACTGGGATAAATGTCGGAC
203733.S7    AGATGTTTTTTATGTGTTTGGATATGATGCT-GTCTG--CTACTGGGATAAATGTCGGAC
M94389.S7    AGATGTTTTTTATGTGTCTTGATATGATGTT-GTCTG--CTGCGGGAATAAATGTTGGAC
203734.S7    AGATGTTCTTTATGTGTTTGGATATGATGCT-GTCTG--CTACTGGGATAAATGTCGGAC
202894.S7    AGATGTTTTTTATGTGTTTGGATATGATGCT-GTCTG--CTACTGGGATAAATGTCGGAC
203826.S7    AGATGTTTTTTATGTGTTTGGATATGATGCT-GTCTG--CTACTGGGATAAATGTCGGAC
A94389.S7    AGATGTTTTTTATGTGTCTTGATATGATGTT-GTCTG--CTGCGGGAATAAATGTTGGAC
202020.S7    AGATGTTCTTTATGTGTTTGGATATGATGCT-GTCTG--CTGCTGGGATAAATGTTGGAC
M9442.S7     AGATGTTTTTTATGTGTCTTGATATGATGTT-GTCTG--CTGCGGGAATAAATGTTGGAC
E94393.S7    AGATGTTTTTTATGTGTCTTGATATGATGTT-GTCTG--CTGCGGGAATAAATGTTGGAC
BTV11.S7GB   AGATGTTTTTTATGTGTTTGGATATGATGCT-GTCTG--CTGCTGGGATAAATGTTGGAC
A9464.S7     AGATGTTTTTTATGTGTCTTGATATGATGTT-GTCTG--CTGCGGGAATAAATGTTGGAC
BTV2.S7GB    AGATGTTTTTTATGTGTCTTGATATGATGTT-GTCTG--CTGCGGGAATAAATGTTGGAC
M94368.S7    AGATGTTTTTTATGTGTCTTGATATGATGTT-GTCTG--CTGCGGGAATAAATGTTGGAC
M94430.S7    AGATGTTTTTTATGTGTCTTGATATGATGTT-GTCTG--CTGCGGGAATAAATGTTGGAC
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BTV17.S7GB  CGATATCGCCAGACTATACTCAACATATGGCTACGATTGGTGTGCTAGCAACACCGGAAA
203853.S7   CGATATCGCCAGACTATACTCAACATATGGCTACGATTGGTGTACTAGCAACACCGGAAA
A945.S7     CGATATCGCCAGATTATACTCAACATATGGCTACAATTGGTGTACTAGCAACGCCGGAAA
BTV11.S7    CGATATCACCAGACTATACTCAACATATGGCTACAATTGGTGTGTTAGCGACACCGGAAA
BTV13.S7GB  CAATCTCACCCGATTATACGCAACATATGGCTACTATAGGTGTGCTGGCAACGCCTGAGA
203732.S7   CGATATCGCCAGACTATACTCAACATATGGCTACGATTGGTGTGCTAGCAACACCGGAAA
201773.S7   CGATATCGCCAGACTATACTCAACATATGGCTACGATTGGTGTGCTAGCAACACCGGAAA
201400.S7   CGATATCGCCAGACTATACTCAACATATGGCTACGATTGGTGTGCTAGCAACACCGGAAA
203843.S7   CGATATCGCCAGACTATACTCAACATATGGCTACGATTGGTGTGCTAGCAACACCGGAAA
270.S7      CGATATCGCCAGACTATACTCAACATATGGCTACGATTGGTGTGCTAGCAACACCGGAAA
201767.S7   CGATATCGCCAGACTATACTCAACATATGGCTACGATTGGTGTGCTAGCAACACCGGAAA
203794.S7   CGATATCGCCAGACTATACTCAACATATGGCTACGATTGGTGTGCTAGCAACACCGGAAA
203733.S7   CGATATCGCCAGACTATACTCAACATATGGCTACGATTGGTGTGCTAGCAACACCGGAAA
M94389.S7   CGATATCGCCAGATTATACTCAACATATGGCTACAATTGGTGTACTAGCAACGCCGGAAA
203734.S7   CGATATCGCCAGACTATACTCAACATATGGCTACGATTGGTGTGCTAGCAACACCGGAAA
202894.S7   CGATATCGCCAGACTATACTCAACATATGGCTACGATTGGTGTGCTAGCAACACCGGAAA
203826.S7   CGATATCGCCAGACTATACTCAACATATGGCTACGATTGGTGTGCTAGCAACACCGGAAA
A94389.S7   CGATATCGCCAGATTATACTCAACATATGGCTACAATTGGTGTACTAGCAACGCCGGAAA
202020.S7   CGATATCGCCAGACTATACTCAACATATGGCTACGATTGGTGTACTAGCAACACCGGAAA
M9442.S7    CGATATCGCCAGATTATACTCAACATATGGCTACAATTGGTGTACTAGCAACGCCGGAAA
E94393.S7   CGATATCGCCAGATTATACTCAACATATGGCTACAATTGGTGTACTAGCAACGCCGGAAA
BTV11.S7GB  CGATATCACCAGACTATACTCAACATATGGCTACAATTGGTGTGTTAGCGACACCGGAAA
A9464.S7    CGATATCGCCAGATTATACTCAACATATGGCTACAATTGGTGTACTAGCAACGCCGGAAA
BTV2.S7GB   CGATATCGCCAGATTATACTCAACATATGGCTACAATTGGTGTACTAGCAACGCCGGAAA
M94368.S7   CGATATCGCCAGATTATACTCAACATATGGCTACAATTGGTGTACTAGCAACGCCGGAAA
M94430.S7   CGATATCGCCAGATTATACTCAACATATGGCTACAATTGGTGTACTAGCAACGCCGGAAA
            * ** ** ** ** ***** ************** ** *****  * ** ** ** ** *

BTV17.S7GB  TACCTTTTACAACGGAAGCGGCGAATGAAATAGCTCGAGTGACTGGGGAGACTTCGACAT
203853.S7   TACCTTTTACAACGGAAGCGGCAAATGAAATAGCTCGAGTGACTGGGGAGACTTCGACAT
A945.S7     TCCCTTTTACAACAGAAGCGGCGAATGAAATAGCACGCGTGACTGGGGAGACTTCGACAT
BTV11.S7    TACCTTTTACAACGGAAGCGGCGAATGAAATAGCACGGGTGACTGGGGAGACTTCGACAT
BTV13.S7GB  TACCGTTCACAACTGAGGCAGCGAATGAGATTGCTCGTGTTACAGGAGAGACTTCAACAT
203732.S7   TACCTTTTACAACGGAAGCGGCGAATGAAATAGCTCGAGTGACTGGGGAGACTTCGACAT
201773.S7   TACCTTTTACAACGGAAGCGGCGAATGAAATAGCTCGAGTGACTGGGGAGACTTCGACAT
201400.S7   TACCTTTTACAACGGAAGCGGCGAATGAAATAGCTCGAGTGACTGGGGAGACTTCGACAT
203843.S7   TACCTTTTACAACGGAAGCGGCGAATGAAATAGCTCGAGTGACTGGGGAGACTTCGACAT
270.S7      TACCTTTTACAACGGAAGCGGCGAATGAAATAGCTCGAGTGACTGGGGAGACTTCGACAT
201767.S7   TACCTTTTACAACGGAAGCGGCGAATGAAATAGCTCGAGTGACTGGGGAGACTTCGACAT
203794.S7   TACCTTTTACAACGGAAGCGGCGAATGAAATAGCTCGAGTGACTGGGGAGACTTCGACAT
203733.S7   TACCTTTTACAACGGAAGCGGCGAATGAAATAGCTCGAGTGACTGGGGAGACTTCGACAT
M94389.S7   TCCCTTTTACAACAGAAGCGGCGAATGAAATAGCACGCGTGACTGGGGAGACTTCGACAT
203734.S7   TACCTTTTACAACGGAAGCGGCGAATGAAATAGCTCGAGTGACTGGGGAGACTTCGACAT
202894.S7   TACCTTTTACAACGGAAGCGGCGAATGAAATAGCTCGAGTGACTGGGGAGACTTCGACAT
203826.S7   TACCTTTTACAACGGAAGCGGCGAATGAAATAGCTCGAGTGACTGGGGAGACTTCGACAT
A94389.S7   TCCCTTTTACAACAGAAGCGGCGAATGAAATAGCACGCGTGACTGGGGAGACTTCGACAT
202020.S7   TACCTTTTACAACGGAAGCGGCAAATGAAATAGCTCGAGTGACTGGGGAGACTTCGACAT
M9442.S7    TCCCTTTTACAACAGAAGCGGCGAATGAAATAGCACGCGTGACTGGGGAGACTTCGACAT
E94393.S7   TCCCTTTTACAACAGAAGCGGCGAATGAAATAGCACGCGTGACTGGGGAGACTTCGACAT
BTV11.S7GB  TACCTTTTACAACGGAAGCGGCGAATGAAATAGCACGGGTGACTGGGGAGACTTCGACAT
A9464.S7    TCCCTTTTACAACAGAAGCGGCGAATGAAATAGCACGCGTGACTGGGGAGACTTCGACAT
BTV2.S7GB   TCCCTTTTACAACAGAAGCGGCGAATGAAATAGCACGCGTGACTGGGGAGACTTCGACAT
M94368.S7   TCCCTTTTACAACAGAAGCGGCGAATGAAATAGCACGCGTGACTGGGGAGACTTCGACAT
M94430.S7   TCCCTTTTACAACAGAAGCGGCGAATGAAATAGCACGCGTGACTGGGGAGACTTCGACAT
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BTV17.S7GB  GGGGGCCAGCGCGTCAGCCTTATGGTTTTTTCCTTGAAACTGAGGAAACCTTCCAACCTG
203853.S7   GGGGGCCAGCGCGTCAGCCTTATGGTTTCTTCCTTGAAACTGAGGAAACCTTCCAACCAG
A945.S7     GGGGGCCGGCGCGTCAGCCTTATGGTTTTTTCCTTGAAACTGAGGAGACTTTCCAACCTG
BTV11.S7    GGGGGCCAGCGCGTCAGCCTTATGGTTTTTTCCTTGAAACTGAAGAAACCTTTCAACCTG
BTV13.S7GB  GGGGACCGGCGCGTCAGCCTTACGGATTTTTCCTTGAAACTGAAGAGGTTTATCAACCTG
203732.S7   GGGGGCCAGCGCGTCAGCCTTATGGTTTTTTCCTTGAAACTGAGGAAACCTTCCAACCTG
201773.S7   GGGGGCCAGCGCGTCAGCCTTATGGTTTTTTCCTTGAAACTGAGGAAACCTTCCAACCTG
201400.S7   GGGGGCCAGCGCGTCAGCCTTATGGTTTTTTCCTTGAAACTGAGGAAACCTTCCAACCTG
203843.S7   GGGGGCCAGCGCGTCAGCCTTATGGTTTTTTCCTTGAAACTGAGGAAACCTTCCAACCTG
270.S7      GGGGGCCAGCGCGTCAGCCTTATGGTTTTTTCCTTGAAACTGAGGAAACCTTCCAACCTG
201767.S7   GGGGGCCAGCGCGTCAGCCTTATGGTTTTTTCCTTGAAACTGAGGAAACCTTCCAACCTG
203794.S7   GGGGGCCAGCGCGTCAGCCTTATGGTTTTTTCCTTGAAACTGAGGAAACCTTCCAACCTG
203733.S7   GGGGGCCAGCGCGTCAGCCTTATGGTTTTTTCCTTGAAACTGAGGAAACCTTCCAACCTG
M94389.S7   GGGGGCCGGCGCGTCAGCCTTATGGTTTTTTCCTTGAAACTGAGGAGACTTTCCAACCTG
203734.S7   GGGGGCCAGCGCGTCAGCCTTATGGTTTTTTCCTTGAAACTGAGGAAACCTTCCAACCTG
202894.S7   GGGGGCCAGCGCGTCAGCCTTATGGGTTTTTCCTTGAAACTGAGGAAACCTTCCAACCTG
203826.S7   GGGGGCCAGCGCGTCAGCCTTATGGTTTTTTCCTTGAAACTGAGGAAACCTTCCAACCTG
A94389.S7   GGGGGCCGGCGCGTCAGCCTTATGGTTTTTTCCTTGAAACTGAGGAGACTTTCCAACCTG
202020.S7   GGGGGCCAGCGCGTCAGCCTTATGGTTTCTTCCTTGAAACTGAGGAAACCTTCCAACCAG
M9442.S7    GGGGGCCGGCGCGTCAGCCTTATGGTTTTTTCCTTGAAACTGAGGAGACTTTCCAACCTG
E94393.S7   GGGGGCCGGCGCGTCAGCCTTATGGTTTTTTCCTTGAAACTGAGGAGACTTTCCAACCTG
BTV11.S7GB  GGGGGCCAGCGCGTCAGCCTTATGGTTTTTTCCTTGAAACTGAAGAAACCTTTCAACCTG
A9464.S7    GGGGGCCGGCGCGTCAGCCTTATGGTTTTTTCCTTGAAACTGAGGAGACTTTCCAACCTG
BTV2.S7GB   GGGGGCCGGCGCGTCAGCCTTATGGTTTTTTCCTTGAAACTGAGGAGACTTTCCAACCTG
M94368.S7   GGGGGCCGGCGCGTCAGCCTTATGGTTTTTTCCTTGAAACTGAGGAGACTTTCCAACCTG
M94430.S7   GGGGGCCGGCGCGTCAGCCTTATGGTTTTTTCCTTGAAACTGAGGAGACTTTCCAACCTG
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BTV17.S7GB  GGAGATGGTTCATGCGCGCCGCTCAAGCAGTAACTGCAGTAGTGTGCGGTCCGGATATGA
203853.S7   GGAGGTGGTTCATGCGCGCCGCTCAAGCAGTAACTGCAGTAGTGTGCGGTCCGGATATGA
A945.S7     GAAGATGGTTCATGCGCGCCGCTCAAGCGGTGACTGCAGTGGTGTGCGGTCCGGATATGA
BTV11.S7    GGAGATGGTTCATGCGTGCCGCTCAAGCGGTAACTGCAGTAGTGTGCGGTCCGGATATGA
BTV13.S7GB  GAAGATGGTTCATGCGAGCTGCTCAAGTTGTTACACCAGTGGTTTGTGGACCAAATATGG
203732.S7   GGAGATGGTTCATGCGCGCCGCTCAAGCAGTAACTGCAGTAGTGTGCGGTCCGGATATGA
201773.S7   GGAGATGGTTCATGCGCGCCGCTCAAGCAGTAACTGCAGTAGTGTGCGGTCCGGATATGA
201400.S7   GGAGATGGTTCATGCGCGCCGCTCAAGCAGTAACTGCAGTAGTGTGCGGTCCGGATATGA
203843.S7   GGAGATGGTTCATGCGCGCCGCTCAAGCAGTAACTGCAGTAGTGTGCGGTCCGGATATGA
270.S7      GGAGATGGTTCATGCGCGCCGCTCAAGCAGTAACTGCAGTAGTGTGCGGTCCGGATATGA
201767.S7   GGAGATGGTTCATGCGCGCCGCTCAAGCAGTAACTGCAGTAGTGTGCGGTCCGGATATGA
203794.S7   GGAGATGGTTCATGCGCGCCGCTCAAGCAGTAACTGCAGTAGTGTGCGGTCCGGATATGA
203733.S7   GGAGATGGTTCATGCGCGCCGCTCAAGCAGTAACTGCAGTAGTGTGCGGTCCGGATATGA
M94389.S7   GAAGATGGTTCATGCGCGCCGCTCAAGCGGTGACTGCAGTGGTGTGCGGTCCGGATATGA
203734.S7   GGAGATGGTTCATGCGCGCCGCTCAAGCAGTAACTGCAGTAGTGTGCGGTCCGGATATGA
202894.S7   GGAGATGGTTCATGCGCGCCGCTCAAGCAGTAACTGCAGTAGTGTGCGGTCCGGATATGA
203826.S7   GGAGATGGTTCATGCGCGCCGCTCAAGCAGTAACTGCAGTAGTGTGCGGTCCGGATATGA
A94389.S7   GAAGATGGTTCATGCGCGCCGCTCAAGCGGTGACTGCAGTGGTGTGCGGTCCGGATATGA
202020.S7   GGAGGTGGTTCATGCGCGCCGCTCAAGCAGTAACTGCAGTAGTGTGCGGTCCGGATATGA
M9442.S7    GAAGATGGTTCATGCGCGCCGCTCAAGCGGTGACTGCAGTGGTGTGCGGTCCGGATATGA
E94393.S7   GAAGATGGTTCATGCGCGCCGCTCAAGCGGTGACTGCAGTGGTGTGCGGTCCGGATATGA
BTV11.S7GB  GGAGATGGTTCATGCGTGCCGCTCAAGCGGTAACTGCAGTAGTGTGCGGTCCGGATATGA
A9464.S7    GAAGATGGTTCATGCGCGCCGCTCAAGCGGTGACTGCAGTGGTGTGCGGTCCGGATATGA
BTV2.S7GB   GAAGATGGTTCATGCGCGCCGCTCAAGCGGTAGCTGCAGTGGTGTGCGGTCCGGATATGA
M94368.S7   GAAGATGGTTCATGCGCGCCGCTCAAGCGGTGACTGCAGTGGTGTGCGGTCCGGATATGA
M94430.S7   GAAGATGGTTCATGCGCGCCGCTCAAGCGGTGACTGCAGTGGTGTGCGGTCCGGATATGA
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BTV17.S7GB  TTCAGGTGTCACTCAATGCTGGAGCGAGAGGGGATGTACAACAGATATTTCAGGGTCGTA
203853.S7   TTCAAGTGTCACTTAATGCTGGAGCGAGAGGAGATGTACAACAGATATTTCAGGGTCGTA
A945.S7     TTCAGGTGTCACTAAATGCTGGAGCGAGAGGGGATGTACAACAGATATTTCAGGGTCGTA
BTV11.S7    TCCAGGTGTCACTAAATGCTGGAGCGAGAGGGGATGTGCAACAAATATTTCAGGGTCGTA
BTV13.S7GB  TTCAGGTTTCACTTAATGCTGGAGCGATAGGTGATGTGCAGCAGATCTTTCAAGGTCGTA
203732.S7   TTCAGGTGTCACTCAATGCTGGAGCGAGAGGGGATGTACAACAGATATTTCAGGGTCGTA
201773.S7   TTCAGGTGTCACTCAATGCTGGAGCGAGAGGGGATGTACAACAGATATTTCAGGGTCGTA
201400.S7   TTCAGGTGTCACTCAATGCTGGAGCGAGAGGGGATGTACAACAGATATTTCAGGGTCGTA
203843.S7   TTCAGGTGTCACTCAATGCTGGAGCGAGAGGGGATGTACAACAGATATTTCAGGGTCGTA
270.S7      TTCAGGTGTCACTCAATGCTGGAGCGAGAGGGGATGTACAACAGATATTTCAGGGTCGTA
201767.S7   TTCAGGTGTCACTCAATGCTGGAGCGAGAGGGGATGTACAACAGATATTTCAGGGTCGTA
203794.S7   TTCAGGTGTCACTCAATGCTGGAGCGAGAGGGGATGTACAACAGATATTTCAGGGTCGTA
203733.S7   TTCAGGTGTCACTCAATGCTGGAGCGAGAGGGGATGTACAACAGATATTTCAGGGTCGTA
M94389.S7   TTCAGGTGTCACTAAATGCTGGAGCGAGAGGGGATGTACAACAGATATTTCAGGGTCGTA
203734.S7   TTCAGGTGTCACTCAATGCTGGAGCGAGAGGGGATGTACAACAGATATTTCAGGGTCGTA
202894.S7   TTCAGGTGTCACTCAATGCTGGAGCGAGAGGGGATGTACAACAGATATTTCAGGGTCGTA
203826.S7   TTCAGGTGTCACTCAATGCTGGAGCGAGAGGGGATGTACAACAGATATTTCAGGGTCGTA
A94389.S7   TTCAGGTGTCACTAAATGCTGGAGCGAGAGGGGATGTACAACAGATATTTCAGGGTCGTA
202020.S7   TTCAAGTGTCACTTAATGCTGGAGCGAGAGGAGATGTACAACAGATATTTCAGGGTCGTA
M9442.S7    TTCAGGTGTCACTAAATGCTGGAGCGAGAGGGGATGTACAACAGATATTTCAGGGTCGTA
E94393.S7   TTCAGGTGTCACTAAATGCTGGAGCGAGAGGGGATGTACAACAGATATTTCAGGGTCGTA
BTV11.S7GB  TCCAGGTGTCACTAAATGCTGGAGCGAGAGGGGATGTGCAACAAATATTTCAGGGTCGTA
A9464.S7    TTCAGGTGTCACTAAATGCTGGAGCGAGAGGGGATGTACAACAGATATTTCAGGGTCGTA
BTV2.S7GB   TTCAGGTGTCACTAAATGCTGGAGCGAGAGGGGATGTACAACAGATATTTCAGGGTCGTA
M94368.S7   TTCAGGTGTCACTAAATGCTGGAGCGAGAGGGGATGTACAACAGATATTTCAGGGTCGTA
M94430.S7   TTCAGGTGTCACTAAATGCTGGAGCGAGAGGGGATGTACAACAGATATTTCAGGGTCGTA
            * ** ** ***** ************* *** ***** ** ** ** ***** *******

BTV17.S7GB  ATGATCCTATGATGATATATTTAGTGTGGAGGAGAATCGAAAACTTTGCGATGGCGCAAG
203853.S7   ATGATCCCATGATGATATATTTAGTGTGGAGGAGAATCGAAAACTTTGCGATGGCGCAAG
A945.S7     ATGATCCCATGATGATATATTTGGTATGGAGAAGAATCGAAAACTTTGCGATGGCGCAAG
BTV11.S7    ATGATCCTATGATGATATATTTAGTGTGGAGGAGAATCGAAAATTTCGCTATGGCGCAAG
BTV13.S7GB  ATGATCCCATGATGATTTATTTAGTTTGGAGACGGATCGAGAACTTCTCCATGCCGCAGG
203732.S7   ATGATCCTATGATGATATATTTAGTGTGGAGGAGAATCGAAAACTTTGCGATGGCGCAAG
201773.S7   ATGATCCTATGATGATATATTTAGTGTGGAGGAGAATCGAAAACTTTGCGATGGCGCAAG
201400.S7   ATGATCCTATGATGATATATTTAGTGTGGAGGAGAATCGAAAACTTTGCGATGGCGCAAG
203843.S7   ATGATCCTATGATGATATATTTAGTGTGGAGGAGAATCGAAAACTTTGCGATGGCGCAAG
270.S7      ATGATCCTATGATGATATATTTAGTGTGGAGGAGAATCGAAAACTTCGCGATGGCGCAAG
201767.S7   ATGATCCTATGATGATATATTTAGTGTGGAGGAGAATCGAAAACTTTGCGATGGCGCAAG
203794.S7   ATGATCCTATGATGATATATTTAGTGTGGAGGAGAATCGAAAACTTTGCGATGGCGCAAG
203733.S7   ATGATCCTATGATGATATATTTAGTGTGGAGGAGAATCGAAAACTTTGCGATGGCGCAAG
M94389.S7   ATGATCCCATGATGATATATTTGGTATGGAGAAGAATCGAAAACTTTGCGATGGCGCAAG
203734.S7   ATGATCCTATGATGATATATTTAGTGTGGAGGAGAATCGAAAACTTTGCGATGGCGCAAG
202894.S7   ATGATCCTATGATGATATATTTAGTGTGGAGGAGAATCGAAAACTTTGCGATGGCGCAAG
203826.S7   ATGATCCCATGATGATATATTTAGTGTGGAGGAGAATCGAAAACTTTGCGATGGCGCAAG
A94389.S7   ATGATCCCATGATGATATATTTGGTATGGAGAAGAATCGAAAACTTTGCGATGGCGCAAG
202020.S7   ATGATCCCATGATGATATATTTAGTGTGGAGGAGAATCGAAAACTTTGCGATGGCGCAAG
M9442.S7    ATGATCCCATGATGATATATTTGGTATGGAGAAGAATCGAAAACTTTGCGATGGCGCAAG
E94393.S7   ATGATCCCATGATGATATATTTGGTATGGAGAAGAATCGAAAACTTTGCGATGGCGCAAG
BTV11.S7GB  ATGATCCTATGATGATATATTTAGTGTGGAGGAGAATCGAAAATTTCGCTATGGCGCAAG
A9464.S7    ATGATCCCATGATGATATATTTGGTATGGAGAAGAATCGAAAACTTTGCGATGGCGCAAG
BTV2.S7GB   ATGATCCCATGATGATATATTTGGTATGGAGAAGAATCGAAAACTTTGCGATGGCGCAAG
M94368.S7   ATGATCCCATGATGATATATTTGGTATGGAGAAGAATCGAAAACTTTGCGATGGCGCAAG
M94430.S7   ATGATCCCATGATGATATATTTGGTATGGAGAAGAATCGAAAACTTTGCGATGGCGCAAG
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BTV17.S7GB  GTAATTCACAACAAACTCAAGCGGG
203853.S7   GTAATTCACAGCAAACTCAAGCGGG
A945.S7     GTAATTCACAGCAAACTCAAGCGGG
BTV11.S7    GTAATTCACAGCAAACTCAAGCGGG
BTV13.S7GB  GTAATTCACAGCGCACGTTAGCTGG
203732.S7   GTAATTCACAGCAAACTCAAGCGGG
201773.S7   GTAATTCACAGCAAACTCAAGCGGG
201400.S7   GTAATTCACAGCAAACTCAAGCGGG
203843.S7   GTAATTCACAGCAAACTCAAGCGGG
270.S7      GTAATTCACAGCAAACTCAAGCGGG
201767.S7   GTAATTCACAGCAAACTCAAGCGGG
203794.S7   GTAATTCACAGCAAACTCAAGCGGG
203733.S7   GTAATTCACAGCAAACTCAAGCGGG
M94389.S7   GTAATTCACAGCAAACTCAAGCGGG
203734.S7   GTAATTCACAGCAAACTCAAGCGGG
202894.S7   GTAATTCACAGCAAACTCAAGCGGG
203826.S7   GTAATTCACAGCAAACTCAAGCGGG
A94389.S7   GTAATTCACAGCAAACTCAAGCGGG
202020.S7   GTAATTCACAGCAAACTCAAGCGGG
M9442.S7    GTAATTCACAGCAAACTCAAGCGGG
E94393.S7   GTAATTCACAGCAAACTCAAGCGGG
BTV11.S7GB  GTAATTCACAGCAAACTCAAGCGGG
A9464.S7    GTAATTCACAGCAAACTCAAGCGGG
BTV2.S7GB   GTAATTCACAGCAAACTCAAGCAGG
M94368.S7   GTAATTCACAGCAAACTCAAGCGGG
M94430.S7   GTAATTCACAGCAAACTCAAGCGGG
            ********** *  **   *** **
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APPENDIX E – Distance analyses for S10

Distance: Number of different nucleotides.
No. of Codons in Subset: 275 of 275
Codon Position(s) Used: 1 2 3
Gap Sites and Missing Information Data: All such sites were removed from the subset data

OTU Labels

1.. BTV17.S10GB
2.. BTV1AUS.S10GB
3.. 202894.S10
4.. 201400.S10
5.. 203733.S10
6.. 203843.S10
7.. 203853.S10
8.. 201773.S10
9.. 202020.S10
10.. 203732.S10
11.. 203734.S10
12.. 203826.S10
13.. BTV10.S10GB
14.. BTV13.S10GB
15.. 201767.S10
16.. EHDV1.S10GB
17.. BTV11-2.S10GB
18.. BTV2-3.S10GB
19.. M94389.S10
20.. EHDV2.S10GB
21.. BTV17-2.S10GB
22.. M9442.S10
23.. BTV11.S10GB
24.. A94389.S10
25.. BTV13-2.S10GB
26.. M94368.S10
27.. 270.S10
28.. E94393.S10
29.. A9464.S10
30.. BTV11.S10
31.. M94430.S10
32.. A945.S10

Distances in the upper-right matrix
'*' indicates an invalid distance value

OTUs	1	2	3	4	5	6	7
1		133.0000	0.0000	0.0000	0.0000	0.0000	0.0000
2			133.0000	133.0000	133.0000	133.0000	133.0000
3				0.0000	0.0000	0.0000	0.0000
4					0.0000	0.0000	0.0000
5						0.0000	0.0000
6							0.0000
7							

OTUs	8	9	10	11	12	13	14
1	3.0000	1.0000	1.0000	1.0000	1.0000	4.0000	3.0000
2	134.0000	132.0000	132.0000	132.0000	132.0000	133.0000	132.0000
3	3.0000	1.0000	1.0000	1.0000	1.0000	4.0000	3.0000
4	3.0000	1.0000	1.0000	1.0000	1.0000	4.0000	3.0000
5	3.0000	1.0000	1.0000	1.0000	1.0000	4.0000	3.0000
6	3.0000	1.0000	1.0000	1.0000	1.0000	4.0000	3.0000

7	3.0000	1.0000	1.0000	1.0000	1.0000	4.0000	3.0000
8		2.0000	2.0000	2.0000	2.0000	7.0000	6.0000
9			0.0000	0.0000	0.0000	5.0000	4.0000
10				0.0000	0.0000	5.0000	4.0000
11					0.0000	5.0000	4.0000
12						5.0000	4.0000
13							5.0000
14							

OTUs	15	16	17	18	19	20	21
1	1.0000	292.0000	141.0000	50.0000	12.0000	295.0000	2.0000
2	132.0000	309.0000	143.0000	130.0000	128.0000	313.0000	135.0000
3	1.0000	292.0000	141.0000	50.0000	12.0000	295.0000	2.0000
4	1.0000	292.0000	141.0000	50.0000	12.0000	295.0000	2.0000
5	1.0000	292.0000	141.0000	50.0000	12.0000	295.0000	2.0000
6	1.0000	292.0000	141.0000	50.0000	12.0000	295.0000	2.0000
7	1.0000	292.0000	141.0000	50.0000	12.0000	295.0000	2.0000
8	2.0000	293.0000	144.0000	51.0000	13.0000	296.0000	5.0000
9	0.0000	291.0000	142.0000	49.0000	11.0000	294.0000	3.0000
10	0.0000	291.0000	142.0000	49.0000	11.0000	294.0000	3.0000
11	0.0000	291.0000	142.0000	49.0000	11.0000	294.0000	3.0000
12	0.0000	291.0000	142.0000	49.0000	11.0000	294.0000	3.0000
13	5.0000	294.0000	140.0000	50.0000	12.0000	298.0000	6.0000
14	4.0000	293.0000	140.0000	51.0000	13.0000	296.0000	5.0000
15		291.0000	142.0000	49.0000	11.0000	294.0000	3.0000
16			310.0000	301.0000	294.0000	27.0000	292.0000
17				141.0000	148.0000	317.0000	141.0000
18					54.0000	306.0000	52.0000
19						297.0000	14.0000
20							295.0000
21							

OTUs	22	23	24	25	26	27	28
1	13.0000	10.0000	12.0000	4.0000	11.0000	11.0000	11.0000
2	129.0000	128.0000	128.0000	133.0000	127.0000	127.0000	127.0000
3	13.0000	10.0000	12.0000	4.0000	11.0000	11.0000	11.0000
4	13.0000	10.0000	12.0000	4.0000	11.0000	11.0000	11.0000
5	13.0000	10.0000	12.0000	4.0000	11.0000	11.0000	11.0000
6	13.0000	10.0000	12.0000	4.0000	11.0000	11.0000	11.0000
7	13.0000	10.0000	12.0000	4.0000	11.0000	11.0000	11.0000
8	14.0000	13.0000	13.0000	7.0000	12.0000	12.0000	12.0000
9	12.0000	11.0000	11.0000	5.0000	10.0000	10.0000	10.0000
10	12.0000	11.0000	11.0000	5.0000	10.0000	10.0000	10.0000
11	12.0000	11.0000	11.0000	5.0000	10.0000	10.0000	10.0000
12	12.0000	11.0000	11.0000	5.0000	10.0000	10.0000	10.0000
13	13.0000	10.0000	12.0000	6.0000	11.0000	11.0000	11.0000
14	14.0000	11.0000	13.0000	1.0000	12.0000	12.0000	12.0000
15	12.0000	11.0000	11.0000	5.0000	10.0000	10.0000	10.0000
16	295.0000	294.0000	294.0000	293.0000	293.0000	293.0000	293.0000
17	149.0000	146.0000	148.0000	141.0000	147.0000	147.0000	147.0000
18	55.0000	54.0000	54.0000	52.0000	53.0000	53.0000	53.0000
19	1.0000	2.0000	0.0000	14.0000	1.0000	1.0000	1.0000
20	298.0000	297.0000	297.0000	296.0000	296.0000	296.0000	296.0000
21	15.0000	12.0000	14.0000	6.0000	13.0000	13.0000	13.0000
22		3.0000	1.0000	15.0000	2.0000	2.0000	2.0000
23			2.0000	12.0000	1.0000	1.0000	1.0000
24				14.0000	1.0000	1.0000	1.0000
25					13.0000	13.0000	13.0000
26						0.0000	0.0000
27							0.0000
28							

OTUs	29	30	31	32
1	12.0000	11.0000	12.0000	12.0000
2	128.0000	127.0000	128.0000	128.0000
3	12.0000	11.0000	12.0000	12.0000
4	12.0000	11.0000	12.0000	12.0000
5	12.0000	11.0000	12.0000	12.0000
6	12.0000	11.0000	12.0000	12.0000
7	12.0000	11.0000	12.0000	12.0000
8	13.0000	12.0000	13.0000	13.0000
9	11.0000	10.0000	11.0000	11.0000
10	11.0000	10.0000	11.0000	11.0000
11	11.0000	10.0000	11.0000	11.0000
12	11.0000	10.0000	11.0000	11.0000
13	12.0000	11.0000	12.0000	12.0000
14	13.0000	12.0000	13.0000	13.0000
15	11.0000	10.0000	11.0000	11.0000
16	294.0000	293.0000	294.0000	294.0000
17	148.0000	147.0000	148.0000	148.0000
18	54.0000	53.0000	54.0000	54.0000
19	0.0000	1.0000	0.0000	0.0000
20	297.0000	296.0000	297.0000	297.0000
21	14.0000	13.0000	14.0000	14.0000
22	1.0000	2.0000	1.0000	1.0000
23	2.0000	1.0000	2.0000	2.0000
24	0.0000	1.0000	0.0000	0.0000
25	14.0000	13.0000	14.0000	14.0000
26	1.0000	0.0000	1.0000	1.0000
27	1.0000	0.0000	1.0000	1.0000
28	1.0000	0.0000	1.0000	1.0000
29		1.0000	0.0000	0.0000
30			1.0000	1.0000
31				0.0000
32				

Distance: Number of different nucleotides (transversions only).
No. of Codons in Subset: 275 of 275
Codon Position(s) Used: 1 2 3
Gap Sites and Missing Information Data: All such sites were removed from the subset data

OTU Labels

1.. BTV17.S10GB
2.. BTV1AUS.S10GB
3.. 202894.S10
4.. 201400.S10
5.. 203733.S10
6.. 203843.S10
7.. 203853.S10
8.. 201773.S10
9.. 202020.S10
10.. 203732.S10
11.. 203734.S10
12.. 203826.S10
13.. BTV10.S10GB
14.. BTV13.S10GB
15.. 201767.S10
16.. EHDV1.S10GB
17.. BTV11-2.S10GB
18.. BTV2-3.S10GB
19.. M94389.S10
20.. EHDV2.S10GB
21.. BTV17-2.S10GB
22.. M9442.S10

23.. BTV11.S10GB
24.. A94389.S10
25.. BTV13-2.S10GB
26.. M94368.S10
27.. 270.S10
28.. E94393.S10
29.. A9464.S10
30.. BTV11.S10
31.. M94430.S10
32.. A945.S10

Distances in the upper-right matrix
'*' indicates an invalid distance value

OTUs	1	2	3	4	5	6	7
1		37.0000	0.0000	0.0000	0.0000	0.0000	0.0000
2			37.0000	37.0000	37.0000	37.0000	37.0000
3				0.0000	0.0000	0.0000	0.0000
4					0.0000	0.0000	0.0000
5						0.0000	0.0000
6							0.0000
7							

OTUs	8	9	10	11	12	13	14
1	2.0000	0.0000	0.0000	0.0000	0.0000	1.0000	0.0000
2	39.0000	37.0000	37.0000	37.0000	37.0000	36.0000	37.0000
3	2.0000	0.0000	0.0000	0.0000	0.0000	1.0000	0.0000
4	2.0000	0.0000	0.0000	0.0000	0.0000	1.0000	0.0000
5	2.0000	0.0000	0.0000	0.0000	0.0000	1.0000	0.0000
6	2.0000	0.0000	0.0000	0.0000	0.0000	1.0000	0.0000
7	2.0000	0.0000	0.0000	0.0000	0.0000	1.0000	0.0000
8		2.0000	2.0000	2.0000	2.0000	3.0000	2.0000
9			0.0000	0.0000	0.0000	1.0000	0.0000
10				0.0000	0.0000	1.0000	0.0000
11					0.0000	1.0000	0.0000
12						1.0000	0.0000
13							1.0000
14							

OTUs	15	16	17	18	19	20	21
1	0.0000	155.0000	44.0000	5.0000	1.0000	156.0000	0.0000
2	37.0000	152.0000	55.0000	38.0000	36.0000	153.0000	37.0000
3	0.0000	155.0000	44.0000	5.0000	1.0000	156.0000	0.0000
4	0.0000	155.0000	44.0000	5.0000	1.0000	156.0000	0.0000
5	0.0000	155.0000	44.0000	5.0000	1.0000	156.0000	0.0000
6	0.0000	155.0000	44.0000	5.0000	1.0000	156.0000	0.0000
7	0.0000	155.0000	44.0000	5.0000	1.0000	156.0000	0.0000
8	2.0000	157.0000	46.0000	7.0000	3.0000	158.0000	2.0000
9	0.0000	155.0000	44.0000	5.0000	1.0000	156.0000	0.0000
10	0.0000	155.0000	44.0000	5.0000	1.0000	156.0000	0.0000
11	0.0000	155.0000	44.0000	5.0000	1.0000	156.0000	0.0000
12	0.0000	155.0000	44.0000	5.0000	1.0000	156.0000	0.0000
13	1.0000	156.0000	43.0000	6.0000	2.0000	157.0000	1.0000
14	0.0000	155.0000	44.0000	5.0000	1.0000	156.0000	0.0000
15		155.0000	44.0000	5.0000	1.0000	156.0000	0.0000
16			153.0000	154.0000	154.0000	3.0000	155.0000
17				45.0000	45.0000	154.0000	44.0000
18					4.0000	155.0000	5.0000
19						155.0000	1.0000
20							156.0000
21							

OTUs	22	23	24	25	26	27	28
1	1.0000	1.0000	1.0000	0.0000	1.0000	1.0000	1.0000
2	36.0000	36.0000	36.0000	37.0000	36.0000	36.0000	36.0000
3	1.0000	1.0000	1.0000	0.0000	1.0000	1.0000	1.0000
4	1.0000	1.0000	1.0000	0.0000	1.0000	1.0000	1.0000
5	1.0000	1.0000	1.0000	0.0000	1.0000	1.0000	1.0000
6	1.0000	1.0000	1.0000	0.0000	1.0000	1.0000	1.0000
7	1.0000	1.0000	1.0000	0.0000	1.0000	1.0000	1.0000
8	3.0000	3.0000	3.0000	2.0000	3.0000	3.0000	3.0000
9	1.0000	1.0000	1.0000	0.0000	1.0000	1.0000	1.0000
10	1.0000	1.0000	1.0000	0.0000	1.0000	1.0000	1.0000
11	1.0000	1.0000	1.0000	0.0000	1.0000	1.0000	1.0000
12	1.0000	1.0000	1.0000	0.0000	1.0000	1.0000	1.0000
13	2.0000	2.0000	2.0000	1.0000	2.0000	2.0000	2.0000
14	1.0000	1.0000	1.0000	0.0000	1.0000	1.0000	1.0000
15	1.0000	1.0000	1.0000	0.0000	1.0000	1.0000	1.0000
16	154.0000	154.0000	154.0000	155.0000	154.0000	154.0000	154.0000
17	45.0000	45.0000	45.0000	44.0000	45.0000	45.0000	45.0000
18	4.0000	4.0000	4.0000	5.0000	4.0000	4.0000	4.0000
19	0.0000	0.0000	0.0000	1.0000	0.0000	0.0000	0.0000
20	155.0000	155.0000	155.0000	156.0000	155.0000	155.0000	155.0000
21	1.0000	1.0000	1.0000	0.0000	1.0000	1.0000	1.0000
22		0.0000	0.0000	1.0000	0.0000	0.0000	0.0000
23			0.0000	1.0000	0.0000	0.0000	0.0000
24				1.0000	0.0000	0.0000	0.0000
25					1.0000	1.0000	1.0000
26						0.0000	0.0000
27							0.0000
28							

OTUs	29	30	31	32
1	1.0000	1.0000	1.0000	1.0000
2	36.0000	36.0000	36.0000	36.0000
3	1.0000	1.0000	1.0000	1.0000
4	1.0000	1.0000	1.0000	1.0000
5	1.0000	1.0000	1.0000	1.0000
6	1.0000	1.0000	1.0000	1.0000
7	1.0000	1.0000	1.0000	1.0000
8	3.0000	3.0000	3.0000	3.0000
9	1.0000	1.0000	1.0000	1.0000
10	1.0000	1.0000	1.0000	1.0000
11	1.0000	1.0000	1.0000	1.0000
12	1.0000	1.0000	1.0000	1.0000
13	2.0000	2.0000	2.0000	2.0000
14	1.0000	1.0000	1.0000	1.0000
15	1.0000	1.0000	1.0000	1.0000
16	154.0000	154.0000	154.0000	154.0000
17	45.0000	45.0000	45.0000	45.0000
18	4.0000	4.0000	4.0000	4.0000
19	0.0000	0.0000	0.0000	0.0000
20	155.0000	155.0000	155.0000	155.0000
21	1.0000	1.0000	1.0000	1.0000
22	0.0000	0.0000	0.0000	0.0000
23	0.0000	0.0000	0.0000	0.0000
24	0.0000	0.0000	0.0000	0.0000
25	1.0000	1.0000	1.0000	1.0000
26	0.0000	0.0000	0.0000	0.0000
27	0.0000	0.0000	0.0000	0.0000
28	0.0000	0.0000	0.0000	0.0000
29		0.0000	0.0000	0.0000
30			0.0000	0.0000
31				0.0000
32				

Distance: Number of different nucleotides (transitions only).
No. of Codons in Subset: 275 of 275
Codon Position(s) Used: 1 2 3
Gap Sites and Missing Information Data: All such sites were removed from the subset data

OTU Labels

1.. BTV17.S10GB
2.. BTV1AUS.S10GB
3.. 202894.S10
4.. 201400.S10
5.. 203733.S10
6.. 203843.S10
7.. 203853.S10
8.. 201773.S10
9.. 202020.S10
10.. 203732.S10
11.. 203734.S10
12.. 203826.S10
13.. BTV10.S10GB
14.. BTV13.S10GB
15.. 201767.S10
16.. EHDV1.S10GB
17.. BTV11-2.S10GB
18.. BTV2-3.S10GB
19.. M94389.S10
20.. EHDV2.S10GB
21.. BTV17-2.S10GB
22.. M9442.S10
23.. BTV11.S10GB
24.. A94389.S10
25.. BTV13-2.S10GB
26.. M94368.S10
27.. 270.S10
28.. E94393.S10
29.. A9464.S10
30.. BTV11.S10
31.. M94430.S10
32.. A945.S10

Distances in the upper-right matrix
'*' indicates an invalid distance value

OTUs	1	2	3	4	5	6	7
1		96.0000	0.0000	0.0000	0.0000	0.0000	0.0000
2			96.0000	96.0000	96.0000	96.0000	96.0000
3				0.0000	0.0000	0.0000	0.0000
4					0.0000	0.0000	0.0000
5						0.0000	0.0000
6							0.0000
7							

OTUs	8	9	10	11	12	13	14
1	1.0000	1.0000	1.0000	1.0000	1.0000	3.0000	3.0000
2	95.0000	95.0000	95.0000	95.0000	95.0000	97.0000	95.0000
3	1.0000	1.0000	1.0000	1.0000	1.0000	3.0000	3.0000
4	1.0000	1.0000	1.0000	1.0000	1.0000	3.0000	3.0000
5	1.0000	1.0000	1.0000	1.0000	1.0000	3.0000	3.0000
6	1.0000	1.0000	1.0000	1.0000	1.0000	3.0000	3.0000
7	1.0000	1.0000	1.0000	1.0000	1.0000	3.0000	3.0000
8		0.0000	0.0000	0.0000	0.0000	4.0000	4.0000
9			0.0000	0.0000	0.0000	4.0000	4.0000

10				0.0000	0.0000	4.0000	4.0000
11					0.0000	4.0000	4.0000
12						4.0000	4.0000
13							4.0000
14							

OTUs	15	16	17	18	19	20	21
1	1.0000	137.0000	97.0000	45.0000	11.0000	139.0000	2.0000
2	95.0000	157.0000	88.0000	92.0000	92.0000	160.0000	98.0000
3	1.0000	137.0000	97.0000	45.0000	11.0000	139.0000	2.0000
4	1.0000	137.0000	97.0000	45.0000	11.0000	139.0000	2.0000
5	1.0000	137.0000	97.0000	45.0000	11.0000	139.0000	2.0000
6	1.0000	137.0000	97.0000	45.0000	11.0000	139.0000	2.0000
7	1.0000	137.0000	97.0000	45.0000	11.0000	139.0000	2.0000
8	0.0000	136.0000	98.0000	44.0000	10.0000	138.0000	3.0000
9	0.0000	136.0000	98.0000	44.0000	10.0000	138.0000	3.0000
10	0.0000	136.0000	98.0000	44.0000	10.0000	138.0000	3.0000
11	0.0000	136.0000	98.0000	44.0000	10.0000	138.0000	3.0000
12	0.0000	136.0000	98.0000	44.0000	10.0000	138.0000	3.0000
13	4.0000	138.0000	97.0000	44.0000	10.0000	141.0000	5.0000
14	4.0000	138.0000	96.0000	46.0000	12.0000	140.0000	5.0000
15		136.0000	98.0000	44.0000	10.0000	138.0000	3.0000
16			157.0000	147.0000	140.0000	24.0000	137.0000
17				96.0000	103.0000	163.0000	97.0000
18					50.0000	151.0000	47.0000
19						142.0000	13.0000
20							139.0000
21							

OTUs	22	23	24	25	26	27	28
1	12.0000	9.0000	11.0000	4.0000	10.0000	10.0000	10.0000
2	93.0000	92.0000	92.0000	96.0000	91.0000	91.0000	91.0000
3	12.0000	9.0000	11.0000	4.0000	10.0000	10.0000	10.0000
4	12.0000	9.0000	11.0000	4.0000	10.0000	10.0000	10.0000
5	12.0000	9.0000	11.0000	4.0000	10.0000	10.0000	10.0000
6	12.0000	9.0000	11.0000	4.0000	10.0000	10.0000	10.0000
7	12.0000	9.0000	11.0000	4.0000	10.0000	10.0000	10.0000
8	11.0000	10.0000	10.0000	5.0000	9.0000	9.0000	9.0000
9	11.0000	10.0000	10.0000	5.0000	9.0000	9.0000	9.0000
10	11.0000	10.0000	10.0000	5.0000	9.0000	9.0000	9.0000
11	11.0000	10.0000	10.0000	5.0000	9.0000	9.0000	9.0000
12	11.0000	10.0000	10.0000	5.0000	9.0000	9.0000	9.0000
13	11.0000	8.0000	10.0000	5.0000	9.0000	9.0000	9.0000
14	13.0000	10.0000	12.0000	1.0000	11.0000	11.0000	11.0000
15	11.0000	10.0000	10.0000	5.0000	9.0000	9.0000	9.0000
16	141.0000	140.0000	140.0000	138.0000	139.0000	139.0000	139.0000
17	104.0000	101.0000	103.0000	97.0000	102.0000	102.0000	102.0000
18	51.0000	50.0000	50.0000	47.0000	49.0000	49.0000	49.0000
19	1.0000	2.0000	0.0000	13.0000	1.0000	1.0000	1.0000
20	143.0000	142.0000	142.0000	140.0000	141.0000	141.0000	141.0000
21	14.0000	11.0000	13.0000	6.0000	12.0000	12.0000	12.0000
22		3.0000	1.0000	14.0000	2.0000	2.0000	2.0000
23			2.0000	11.0000	1.0000	1.0000	1.0000
24				13.0000	1.0000	1.0000	1.0000
25					12.0000	12.0000	12.0000
26						0.0000	0.0000
27							0.0000
28							

OTUs	29	30	31	32
1	11.0000	10.0000	11.0000	11.0000
2	92.0000	91.0000	92.0000	92.0000
3	11.0000	10.0000	11.0000	11.0000

4	11.0000	10.0000	11.0000	11.0000
5	11.0000	10.0000	11.0000	11.0000
6	11.0000	10.0000	11.0000	11.0000
7	11.0000	10.0000	11.0000	11.0000
8	10.0000	9.0000	10.0000	10.0000
9	10.0000	9.0000	10.0000	10.0000
10	10.0000	9.0000	10.0000	10.0000
11	10.0000	9.0000	10.0000	10.0000
12	10.0000	9.0000	10.0000	10.0000
13	10.0000	9.0000	10.0000	10.0000
14	12.0000	11.0000	12.0000	12.0000
15	10.0000	9.0000	10.0000	10.0000
16	140.0000	139.0000	140.0000	140.0000
17	103.0000	102.0000	103.0000	103.0000
18	50.0000	49.0000	50.0000	50.0000
19	0.0000	1.0000	0.0000	0.0000
20	142.0000	141.0000	142.0000	142.0000
21	13.0000	12.0000	13.0000	13.0000
22	1.0000	2.0000	1.0000	1.0000
23	2.0000	1.0000	2.0000	2.0000
24	0.0000	1.0000	0.0000	0.0000
25	13.0000	12.0000	13.0000	13.0000
26	1.0000	0.0000	1.0000	1.0000
27	1.0000	0.0000	1.0000	1.0000
28	1.0000	0.0000	1.0000	1.0000
29		1.0000	0.0000	0.0000
30			1.0000	1.0000
31				0.0000
32				

APPENDIX F – Multiple Alignment file for S10

```
BTV17.S10GB       GTTAAAAAGTG-TCGCTGCCATGCTATCCGGGCTGATCCAAAGGTTCGAGGAAGAAAAAA
BTV1AUS.S10GB     GTTAAAAAGTG-TCGCTGCCATGCTATCCGGGCTGATCCAAAGGTTCGAGGAAGAAAAAA
202894.S10        GTTAAAAAGTG-TCGCTGCCATGCTATCCGGGCTGATCCAAAGGTTCGAGGAAGAAAAAA
201400.S10        GTTAAAAAGTG-TCGCTGCCATGCTATCCGGGCTGATCCAAAGGTTCGAGGAAGAAAAAA
203733.S10        GTTAAAAAGTG-TCGCTGCCATGCTATCCGGGCTGATCCAAAGGTTCGAGGAAGAAAAAA
203843.S10        GTTAAAAAGTG-TCGCTGCCATGCTATCCGGGCTGATCCAAAGGTTCGAGGAAGAAAAAA
203853.S10        GTTAAAAAGTG-TCGCTGCCATGCTATCCGGGCTGATCCAAAGGTTCGAGGAAGAAAAAA
201773.S10        GTTAAAAAGTG-TCGCTGCCATGCTATCCGGGCTGATCCAAAGGTTCGAGGAAGAAAAAA
202020.S10        GTTAAAAAGTG-TCGCTGCCATGCTATCCGGGCTGATCCAAAGGTTCGAGGAAGAAAAAA
203732.S10        GTTAAAAAGTG-TCGCTGCCATGCTATCCGGGCTGATCCAAAGGTTCGAGGAAGAAAAAA
203734.S10        GTTAAAAAGTG-TCGCTGCCATGCTATCCGGGCTGATCCAAAGGTTCGAGGAAGAAAAAA
203826.S10        GTTAAAAAGTG-TCGCTGCCATGCTATCCGGGCTGATCCAAAGGTTCGAGGAAGAAAAAA
BTV10.S10GB       GTTAAAAAGTG-TCGCTGCCATGCTATCCGGGCTGATCCAAAGGTTCGAGGAAGAAAAAA
BTV13.S10GB       GTTAAAAAGTG-TCGCTGCCATGCTATCCGGGCTGATCCAAAGGTTCGAGGAAGAAAAAA
201767.S10        GTTAAAAAGTG-TCGCTGCCATGCTATCCGGGCTGATCCAAAGGTTCGAGGAAGAAAAAA
EHDV1.S10GB       GTTAAAAAGAGGTTGGCATCATGCTATCCAGGTTGGTTCCAGGTGTTGA--AACTAGAAT
BTV11-2.S10GB     GTTAAAAAGTG-TCGCTGTCATGCTATCCGGGCTGATCCAAAGGTTCGAAGAAGAAAAGA
BTV2-3.S10GB      GTTAAAAAGTG-TCGCTGCCATGCTATCCGGGCTGATCCAAAGGTTCGAGGAAGAAAAAA
M94389.S10        GTTAAAA-GTG-TCGCTGCCATGCTATCCGGGCTGATCCAAAGGTTCGAGGAAGAAAAAA
EHDV2.S10GB       GTTAAAAAGAGGTTGGCATCATGCTATCCAGGTTGGTTCCAGGTGTTGA--AACTAGAAT
BTV17-2.S10GB     GTTAAAAAGTG-TCGCTGCCATGCTATCCGGGCTGATCCAAAGGTTCGAGGAAGAAAAAA
M9442.S10         GTTAAAAAGTG-TCGCTGCCATGCTATCCGGGCTGATCCAAAGGTTCGAGGAAGAAAAAA
BTV11.S10GB       GTTAAAAAGTG-TCGCTGCCATGCTATCCGGGCTGATCCAAAGGTTCGAGGAAGAAAAAA
A94389.S10        GTTAAAAAGTG-TCGCTGCCATGCTATCCGGGCTGATCCAAAGGTTCGAGGAAGAAAAAA
BTV13-2.S10GB     GTTAAAAAGTG-TCGCTGCCATGCTATCCGGGCTGATCCAAAGGTTCGAGGAAGAAAAAA
M94368.S10        GTTAAAAAGTG-TCGCTGCCATGCTATCCGGGCTGATCCAAAGGTTCGAGGAAGAAAAAA
270.S10           GTTAAAAAGTG-TCGCTGCCATGCTATCCGGGCTGATCCAAAGGTTCGAGGAAGAAAAAA
E94393.S10        GTTAAAAAGTG-TCGCTGCCATGCTATCCGGGCTGATCCAAAGGTTCGAGGAAGAAAAAA
A9464.S10         GTTAAAAAGTG-TCGCTGCCATGCTATCCGGGCTGATCCAAAGGTTCGAGGAAGAAAAAA
BTV11.S10         GTTAAAAAGTG-TCGCTGCCATGCTATCCGGGCTGATCCAAAGGTTCGAGGAAGAAAAAA
M94430.S10        GTTAAAAAGTG-TCGCTGCCATGCTATCCGGGCTGATCCAAAGGTTCGAGGAAGAAAAAA
A945.S10          GTTAAAAAGTG-TCGCTGCCATGCTATCCGGGCTGATCCAAAGGTTCGAGGAAGAAAAAA
                  ******* * * * *    ********** ** ** * * * * *  * **  **  * *

BTV17.S10GB       TGAAACATAATCAGGAACGGGTTGAAGAGTTGAGTTTGGTCCGTGTGGATGATACAATTT
BTV1AUS.S10GB     TGAAACATAACCAGGAGAGAGTTGAAGAACTAAGTCTAGTACGCGTGGATGATACAATTT
202894.S10        TGAAACATAATCAGGAACGGGTTGAAGAGTTGAGTTTGGTCCGTGTGGATGATACAATTT
201400.S10        TGAAACATAATCAGGAACGGGTTGAAGAGTTGAGTTTGGTCCGTGTGGATGATACAATTT
203733.S10        TGAAACATAATCAGGAACGGGTTGAAGAGTTGAGTTTGGTCCGTGTGGATGATACAATTT
203843.S10        TGAAACATAATCAGGAACGGGTTGAAGAGTTGAGTTTGGTCCGTGTGGATGATACAATTT
203853.S10        TGAAACATAATCAGGAACGGGTTGAAGAGTTGAGTTTGGTCCGTGTGGATGATACAATTT
201773.S10        TGAAACATAATCAGGAACGGGTTGAAGAGTTGAGTTTGGTCCGTGTGGATGATACAATTT
202020.S10        TGAAACATAATCAGGAACGGGTTGAAGAGTTGAGTTTGGTCCGTGTGGATGATACAATTT
203732.S10        TGAAACATAATCAGGAACGGGTTGAAGAGTTGAGTTTGGTCCGTGTGGATGATACAATTT
203734.S10        TGAAACATAATCAGGAACGGGTTGAAGAGTTGAGTTTGGTCCGTGTGGATGATACAATTT
203826.S10        TGAAACATAATCAGGAACGGGTTGAAGAGTTGAGTTTGGTCCGTGTGGATGATACAATTT
BTV10.S10GB       TGAAACATAATCAGGAACGGGTTGAAGAGTTGAGTTTGGTCCGTGTGGATGATACAATTT
BTV13.S10GB       TGAAACATAATCAGGAACGGGTTGAAGAGTTGAGTTTGGTCCGTGTGGATGATACAATTT
201767.S10        TGAAACATAATCAGGAACGGGTTGAAGAGTTGAGTTTGGTCCGTGTGGATGATACAATTT
EHDV1.S10GB       CGAAATGAAGGCAAG-------CGATGAAGTGAGTTTAGTGCCT-TATCAGGAACAGG--
BTV11-2.S10GB     TGAAACACAATCAAGATAGAGTCGAAGAGCCGAGTCAGGTCCGTGTAGATGATACCATTT
BTV2-3.S10GB      TGAAACATAATCAGGAACGGGTTGAAGAACTGAGTTTAGTTCGTGTGGATGACGCGATTT
M94389.S10        TGAAACATAATCAGGAACGGGTTGAAGAGTTGAGTTTGGTCCGTGTGGATGATACAATTT
EHDV2.S10GB       CGAAATGAAGGCAAG-------CGATGAAGTGAGTTTAGTGCCT-TATCAGGAGCAGGT-
BTV17-2.S10GB     TGAAACATAATCAGGAACGGGTTGAAGAGTTGAGTTTGGTCCGTGTGGATGATACAATTT
M9442.S10         TGAAACATAATCAGGAACGGGTTGAAGAGTTGAGTTTGGTCCGTGTGGATGATACAATTT
BTV11.S10GB       TGAAACATAATCAGGAACGGGTTGAAGAGTTGAGTTTGGTCCGTGTGGATGATACAATTT
A94389.S10        TGAAACATAATCAGGAACGGGTTGAAGAGTTGAGTTTGGTCCGTGTGGATGATACAATTT
BTV13-2.S10GB     TGAAACATAATCAGGAGCGGGTTGAAGAGTTGAGTTTGGTCCATGTGGATGATACAATTT
M94368.S10        TGAAACATAATCAGGAACGGGTTGAAGAGTTGAGTTTGGTCCGTGTGGATGATACAATTT
270.S10           TGAAACATAATCAGGAACGGGTTGAAGAGTTGAGTTTGGTCCGTGTGGATGATACAATTT
E94393.S10        TGAAACATAATCAGGAACGGGTTGAAGAGTTGAGTTTGGTCCGTGTGGATGATACAATTT
A9464.S10         TGAAACATAATCAGGAACGGGTTGAAGAGTTGAGTTTGGTCCGTGTGGATGATACAATTT
BTV11.S10         TGAAACATAATCAGGAACGGGTTGAAGAGTTGAGTTTGGTCCGTGTGGATGATACAATTT
M94430.S10        TGAAACATAATCAGGAACGGGTTGAAGAGTTGAGTTTGGTCCGTGTGGATGATACAATTT
A945.S10          TGAAACATAATCAGGAACGGGTTGAAGAGTTGAGTTTGGTCCGTGTGGATGATACAATTT
                  ****   *  ** *        ** **    ***   ** *  *    *   *
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BTV17.S10GB         CTCAGCCGCCAAGATATGCTCCGAGCGCGCCAATGCCATCATCTATGCCCACTGTTGCAC
BTV1AUS.S10GB       CACAACCACCGAGGTATGCTCCGAGTGCCCCGATGCCGTCATCAATGCCGACCGTTGCCC
202894.S10          CTCAGCCGCCAAGATATGCTCCGAGCGCGCCAATGCCATCATCTATGCCCACTGTTGCAC
201400.S10          CTCAGCCGCCAAGATATGCTCCGAGCGCGCCAATGCCATCATCTATGCCCACTGTTGCAC
203733.S10          CTCAGCCGCCAAGATATGCTCCGAGCGCGCCAATGCCATCATCTATGCCCACTGTTGCAC
203843.S10          CTCAGCCGCCAAGATATGCTCCGAGCGCGCCAATGCCATCATCTATGCCCACTGTTGCAC
203853.S10          CTCAGCCGCCAAGATATGCTCCGAGCGCGCCAATGCCATCATCTATGCCCACTGTTGCAC
201773.S10          CTCAGCCGCCAAGATATGCTCCGAGCGCGCCAATGCCATCATCTATGCCCACTGTTGCAC
202020.S10          CTCAGCCGCCAAGATATGCTCCGAGCGCGCCAATGCCATCATCTATGCCCACTGTTGCAC
203732.S10          CTCAGCCGCCAAGATATGCTCCGAGCGCGCCAATGCCATCATCTATGCCCACTGTTGCAC
203734.S10          CTCAGCCGCCAAGATATGCTCCGAGCGCGCCAATGCCATCATCTATGCCCACTGTTGCAC
203826.S10          CTCAGCCGCCAAGATATGCTCCGAGCGCGCCAATGCCATCATCTATGCCCACTGTTGCAC
BTV10.S10GB         ATCAGCCGCCAAGATATGCTCCGAGCGCGCCAATGCCATCATCTATGCCTACTGTTGCAC
BTV13.S10GB         CTCAGCCGCCAAGATATGCTCCGAGCGCGCCAATGCCATCATCTATGCCCACTGTTGCAC
201767.S10          CTCAGCCGCCAAGATATGCTCCGAGCGCGCCAATGCCATCATCTATGCCCACTGTTGCAC
EHDV1.S10GB         CGAGACCGCCCAGCTATGTGCCATCGGCCCCAATACCAACAGCAATGCCGAGAGTCGCGT
BTV11-2.S10GB       CTCAACCACCAAGGTATGCCCCGAGTGCGCCGATGCCATCATCTATGCCAACGGTTGCCC
BTV2-3.S10GB        CTCAACCACCAAGATATGCTCCGAGCGCGCCAATGCCATCGTCGATGCCAACAGTTGCAC
M94389.S10          CTCAGCCGCCAAGATATGCTCCGAGCGCGCCAATGCCATCGTCAATGCCTACTGTTGCAC
EHDV2.S10GB         -GAGACCGCCCAGTTATGTGCCATCGGCTCCAATACCAACAGCAATGCCGAGAGTCGCGT
BTV17-2.S10GB       CTCAGCCGCCAAGATATGCTCCGAGCGCGCCAATGCCATCATCTATGCCCACTGTTGCAC
M9442.S10           CTCAGCCGCCAAGATATGCTCCGAGCGCGCCAATGCCATCGTCAATGCCTACTGTTGCAC
BTV11.S10GB         CTCAGCCGCCAAGATATGCTCCGAGCGCGCCAATGCCATCGTCAATGCCTACTGTTGCAC
A94389.S10          CTCAGCCGCCAAGATATGCTCCGAGCGCGCCAATGCCATCGTCAATGCCTACTGTTGCAC
BTV13-2.S10GB       CTCAGCCGCCAAGATATGCTCCGAGCGCGCCAATGCCATCATCTATGCCCACTGTTGCAC
M94368.S10          CTCAGCCGCCAAGATATGCTCCGAGCGCGCCAATGCCATCGTCAATGCCTACTGTTGCAC
270.S10             CTCAGCCGCCAAGATATGCTCCGAGCGCGCCAATGCCATCGTCAATGCCTACTGTTGCAC
E94393.S10          CTCAGCCGCCAAGATATGCTCCGAGCGCGCCAATGCCATCGTCAATGCCTACTGTTGCAC
A9464.S10           CTCAGCCGCCAAGATATGCTCCGAGCGCGCCAATGCCATCGTCAATGCCTACTGTTGCAC
BTV11.S10           CTCAGCCGCCAAGATATGCTCCGAGCGCGCCAATGCCATCGTCAATGCCTACTGTTGCAC
M94430.S10          CTCAGCCGCCAAGATATGCTCCGAGCGCGCCAATGCCATCGTCAATGCCTACTGTTGCAC
A945.S10            CTCAGCCGCCAAGATATGCTCCGAGCGCGCCAATGCCATCGTCAATGCCTACTGTTGCAC
                         ** ** ** ****  **    ** ** ** **  *  * ***** *  ** **

BTV17.S10GB         TTGAAATCTTGGACAAAGCGATGTCAAATACAACGGGTGCTACGCAAACACAAAAAGCGG
BTV1AUS.S10GB       TTGAAATATTGGATAAAGCGATGTCAAACACTACTGGTGCTACGCAAACACAAAAGGCGG
202894.S10          TTGAAATCTTGGACAAAGCGATGTCAAATACAACGGGTGCTACGCAAACACAAAAAGCGG
201400.S10          TTGAAATCTTGGACAAAGCGATGTCAAATACAACGGGTGCTACGCAAACACAAAAAGCGG
203733.S10          TTGAAATCTTGGACAAAGCGATGTCAAATACAACGGGTGCTACGCAAACACAAAAAGCGG
203843.S10          TTGAAATCTTGGACAAAGCGATGTCAAATACAACGGGTGCTACGCAAACACAAAAAGCGG
203853.S10          TTGAAATCTTGGACAAAGCGATGTCAAATACAACGGGTGCTACGCAAACACAAAAAGCGG
201773.S10          TTGAAATCTTGGACAAAGCGATGTCAAATACAACGGGTGCTACGCAAACACAAAAAGCGG
202020.S10          TTGAAATCTTGGACAAAGCGATGTCAAATACAACGGGTGCTACGCAAACACAAAAAGCGG
203732.S10          TTGAAATCTTGGACAAAGCGATGTCAAATACAACGGGTGCTACGCAAACACAAAAAGCGG
203734.S10          TTGAAATCTTGGACAAAGCGATGTCAAATACAACGGGTGCTACGCAAACACAAAAAGCGG
203826.S10          TTGAAATCTTGGACAAAGCGATGTCAAATACAACGGGTGCTACGCAAACACAAAAAGCGG
BTV10.S10GB         TTGAAATCTTGGACAAAGCGATGTCAAATACAACGGGTGCTACGCAAACACAAAAAGCGG
BTV13.S10GB         TTGAAATCTTGGACAAAGCGATGTCAAATACAACGGGTGCTACGCAAACACAAAAAGCGG
201767.S10          TTGAAATCTTGGACAAAGCGATGTCAAATACAACGGGTGCTACGCAAACACAAAAAGCGG
EHDV1.S10GB         TGGATATATTGGACAAAGCGATGTCAAATCAAACGGGCGCAACTATGGCACAAAAAGTAG
BTV11-2.S10GB       TTGAAATATTGGACAAAGCGATGTCAAACACAACTGGTGCAACGCAAACACAAAAAGCTG
BTV2-3.S10GB        TTGAAATCTTGGACAAAGCGATGTCAAATACAACGGGTGCTACGCAAACACAAAAAGCGG
M94389.S10          TTGAAATCTTGGACAAAGCGATGTCAAATACAACGGGTGCTACGCAAACACAAAAGGCGG
EHDV2.S10GB         TGGATATATTGGACAAAGCGATGTCGAATCAAACGGGCGCAACTATGGCACAAAAAGTAG
BTV17-2.S10GB       TTGAAATCTTGGACAAAGCGATGTCAAATACAACGGGTGCTACGCAAACACAAAAAGCGG
M9442.S10           TTGAAATCTTGGACAAAGCGATGTCAAATACAACGGGTGCTACGCAAACACAAAAGGCGG
BTV11.S10GB         TTGAAATCTTGGACAAAGCGATGTCAAATACAACGGGTGCTACGCAAACACAAAAGGCGG
A94389.S10          TTGAAATCTTGGACAAAGCGATGTCAAATACAACGGGTGCTACGCAAACACAAAAGGCGG
BTV13-2.S10GB       TTGAAATCTTGGACAAAGCGATGTCAAATACAACGGGTGCTACGCAAACACAAAAAGCGG
M94368.S10          TTGAAATCTTGGACAAAGCGATGTCAAATACAACGGGTGCTACGCAAACACAAAAGGCGG
270.S10             TTGAAATCTTGGACAAAGCGATGTCAAATACAACGGGTGCTACGCAAACACAAAAGGCGG
E94393.S10          TTGAAATCTTGGACAAAGCGATGTCAAATACAACGGGTGCTACGCAAACACAAAAGGCGG
A9464.S10           TTGAAATCTTGGACAAAGCGATGTCAAATACAACGGGTGCTACGCAAACACAAAAGGCGG
BTV11.S10           TTGAAATCTTGGACAAAGCGATGTCAAATACAACGGGTGCTACGCAAACACAAAAGGCGG
M94430.S10          TTGAAATCTTGGACAAAGCGATGTCAAATACAACGGGTGCTACGCAAACACAAAAGGCGG
A945.S10            TTGAAATCTTGGACAAAGCGATGTCAAATACAACGGGTGCTACGCAAACACAAAAGGCGG
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BTV17.S10GB         AGAAGGCTGCATTCGCATCGTACGCGGAAGCGTTTCGTGATGATGTGAGGTTAAGACAAA
BTV1AUS.S10GB       AAAAAGCTGCATTCGCATCGTACGCAGAAGCGTTTCGTGATGATGTGAGGCTGAGGCAGA
202894.S10          AGAAGGCTGCATTCGCATCGTACGCGGAAGCGTTTCGTGATGATGTGAGGTTAAGACAAA
201400.S10          AGAAGGCTGCATTCGCATCGTACGCGGAAGCGTTTCGTGATGATGTGAGGTTAAGACAAA
203733.S10          AGAAGGCTGCATTCGCATCGTACGCGGAAGCGTTTCGTGATGATGTGAGGTTAAGACAAA
203843.S10          AGAAGGCTGCATTCGCATCGTACGCGGAAGCGTTTCGTGATGATGTGAGGTTAAGACAAA
203853.S10          AGAAGGCTGCATTCGCATCGTACGCGGAAGCGTTTCGTGATGATGTGAGGTTAAGACAAA
201773.S10          AGAAGGCTGCATTCGCATCGTACGCGGAAGCGTTTCGTGATGATGTGAGGTTAAGACAAA
202020.S10          AGAAGGCTGCATTCGCATCGTACGCGGAAGCGTTTCGTGATGATGTGAGGTTAAGACAAA
203732.S10          AGAAGGCTGCATTCGCATCGTACGCGGAAGCGTTTCGTGATGATGTGAGGTTAAGACAAA
203734.S10          AGAAGGCTGCATTCGCATCGTACGCGGAAGCGTTTCGTGATGATGTGAGGTTAAGACAAA
203826.S10          AGAAGGCTGCATTCGCATCGTACGCGGAAGCGTTTCGTGATGATGTGAGGTTAAGACAAA
BTV10.S10GB         AGAAGGCTGCATTCGCATCGTACGCGGAAGCGTTTCGTGATGATGTGAGGTTAAGACAAA
BTV13.S10GB         AGAAGGCTGCATTCGCATCGTACGCGGAAGCGTTTCGTGATGATGTGAGGTTAAGACAAA
201767.S10          AGAAGGCTGCATTCGCATCGTACGCGGAAGCGTTTCGTGATGATGTGAGGTTAAGACAAA
EHDV1.S10GB         AGAAGGTTGCTTATGCTTCGTATGCGGAGGCGTTCAGAGATGATTTAAGATTGCGTCAAA
BTV11-2.S10GB       AGAAGGCTGCATTCGCATCGTACGCGGGAGTAGCTCGTGATGATGTGAGATTGAGACAGA
BTV2-3.S10GB        AGAAGGCTGCATTCGCATCGTACGCGGAAGCGTTTCGTGATGATGTGAGGCTGAGACAAA
M94389.S10          AAAAGGCTGCATTCGCATCGTACGCGGAAGCGTTTCGTGATGATGTGAGGTTAAGGCAAA
EHDV2.S10GB         AGAAGGTTGCTTATGCTTCGTATGCGGAGGCGTTCAGAGACGATTTAAGATTGCGTCAAA
BTV17-2.S10GB       AGAAGGCTGCATTCGCATCGTACGCGGAAGCGTTTCGTGATGATGTGAGGTTAAGACAAA
M9442.S10           AAAAGGCTGCATTCGCATCGTACGCGGAAGCGTTTCGTGATGATGTGAGGTTAAGGCAAA
BTV11.S10GB         AAAAGGCTGCATTCGCATCGTACGCGGAAGCGTTTCGTGATGATGTGAGGTTAAGGCAAA
A94389.S10          AAAAGGCTGCATTCGCATCGTACGCGGAAGCGTTTCGTGATGATGTGAGGTTAAGGCAAA
BTV13-2.S10GB       AGAAGGCTGCATTCGCATCGTACGCGGAAGCGTTTCGTGATGATGTGAGGTTAAGACAAA
M94368.S10          AAAAGGCTGCATTCGCATCGTACGCGGAAGCGTTTCGTGATGATGTGAGGTTAAGGCAAA
270.S10             AAAAGGCTGCATTCGCATCGTACGCGGAAGCGTTTCGTGATGATGTGAGGTTAAGGCAAA
E94393.S10          AAAAGGCTGCATTCGCATCGTACGCGGAAGCGTTTCGTGATGATGTGAGGTTAAGGCAAA
A9464.S10           AAAAGGCTGCATTCGCATCGTACGCGGAAGCGTTTCGTGATGATGTGAGGTTAAGGCAAA
BTV11.S10           AAAAGGCTGCATTCGCATCGTACGCGGAAGCGTTTCGTGATGATGTGAGGTTAAGGCAAA
M94430.S10          AAAAGGCTGCATTCGCATCGTACGCGGAAGCGTTTCGTGATGATGTGAGGTTAAGGCAAA
A945.S10            AAAAGGCTGCATTCGCATCGTACGCGGAAGCGTTTCGTGATGATGTGAGGTTAAGGCAAA
                    * ** * *** *  ** ***** ** * *      * ** *** * **  *  * ** *

BTV17.S10GB         TTAAGCGACATGTGAATGAGCAAATTTTACCAAAGTTGAAAAGTGACCTAGGAGGCTTAA
BTV1AUS.S10GB       TCAACCGTCATGTTAATGAACAGATTTTCCCAAAGTTAAAGAGTGATTTAGGCGGTTTAA
202894.S10          TTAAGCGACATGTGAATGAGCAAATTTTACCAAAGTTGAAAAGTGACCTAGGAGGCTTAA
201400.S10          TTAAGCGACATGTGAATGAGCAAATTTTACCAAAGTTGAAAAGTGACCTAGGAGGCTTAA
203733.S10          TTAAGCGACATGTGAATGAGCAAATTTTACCAAAGTTGAAAAGTGACCTAGGAGGCTTAA
203843.S10          TTAAGCGACATGTGAATGAGCAAATTTTACCAAAGTTGAAAAGTGACCTAGGAGGCTTAA
203853.S10          TTAAGCGACATGTGAATGAGCAAATTTTACCAAAGTTGAAAAGTGACCTAGGAGGCTTAA
201773.S10          TTAAGCGACATGTGAATGAGCAAATTTTACCAAAGTTGAAAAGTGACCTAGGAGGCTTAA
202020.S10          TTAAGCGACATGTGAATGAGCAAATTTTACCAAAGTTGAAAAGTGACCTAGGAGGCTTAA
203732.S10          TTAAGCGACATGTGAATGAGCAAATTTTACCAAAGTTGAAAAGTGACCTAGGAGGCTTAA
203734.S10          TTAAGCGACATGTGAATGAGCAAATTTTACCAAAGTTGAAAAGTGACCTAGGAGGCTTAA
203826.S10          TTAAGCGACATGTGAATGAGCAAATTTTACCAAAGTTGAAAAGTGACCTAGGAGGCTTAA
BTV10.S10GB         TTAAGCGACATGTGAATGAGCAAATTTTACCAAAGTTGAAAAGTGACCTAGGAGGCTTAA
BTV13.S10GB         TTAAGCGACATGTGAATGAGCAAATTTTACCAAAGTTGAAAAGTGACCTAGGAGGCTTAA
201767.S10          TTAAGCGACATGTGAATGAGCAAATTTTACCAAAGTTGAAAAGTGACCTAGGAGGCTTAA
EHDV1.S10GB         TTAAAAAGCATGTAAACGAGCAAATATTACCTAAAATGCGTGTGGAGTTGACAGCGATGA
BTV11-2.S10GB       TCAAACGCCATGTGAACGAGCAGATTTTACCAAAATTAAAAAGTGATCTAAGTGGATTGA
BTV2-3.S10GB        TTAAACGACATGTGAATGAACAGATTCTGCCAAAATTGAAAAGTGACCTTGGAGGCTTAA
M94389.S10          TTAAGCGACATGTGAATGAGCAAATTTTACCAAAGTTGAAAAGTGACCTAGGAGGCTTAA
EHDV2.S10GB         TTAAGAAGCATGTAAACGAGCAAGTATTACCCAAAATGCGTGTAGAGTTGACAGCGATGA
BTV17-2.S10GB       TTAAGCGACATGTGAATGAGCAAATTTCACCAAAGTTGAAAAGTGACCTAGGAGGCTTAA
M9442.S10           TTAAGCGACATGTGAATGAGCAAATTTTACCAAAGTTGAAAAGTGACCTAGGAGGCTTAA
BTV11.S10GB         TTAAGCGACATGTGAATGAGCAAATTTTACCAAAGTTGAAAAGTGACCTAGGAGGCTTAA
A94389.S10          TTAAGCGACATGTGAATGAGCAAATTTTACCAAAGTTGAAAAGTGACCTAGGAGGCTTAA
BTV13-2.S10GB       TTAAGCGACATGTGAATGAGCAAATTTTACCAAAGTTGAAAAGTGACCTAGGAGGCTTAA
M94368.S10          TTAAGCGACATGTGAATGAGCAAATTTTACCAAAGTTGAAAAGTGACCTAGGAGGCTTAA
270.S10             TTAAGCGACATGTGAATGAGCAAATTTTACCAAAGTTGAAAAGTGACCTAGGAGGCTTAA
E94393.S10          TTAAGCGACATGTGAATGAGCAAATTTTACCAAAGTTGAAAAGTGACCTAGGAGGCTTAA
A9464.S10           TTAAGCGACATGTGAATGAGCAAATTTTACCAAAGTTGAAAAGTGACCTAGGAGGCTTAA
BTV11.S10           TTAAGCGACATGTGAATGAGCAAATTTTACCAAAGTTGAAAAGTGACCTAGGAGGCTTAA
M94430.S10          TTAAGCGACATGTGAATGAGCAAATTTTACCAAAGTTGAAAAGTGACCTAGGAGGCTTAA
A945.S10            TTAAGCGACATGTGAATGAGCAAATTTTACCAAAGTTGAAAAGTGACCTAGGAGGCTTAA
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BTV17.S10GB         AGAAGAAAAGAGCTATTATACATATGACACTGCTGATAGCGGCAGTCGTTGCTCTGCTGA
BTV1AUS.S10GB       AGAAAAAGAGAGCTATCATACACATGACGTTGTTGGTCGCCGCTGTCGTAGCGTTGTTGA
202894.S10          AGAAGAAAAGAGCTATTATACATATGACACTGCTGATAGCGGCAGTCGTTGCTCTGCTGA
201400.S10          AGAAGAAAAGAGCTATTATACATATGACACTGCTGATAGCGGCAGTCGTTGCTCTGCTGA
203733.S10          AGAAGAAAAGAGCTATTATACATATGACACTGCTGATAGCGGCAGTCGTTGCTCTGCTGA
203843.S10          AGAAGAAAAGAGCTATTATACATATGACACTGCTGATAGCGGCAGTCGTTGCTCTGCTGA
203853.S10          AGAAGAAAAGAGCTATTATACATATGACACTGCTGATAGCGGCAGTCGTTGCTCTGCTGA
201773.S10          AGAAGAAAAGAGCTATTATACATATGACACTGCTGATAGCGGCAGTCGTTGCTCTGCTGA
202020.S10          AGAAGAAAAGAGCTATTATACATATGACACTGCTGATAGCGGCAGTCGTTGCTCTGCTGA
203732.S10          AGAAGAAAAGAGCTATTATACATATGACACTGCTGATAGCGGCAGTCGTTGCTCTGCTGA
203734.S10          AGAAGAAAAGAGCTATTATACATATGACACTGCTGATAGCGGCAGTCGTTGCTCTGCTGA
203826.S10          AGAAGAAAAGAGCTATTATACATATGACACTGCTGATAGCGGCAGTCGTTGCTCTGCTGA
BTV10.S10GB         AGAAGAAAAGAGCTATTATACATATGACACTGCTGATAGCTGCAGTCGTTGCTCTGCTGA
BTV13.S10GB         AGAAGAAAAGAGCTATTATACATATGACACTGCTGATAGCGGCAGTCGTTGCTCTGCTGA
201767.S10          AGAAGAAAAGAGCTATTATACATATGACACTGCTGATAGCGGCAGTCGTTGCTCTGCTGA
EHDV1.S10GB         AACGGCGACGAGCGATGGCTCATATGGTCTTGATCATTGCAGCTGTGGTAGCGTTGATAA
BTV11-2.S10GB       AGAAGAAGCGAGCAATCATACACACTACTCTATTGGTAGCTGCTGTGGTTGCTCTGTTGA
BTV2-3.S10GB        AGAAGAAGAGAGCCATCATACATATGACATTGCTGATAGCAGCGGTTGTCGCTCTGCTGA
M94389.S10          AGAAGAAAAGAGCTATTATACATGTGACACTGCTGATAGCGGCAGTCGTTGCTCTGCTGA
EHDV2.S10GB         AACGACGACGAGCGATGGCTCATATGATCTTGATAATTGCGGCTGTGGTAGCGTTGATAA
BTV17-2.S10GB       AGAAGAAAAGAGCTATTATACATATGACACTGCTGATAGCGGCAGTCGTTGCTCTGCTGA
M9442.S10           AGAAGAAAAGAGCTATTATACATGTGACACTGCTGATAGCGGCAGTCGTTGCTCTGCTGA
BTV11.S10GB         AGAAGAAAAGAGCTATTATACATATGACACTGCTGATAGCGGCAGTCGTTGCTCTGCTGA
A94389.S10          AGAAGAAAAGAGCTATTATACATGTGACACTGCTGATAGCGGCAGTCGTTGCTCTGCTGA
BTV13-2.S10GB       AGAAGAAAAGAGCTATTATACATATGACACTGCTGATAGCGGCAGTCGTTGCTCTGCTGA
M94368.S10          AGAAGAAAAGAGCTATTATACATATGACACTGCTGATAGCGGCAGTCGTTGCTCTGCTGA
270.S10             AGAAGAAAAGAGCTATTATACATATGACACTGCTGATAGCGGCAGTCGTTGCTCTGCTGA
E94393.S10          AGAAGAAAAGAGCTATTATACATATGACACTGCTGATAGCGGCAGTCGTTGCTCTGCTGA
A9464.S10           AGAAGAAAAGAGCTATTATACATGTGACACTGCTGATAGCGGCAGTCGTTGCTCTGCTGA
BTV11.S10           AGAAGAAAAGAGCTATTATACATATGACACTGCTGATAGCGGCAGTCGTTGCTCTGCTGA
M94430.S10          AGAAGAAAAGAGCTATTATACATGTGACACTGCTGATAGCGGCAGTCGTTGCTCTGCTGA
A945.S10            AGAAGAAAAGAGCTATTATACATGTGACACTGCTGATAGCGGCAGTCGTTGCTCTGCTGA
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BTV17.S10GB         CATCAGTTTGTACG-TTGTCGAGCGATATGAGCGTGGCATTTAAATTAAATGGTACATCA
BTV1AUS.S10GB       CATCGGTATGCACA-CTTTCAAGTGATATGAGTGTGGCATTTAAGCTTAATGGTACATCA
202894.S10          CATCAGTTTGTACG-TTGTCGAGCGATATGAGCGTGGCATTTAAATTAAATGGTACATCA
201400.S10          CATCAGTTTGTACG-TTGTCGAGCGATATGAGCGTGGCATTTAAATTAAATGGTACATCA
203733.S10          CATCAGTTTGTACG-TTGTCGAGCGATATGAGCGTGGCATTTAAATTAAATGGTACATCA
203843.S10          CATCAGTTTGTACG-TTGTCGAGCGATATGAGCGTGGCATTTAAATTAAATGGTACATCA
203853.S10          CATCAGTTTGTACG-TTGTCGAGCGATATGAGCGTGGCATTTAAATTAAATGGTACATCA
201773.S10          CATCAGTTTGTACG-TTGTCGAGCGATATGAGCGTGGCATTTAAATTAAATGGTACATCA
202020.S10          CATCAGTTTGTACG-TTGTCGAGCGATATGAGCGTGGCATTTAAATTAAATGGTACATCA
203732.S10          CATCAGTTTGTACG-TTGTCGAGCGATATGAGCGTGGCATTTAAATTAAATGGTACATCA
203734.S10          CATCAGTTTGTACG-TTGTCGAGCGATATGAGCGTGGCATTTAAATTAAATGGTACATCA
203826.S10          CATCAGTTTGTACG-TTGTCGAGCGATATGAGCGTGGCATTTAAATTAAATGGTACATCA
BTV10.S10GB         CATCAGTTTGTACG-TTGTCGAGCGATATGAGCGTGGCATTTAAATTAAATGGTACATCA
BTV13.S10GB         CATCAGTTTGTACG-TTGTCGAGCGATATGAGCGTGGCATTTAAATTAAATGGTACATCA
201767.S10          CATCAGTTTGTACG-TTGTCGAGCGATATGAGCGTGGCATTTAAATTAAATGGTACATCA
EHDV1.S10GB         CGTCAGCCAGTACG-CTGACGAGTGATTTGGGTGTAATTCTAAAACATAATACAACAACG
BTV11-2.S10GB       CATCAGTTTGCACC-CTCTCAAGCGATATGAGTGTGGCCTTTAAGATAAACGGAACTAAG
BTV2-3.S10GB        CATCGGTTTGTACG-TTGTCGAGCGATATGAGCGTGGCGTTTAAGTTAAATGGTACATCA
M94389.S10          CATCAGTTTGTACG-TTGTCGAGCGATATGAGCGTGGCATTTAAATTAAATGGTACATCA
EHDV2.S10GB         CGTCAGCCAGTACG-TTGACGAGTGACTTGGGTGTAATTCTAAAACATAATACAACAACG
BTV17-2.S10GB       CATCAGTTTGTACG-TTGTCGAGCGATATGAGCGTGGCATTTAAATTAAATGGTACATCA
M9442.S10           CATCAGTTTGTACGGTTGTCGAGCAATATGAGCGTGGCATTTAAATTAAATGGTACATCA
BTV11.S10GB         CATCAGTTTGTACG-TTGTCGAGCGATATGAGCGTGGCATTTAAATTAAATGGTACATCA
A94389.S10          CATCAGTTTGTACG-TTGTCGAGCGATATGAGCGTGGCATTTAAATTAAATGGTACATCA
BTV13-2.S10GB       CATCAGTTTGTACG-TTGTCGAGCGATATGAGCGTGGCATTTAAATTAAATGGTACATCA
M94368.S10          CATCAGTTTGTACG-TTGTCGAGCGATATGAGCGTGGCATTTAAATTAAATGGTACATCA
270.S10             CATCAGTTTGTACG-TTGTCGAGCGATATGAGCGTGGCATTTAAATTAAATGGTACATCA
E94393.S10          CATCAGTTTGTACG-TTGTCGAGCGATATGAGCGTGGCATTTAAATTAAATGGTACATCA
A9464.S10           CATCAGTTTGTACG-TTGTCGAGCGATATGAGCGTGGCATTTAAATTAAATGGTACATCA
BTV11.S10           CATCAGTTTGTACG-TTGTCGAGCGATATGAGCGTGGCATTTAAATTAAATGGTACATCA
M94430.S10          CATCAGTTTGTACG-TTGTCGAGCGATATGAGCGTGGCATTTAAATTAAATGGTACATCA
A945.S10            CATCAGTTTGTACG-TTGTCGAGCGATATGAGCGTGGCATTTAAATTAAATGGTACATCA
                    * ** *   * **   *  * **  *  ** * **     * **    **    **
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BTV17.S10GB       GCTGAAATACCTCAGTGGTTTAAGAGTTTGAATCCAATGTTGGGTGTGGTAAATCTGGGA
BTV1AUS.S10GB     GCGGAAATACCACAGTGGTTTAAGAGTCTAAATCCCATGCTTGGAGTAGTGAATCTGGGT
202894.S10        GCTGAAATACCTCAGTGGTTTAAGAGTTTGAATCCAATGTTGGGTGTGGTAAATCTGGGA
201400.S10        GCTGAAATACCTCAGTGGTTTAAGAGTTTGAATCCAATGTTGGGTGTGGTAAATCTGGGA
203733.S10        GCTGAAATACCTCAGTGGTTTAAGAGTTTGAATCCAATGTTGGGTGTGGTAAATCTGGGA
203843.S10        GCTGAAATACCTCAGTGGTTTAAGAGTTTGAATCCAATGTTGGGTGTGGTAAATCTGGGA
203853.S10        GCTGAAATACCTCAGTGGTTTAAGAGTTTGAATCCAATGTTGGGTGTGGTAAATCTGGGA
201773.S10        GCTGAAATACCTCAGTGGTTTAAGAGTTTGAATCCAATGTTGGGTGTGGTAAATCTGGGA
202020.S10        GCTGAAATACCTCAGTGGTTTAAGAGTTTGAATCCAATGTTGGGTGTGGTAAATCTGGGA
203732.S10        GCTGAAATACCTCAGTGGTTTAAGAGTTTGAATCCAATGTTGGGTGTGGTAAATCTGGGA
203734.S10        GCTGAAATACCTCAGTGGTTTAAGAGTTTGAATCCAATGTTGGGTGTGGTAAATCTGGGA
203826.S10        GCTGAAATACCTCAGTGGTTTAAGAGTTTGAATCCAATGTTGGGTGTGGTAAATCTGGGA
BTV10.S10GB       GCTGAAATACCTCAGTGGTTTAAGAGTTTGAATCCAATGTTGGGTGTGGTAAATCTGGGA
BTV13.S10GB       GCTGAAATACCTCAGTGGTTTAAGAGTTTGAATCCAATGTTGGGTGTGGTAAATCTGGGA
201767.S10        GCTGAAATACCTCAGTGGTTTAAGAGTTTGAATCCAATGTTGGGTGTGGTAAATCTGGGA
EHDV1.S10GB       GAGGCGATACAAACGTATATTAAACCCTTTTGTGCGGCTTTCGGAATAATAAATTTAGCC
BTV11-2.S10GB     ACAGAAGTGCCTTCATGGTTTAAAAGCCTTAACCCGATGCTTGGCGTTGTTAATTTGGGA
BTV2-3.S10GB      GCTGAAATACCTCAATGGTTTAAGAGTTTGAATCCGATGTTGGGTGTGGTGAATGTGGGG
M94389.S10        GCTGAAATACCTCAGTGGTTTAAGAGTTTGAATCCAATGTTGGGTGTGGTAAATCTGGGA
EHDV2.S10GB       GAGGCGATACAGACGTATATTAAACCCTTTTGTGCGGCTTTCGGAATAATAAATTTAGCC
BTV17-2.S10GB     GCTGAAATACCTCAGTGGTTTAAGAGTTTGAATCCAATGTTGGGTGTGGTAAATCTGGGA
M9442.S10         GCTGAAATACCTCAGTGGTTTAAGAGTTTGAATCCAATGTTGGGTGTGGTAAATCTGGGA
BTV11.S10GB       GCTGAAATACCTCAGTGGTTTAAGAGTTTGAATCCAATGTTGGGTGTGGTAAATCTGGGA
A94389.S10        GCTGAAATACCTCAGTGGTTTAAGAGTTTGAATCCAATGTTGGGTGTGGTAAATCTGGGA
BTV13-2.S10GB     GCTGAAATACCTCAGTGGTTTAAGAGTTTGAATCCAATGTTGGGTGTGGTAAATCTGGGA
M94368.S10        GCTGAAATACCTCAGTGGTTTAAGAGTTTGAATCCAATGTTGGGTGTGGTAAATCTGGGA
270.S10           GCTGAAATACCTCAGTGGTTTAAGAGTTTGAATCCAATGTTGGGTGTGGTAAATCTGGGA
E94393.S10        GCTGAAATACCTCAGTGGTTTAAGAGTTTGAATCCAATGTTGGGTGTGGTAAATCTGGGA
A9464.S10         GCTGAAATACCTCAGTGGTTTAAGAGTTTGAATCCAATGTTGGGTGTGGTAAATCTGGGA
BTV11.S10         GCTGAAATACCTCAGTGGTTTAAGAGTTTGAATCCAATGTTGGGTGTGGTAAATCTGGGA
M94430.S10        GCTGAAATACCTCAGTGGTTTAAGAGTTTGAATCCAATGTTGGGTGTGGTAAATCTGGGA
A945.S10          GCTGAAATACCTCAGTGGTTTAAGAGTTTGAATCCAATGTTGGGTGTGGTAAATCTGGGA
                     *   * *     *   ****     *     *     * **  *  * *** * *

BTV17.S10GB       GCTACCTTCCTGATGATGGTCTGTGCAAAAAGTGAAAGAAGCTTGAATCAGCAAATTGAT
BTV1AUS.S10GB     GCGACTTTCATTATGATGGTTTGTGCGAAGAGCGAAAGAGGGTTAAATCAGCAGATTGAC
202894.S10        GCTACCTTCCTGATGATGGTCTGTGCAAAAAGTGAAAGAAGCTTGAATCAGCAAATTGAT
201400.S10        GCTACCTTCCTGATGATGGTCTGTGCAAAAAGTGAAAGAAGCTTGAATCAGCAAATTGAT
203733.S10        GCTACCTTCCTGATGATGGTCTGTGCAAAAAGTGAAAGAAGCTTGAATCAGCAAATTGAT
203843.S10        GCTACCTTCCTGATGATGGTCTGTGCAAAAAGTGAAAGAAGCTTGAATCAGCAAATTGAT
203853.S10        GCTACCTTCCTGATGATGGTCTGTGCAAAAAGTGAAAGAAGCTTGAATCAGCAAATTGAT
201773.S10        GCTACCTTCCTGATGATGGTCTGTGCAAAAAGTGAAAGAAGCTTGAATCAGCAAATTGAT
202020.S10        GCTACCTTCCTGATGATGGTCTGTGCAAAAAGTGAAAGAAGCTTGAATCAGCAAATTGAT
203732.S10        GCTACCTTCCTGATGATGGTCTGTGCAAAAAGTGAAAGAAGCTTGAATCAGCAAATTGAT
203734.S10        GCTACCTTCCTGATGATGGTCTGTGCAAAAAGTGAAAGAAGCTTGAATCAGCAAATTGAT
203826.S10        GCTACCTTCCTGATGATGGTCTGTGCAAAAAGTGAAAGAAGCTTGAATCAGCAAATTGAT
BTV10.S10GB       GCTACCTTCCTGATGATGGTTTGTGCAAAAAGTGAAAGAAGCTTGAATCAGCAAATTGAT
BTV13.S10GB       GCTACCTTCCTGATGATGGTTTGTGCAAAAAGTGAAAGGAGCTTGAATCAGCAAATTGAT
201767.S10        GCTACCTTCCTGATGATGGTCTGTGCAAAAAGTGAAAGAAGCTTGAATCAGCAAATTGAT
EHDV1.S10GB       GCGACAATGGTGATGATGTTCATGGCGAAAAATGAAAAGATTATCAATCAACAAATAGAT
BTV11-2.S10GB     GCAACTTTTTTGATGATGGTTTGCGCAAAGAGTGAGAGAGCCTTGAACCAGCAGATAGAT
BTV2-3.S10GB      GCTACCTTTCTGATGATGGTTTGCGCAAAAAGTGAGAGAAGCTTGAATCAGCAAATTGAT
M94389.S10        GCTACCTTCCTGATGATGGTTTGTGCGAAAAGTGAAAGAAGCTTGAATCAGCAAATTGAT
EHDV2.S10GB       GCAACGATGGTGATGATGTTCATGGCGAAAAATGAAAAGATTATCAATCACGAAATAGAC
BTV17-2.S10GB     GCTACCTTCCTGATGATGGTCTGTGCAAAAAGTGAAAGAAGCTTGAATCAGCAAATTGAT
M9442.S10         GCTACCTTCCTGATGATGGTTTGTGCGAAAAGTGAAAGAAGCTTGAATCAGCAAATTGAT
BTV11.S10GB       GCTACCTTCCTGATGATGGTTTGTGCGAAAAGTGAAAGAAGCTTGAATCAGCAAATTGAT
A94389.S10        GCTACCTTCCTGATGATGGTTTGTGCGAAAAGTGAAAGAAGCTTGAATCAGCAAATTGAT
BTV13-2.S10GB     GCTACCTTCCTGATGATGGTTTGTGCAAAAAGTGAAAGGAGCTTGAATCAGCAAATTGAT
M94368.S10        GCTACCTTCCTGATGATGGTTTGTGCGAAAAGTGAAAGAAGCTTGAATCAGCAAATTGAT
270.S10           GCTACCTTCCTGATGATGGTTTGTGCGAAAAGTGAAAGAAGCTTGAATCAGCAAATTGAT
E94393.S10        GCTACCTTCCTGATGATGGTTTGTGCGAAAAGTGAAAGAAGCTTGAATCAGCAAATTGAT
A9464.S10         GCTACCTTCCTGATGATGGTTTGTGCGAAAAGTGAAAGAAGCTTGAATCAGCAAATTGAT
BTV11.S10         GCTACCTTCCTGATGATGGTTTGTGCGAAAAGTGAAAGAAGCTTGAATCAGCAAATTGAT
M94430.S10        GCTACCTTCCTGATGATGGTTTGTGCGAAAAGTGAAAGAAGCTTGAATCAGCAAATTGAT
A945.S10          GCTACCTTCCTGATGATGGTTTGTGCGAAAAGTGAAAGAAGCTTGAATCAGCAAATTGAT
                  ** **  *  * ****** *    ** ** *  ** *      * ** **  * ** **
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BTV17.S10GB         ATGATTAAGAAGGAAGTGATGAAGAAGCAATCGTATAATGATGCGGTGAGGATGAGTTTT
BTV1AUS.S10GB       ATGATTAAGAAGGAAGTTATGAAAAAACAATCATATAATGACGCAGTGAGGATGAGTTTC
202894.S10          ATGATTAAGAAGGAAGTGATGAAGAAGCAATCGTATAATGATGCGGTGAGGATGAGTTTT
201400.S10          ATGATTAAGAAGGAAGTGATGAAGAAGCAATCGTATAATGATGCGGTGAGGATGAGTTTT
203733.S10          ATGATTAAGAAGGAAGTGATGAAGAAGCAATCGTATAATGATGCGGTGAGGATGAGTTTT
203843.S10          ATGATTAAGAAGGAAGTGATGAAGAAGCAATCGTATAATGATGCGGTGAGGATGAGTTTT
203853.S10          ATGATTAAGAAGGAAGTGATGAAGAAGCAATCGTATAATGATGCGGTGAGGATGAGTTTT
201773.S10          ATGATTAAGAAGGAAGTGATGAAGAAGCAATCGTATAATGATGCGGTGAGGATGAGTTTT
202020.S10          ATGATTAAGAAGGAAGTGATGAAGAAGCAATCGTATAATGATGCGGTGAGGATGAGTTTT
203732.S10          ATGATTAAGAAGGAAGTGATGAAGAAGCAATCGTATAATGATGCGGTGAGGATGAGTTTT
203734.S10          ATGATTAAGAAGGAAGTGATGAAGAAGCAATCGTATAATGATGCGGTGAGGATGAGTTTT
203826.S10          ATGATTAAGAAGGAAGTGATGAAGAAGCAATCGTATAATGATGCGGTGAGGATGAGTTTT
BTV10.S10GB         ATGATTAAGAAGGAAGTGATGAAGAAGCAATCGTATAATGATGCGGTGAGGATGAGTTTT
BTV13.S10GB         ATGATTAAGAAGGAAGTGATGAAGAAGCAATCGTATAATGATGCGGTGAGGATGAGTTTT
201767.S10          ATGATTAAGAAGGAAGTGATGAAGAAGCAATCGTATAATGATGCGGTGAGGATGAGTTTT
EHDV1.S10GB         CACACGAGGAAGGAAATAATGAAGAAGGATGCATACAATGAGGCAGTACGAATGAGTGTG
BTV11-2.S10GB       ATGATAAAGAAGGAAGTGATGAAAAAACAATCTTATAATGACGCGGTGAGAATGAGTTTT
BTV2-3.S10GB        ATGATTAAGAAGGAGGTGATGAAGAAGCAATCGTATAATGATGCGGTGAGGATGAGTTTT
M94389.S10          ATGATTAAGAAGGAAGTGATGAAGAAGCAATCGTATAATGATGCGGTGAGGATGAGTTTT
EHDV2.S10GB         CATACGAGGAAGGAAATAATGAAGAAAGATGCATATAATGAGGCAGTACGAATGAGTGTG
BTV17-2.S10GB       ATGATTAAGAAGGAAGTGATGAAGAAGCAATCGTATAATGATGCGGTGAGAATGAGTTTT
M9442.S10           ATGATTAAGAAGGAAGTGATGAAGAAGCAATCGTATAATGATGCGGTGAGGATGAGTTTT
BTV11.S10GB         ATGATTAAGAAGGAAGTGATGAAGAAGCAATCGTATAATGATGCGGTGAGGATGAGTTTT
A94389.S10          ATGATTAAGAAGGAAGTGATGAAGAAGCAATCGTATAATGATGCGGTGAGGATGAGTTTT
BTV13-2.S10GB       ATGATTAAGAAGGAAGTGATGAAGAAGCAATCGTATAATGATGCGGTGAGGATGAGTTTT
M94368.S10          ATGATTAAGAAGGAAGTGATGAAGAAGCAATCGTATAATGATGCGGTGAGGATGAGTTTT
270.S10             ATGATTAAGAAGGAAGTGATGAAGAAGCAATCGTATAATGATGCGGTGAGGATGAGTTTT
E94393.S10          ATGATTAAGAAGGAAGTGATGAAGAAGCAATCGTATAATGATGCGGTGAGGATGAGTTTT
A9464.S10           ATGATTAAGAAGGAAGTGATGAAGAAGCAATCGTATAATGATGCGGTGAGGATGAGTTTT
BTV11.S10           ATGATTAAGAAGGAAGTGATGAAGAAGCAATCGTATAATGATGCGGTGAGGATGAGTTTT
M94430.S10          ATGATTAAGAAGGAAGTGATGAAGAAGCAATCGTATAATGATGCGGTGAGGATGAGTTTT
A945.S10            ATGATTAAGAAGGAAGTGATGAAGAAGCAATCGTATAATGATGCGGTGAGGATGAGTTTT
                      *  * ******  * ***** **  *  * ** ***** ** **  * ****** *

BTV17.S10GB         ACGGAATTTTCATCAGTCCCGCTAGATGGTTTTGAATTACCATTAACCTAAGGTCAGTAG
BTV1AUS.S10GB       ACAGAGTTCTCGTCAGTCCCGCTAGATGGTTTCGAACTGCCATTAACCTGAGATCAGTAG
202894.S10          ACGGAATTTTCATCAGTCCCGCTAGATGGTTTTGAATTACCATTAACCTAAGGTCAGTAG
201400.S10          ACGGAATTTTCATCAGTCCCGCTAGATGGTTTTGAATTACCATTAACCTAAGGTCAGTAG
203733.S10          ACGGAATTTTCATCAGTCCCGCTAGATGGTTTTGAATTACCATTAACCTAAGGTCAGTAG
203843.S10          ACGGAATTTTCATCAGTCCCGCTAGATGGTTTTGAATTACCATTAACCTAAGGTCAGTAG
203853.S10          ACGGAATTTTCATCAGTCCCGCTAGATGGTTTTGAATTACCATTAACCTAAGGTCAGTAG
201773.S10          ACGGAATTTTCATCAGTCCCGCTAGATGGTTTTGAATTACCATTAACCTAAGGTCAGTAG
202020.S10          ACGGAATTTTCATCAGTCCCGCTAGATGGTTTTGAATTACCATTAACCTAAGGTCAGTAG
203732.S10          ACGGAATTTTCATCAGTCCCGCTAGATGGTTTTGAATTACCATTAACCTAAGGTCAGTAG
203734.S10          ACGGAATTTTCATCAGTCCCGCTAGATGGTTTTGAATTACCATTAACCTAAGGTCAGTAG
203826.S10          ACGGAATTTTCATCAGTCCCGCTAGATGGTTTTGAATTACCATTAACCTAAGGTCAGTAG
BTV10.S10GB         ACGGAATTTTCATCAGTCCCGCTAGATGGTTTTGAATTACCATTAACCTAAGGTCGGTAG
BTV13.S10GB         ACGGAATTTTCATCAGTCCCGCTAGATGGTTTTGAATTGCCATTAACCTAAGGTCAGTAG
201767.S10          ACGGAATTTTCATCAGTCCCGCTAGATGGTTTT-AATTACCATTAACCTAAGGTCAGTAG
EHDV1.S10GB         ACAGAGTTCAGTGGGATCCCGCTAGATGGATTTGACATACCA----CCGGAACTCACCAG
BTV11-2.S10GB       ACAGAGTTCTCGTCAATCCCGCTAGATGGTTTAGAAATGCCATTAACCTGAGGACAGTAG
BTV2-3.S10GB        ACGGAATTTTCATCAGTTCCGCTAGATGGTTTCGAATTACCATTAACCTGAGATCAGTAG
M94389.S10          ACAGAATTTTCATCAGTCCCGCTAGATGGTTTTGAATCACCATTAACCTAAGGTCAGTAG
EHDV2.S10GB         ACAGAGTTCAGTGGGATCCCGCTAGATGGATTTGACATACCG----CCGGAACTCACCAG
BTV17-2.S10GB       ACGGAATTTTCATCAGTCCCGCTAGATGGTTTTGAATTACCATTAACCTAAGGTCAGTAG
M9442.S10           ACAGAATTTTCATCAGTCCCGCTAGATGGTTTTGAATCACCATTAACCTAAGGTCAGTAG
BTV11.S10GB         ACAGAATTTTCATCAGTCCCGCTAGATGGTTTTGAATCACCATTAACCTAAGGTCAGTAG
A94389.S10          ACAGAATTTTCATCAGTCCCGCTAGATGGTTTTGAATCACCATTAACCTAAGGTCAGTAG
BTV13-2.S10GB       ACGGAATTTTCATCAGTCCCGCTAGATGGTTTTGAATTGCCATTAACCTAAGGTCAGTAG
M94368.S10          ACAGAATTTTCATCAGTCCCGCTAGATGGTTTTGAATCACCATTAACCTAAGGTCAGTAG
270.S10             ACAGAATTTTCATCAGTCCCGCTAGATGGTTTTGAATCACCATTAACCTAAGGTCAGTAG
E94393.S10          ACAGAATTTTCATCAGTCCCGCTAGATGGTTTTGAATCACGATTAACCTAAGGTCAGTAG
A9464.S10           ACAGAATTTTCATCAGTCCCGCTAGATGGTTTTGAATCACCATTAACCTAAGGTCAGTAG
BTV11.S10           ACAGAATTTTCATCAGTCCCGCTAGATGGTTTTGAATCACCATTAACCTAAGGTCAGTAG
M94430.S10          ACAGAATTTTCATCAGTCCCGCTAGATGGTTTTGAATCACCATTAACCTAAGGTCAGTAG
A945.S10            ACAGAATTTTCATCAGTCCCGCTAGATGGTTTTGAATCACCATTAACCTAAGGTCAGTAG
                    ** ** **        * ************ **  *    **     **  *   *   **
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BTV17.S10GB         GTAGAGTGGCGCCCCAAGGTCCGTATCGTGTAGAGT-GGTTGATCTCACGATGCGGACTC
BTV1AUS.S10GB       GTAGAGTTGCGCCCCAAGGTTTGCACCGCATGGAGT-GGTTGATCCAGAGATGCAAATTC
202894.S10          GTAGAGTGGCGCCCCAAGGTCCGTATCGTGTAGAGT-GGTTGATCTCACGATGCGGACTC
201400.S10          GTAGAGTGGCGCCCCAAGGTCCGTATCGTGTAGAGT-GGTTGATCTCACGATGCGGACTC
203733.S10          GTAGAGTGGCGCCCCAAGGTCCGTATCGTGTAGAGT-GGTTGATCTCACGATGCGGACTC
203843.S10          GTAGAGTGGCGCCCCAAGGTCCGTATCGTGTAGAGT-GGTTGATCTCACGATGCGGACTC
203853.S10          GTAGAGTGGCGCCCCAAGGTCCGTATCGTGTAGAGT-GGTTGATCTCACGATGCGGACTC
201773.S10          GTAGAGTGGCGCCCCAAGGTCCGTATCGTGTAGAGT-GGTTGATCTCACGATGCGGACTC
202020.S10          GTAGAGTGGCGCCCCAAGGTCCGTATCGTGTAGAGT-GGTTGATCTCACGATGCGGACTC
203732.S10          GTAGAGTGGCGCCCCAAGGTCCGTATCGTGTAGAGT-GGTTGATCTCACGATGCGGACTC
203734.S10          GTAGAGTGGCGCCCCAAGGTCCGTATCGTGTAGAGT-GGTTGATCTCACGATGCGGACTC
203826.S10          GTAGAGTGGCGCCCCAAGGTCCGTATCGTGTAGAGT-GGTTGATCTCACGATGCGGACTC
BTV10.S10GB         GTAGAGTGGCGCCCCAAGGTCCGTATCGTGTAGAGT-GGTTGATCTCACGATGCGGACTC
BTV13.S10GB         GTAGAGTGGCGCCCCAAGGTCCGTATCGTGTAGAGT-GGTTGATCTCACGATGCGGACTC
201767.S10          GTAGAGTGGCGCCCCAAGGTCCGTATCGTGTAGAGT-GGTTGATCTCACGATGCGGACTC
EHDV1.S10GB         ATAGAGTGAAGCTCCAAGATCACTATGTCGTGGGATTGGCAGGTCCCGGAGTAGTGATTC
BTV11-2.S10GB       GTAGAGTGGCGCCCCGAGGTTTACGTCGTGCAGGGT-GGTTGATCTCGCGGCGTACATTC
BTV2-3.S10GB        GTAGAGTGGCGCCCCGAGGTTCGTATCGTGTAGAGT-GGTTGATCTCACGATGCGGACTC
M94389.S10          GTAGAGTGGCGCCCCAAGGTCCGTATCGTGTAGAGT-GGTTGATCTCACGATGCGGACTC
EHDV2.S10GB         ATAGAGTGAAGCTCCAAGGTCACTATGTCGTGGGATTGGCAGGTCCCGGAGTAGTGATTC
BTV17-2.S10GB       GTAGAGTGGCGCCCCAAGGTCCGTATCGTGTAGAGT-GGTTGATCTCACGATGCGGACTC
M9442.S10           GTAGAGTGGCGCCCCAAGGTCCGTATCGTGTAGAGT-GGTTGATCTCACGATGCGGACTC
BTV11.S10GB         GTAGAGTGGCGCCCCAAGGTCCGTATCGTGTAGAGT-GGTTGATCTCACGATGCGGACTC
A94389.S10          GTAGAGTGGCGCCCCAAGGTCCGTATCGTGTAGAGT-GGTTGATCTCACGATGCGGACTC
BTV13-2.S10GB       GTAGAGTGGCGCCCCAAGGTCCGTATCGTGTAGAGT-GGTTGATCTCACGATGCGGACTC
M94368.S10          GTAGAGTGGCGCCCCAAGGTCCGTATCGTGTAGAGT-GGTTGATCTCACGATGCGGACTC
270.S10             GTAGAGTGGCGCCCCAAGGTCCGTATCGTGTAGAGT-GGTTGATCTCACGATGCGGACTC
E94393.S10          GTAGAGTGGCGCCCCAAGGTCCGTATCGTGTAGAGT-GGTTGATCTCACGATGCGGACTC
A9464.S10           GTAGAGTGGCGCCCCAAGGTCCGTATCGTGTAGAGT-GGTTGATCTCACGATGCGGACTC
BTV11.S10           GTAGAGTGGCGCCCCAAGGTCCGTATCGTGTAGAGT-GGTTGATCTCACGATGCGGACTC
M94430.S10          GTAGAGTGGCGCCCCAAGGTCCGTATCGTGTAGAGT-GGTTGATCTCACGATGCGGACTC
A945.S10            GTAGAGTGGCGCCCCAAGGTCCGTATCGTGTAGAGT-GGTTGATCTCACGATGCGGACTC
                     ******   ** ** ** *          *  * **  * **          * **

BTV17.S10GB         CTACTGCTGTCTAACGGGGGAGGGTACGCGGCGCT--ACACACTTAC
BTV1AUS.S10GB       CTACTGCTGTATAACGGGGGAGGGTGTGCGGCGCT--ATACACTTAC
202894.S10          CTACTGCTGTCTAACGGGGGAGGGTACGCGGCGCT--ACACACTTAC
201400.S10          CTACTGCTGTCTAACGGGGGAGGGTACGCGGCGCT--ACACACTTAC
203733.S10          CTACTGCTGTCTAACGGGGGAGGGTACGCGGCGCT--ACACACTTAC
203843.S10          CTACTGCTGTCTAACGGGGGAGGGTACGCGGCGCT--ACACACTTAC
203853.S10          CTACTGCTGTCTAACGGGGGAGGGTACGCGGCGCT--ACACACTTAC
201773.S10          CTACTGCTGTCTAACGGGGGAGGGTATGCGGCGCA--TCACACTTAC
202020.S10          CTACTGCTGTCTAACGGGGGAGGGTATGCGGCGCT--ACACACTTAC
203732.S10          CTACTGCTGTCTAACGGGGGAGGGTATGCGGCGCT--ACACACTTAC
203734.S10          CTACTGCTGTCTAACGGGGGAGGGTATGCGGCGCT--ACACACTTAC
203826.S10          CTACTGCTGTCTAACGGGGGAGGGTATGCGGCGCT--ACACACTTAC
BTV10.S10GB         CTACTGCTGTCTAACGGGGGAGGGTACGCGGCGCT--ACACACTTAC
BTV13.S10GB         CTACTGCTGTCTAACGGGGGAGGGTACGCGGCGCT--ACACACTTAC
201767.S10          CTACTGCTGTCTAACGGGGGAGGGTATGCGGCGCT--ACACACTTAC
EHDV1.S10GB         CCACCGCTGTATAACGGGGGAGG-TATGCCATCCTCGACACACTTAC
BTV11-2.S10GB       CCACTGCTGTATAACGGGGGAGGGTGCGCGGCGCT--ACACACTTAC
BTV2-3.S10GB        CTACTGCTGTCTAACGGGGGAGGGTATGCGGCGCT--ACACACTTAC
M94389.S10          CTACTGCTGTCTAACGGGGGAGGGTATGCGGCGCT--ACACACTTAC
EHDV2.S10GB         CTACCGCTGTATAACGGGGGAGG-TATGCCATCCTCGACACACTTAC
BTV17-2.S10GB       CTACTGCTGTCTAACGGGGGAGGGTACGCGGCGCT--ACACACTTAC
M9442.S10           CTACTGCTGTCTAACGGGGGAGGGTATGCGGCGCT--ACACACTTAC
BTV11.S10GB         CTACTGCTGTCTAACGGGGGAGGGTACGCGGCGCT--ACACACTTAC
A94389.S10          CTACTGCTGTCTAACGGGGGAGGGTATGCGGCGCT--ACACACTTAC
BTV13-2.S10GB       CTACTGCTGTCTAACGGGGGAGGGTACGCGGCGCT--ACACACTTAC
M94368.S10          CTACTGCTGTCTAACGGGGGAGGGTATGCGGCGCT--ACACACTTAC
270.S10             CTACTGCTGTCTAACGGGGGAGGGTATGCGGCGCT--ACACACTTAC
E94393.S10          CTACTGCTGTCTAACGGGGGAGGGTATGCGGCGCT--ACACACTTAC
A9464.S10           CTACTGCTGTCTAACGGGGGAGGGTATGCGGCGCT--ACACACTTAC
BTV11.S10           CTACTGCTGTCTAACGGGGGAGGGTATGCGGCGCT--ACACACTTAC
M94430.S10          CTACTGCTGTCTAACGGGGGAGGGTATGCGGCGCT--ACACACTTAC
A945.S10            CTACTGCTGTCTAACGGGGGAGGGTATGCGGCGCT--ACACACTTAC
                    * ** ***** ************ *  **    *     ********
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APPENDIX G: *Culicoides* spp. collected and assayed for BTV in 1996. See footnotes for explanation.

NUMBER	DATE COLL.	SITE	SPP.	# IN POOL	ELISA	MOS. BHK	ECE. BHK	BHK
A-96-1	4/9/96	Howard	C.v.	30	neg.	neg.	neg.	neg.
A-96-2	4/9/96	Howard	C.crep.	30	pos.	neg.	neg.	neg.
A-96-3	4/9/96	Howard	C.v.	30	pos.	neg.	neg.	neg.
A-96-4	4/9/96	Howard	C.v.	30	neg.	neg.	neg.	neg.
A-96-5	4/9/96	Howard	C.v.	30	pos.	neg.	neg.	neg.
A-96-6	4/9/96	Howard	C.crep.	30	pos.	neg.	neg.	neg.
A-96-7	4/9/96	Howard	C.v.	30	pos.	neg.	neg.	neg.
A-96-8	4/9/96	Howard	Unknown	8	neg.	neg.	neg.	neg.
A-96-9	4/9/96	Howard	C.v.	30	pos.	neg.	neg.	neg.
A-96-10	4/9/96	Howard	C.v.	30	pos.	neg.	neg.	neg.
A-96-11	4/9/96	Howard	C.crep.	30	pos.	neg.	neg.	neg.
A-96-12	4/9/96	Howard	C.crep.	30	pos.	neg.	neg.	neg.
A-96-13	4/9/96	Howard	C.v.	30	pos.	neg.	neg.	neg.
A-96-14	4/9/96	Howard	C.v.	30	pos.	neg.	neg.	neg.
A-96-15	4/9/96	Howard	C.v.	30	neg.	neg.	neg.	neg.
A-96-16	4/9/96	Howard	C.v.	30	pos.	neg.	neg.	neg.
A-96-17	4/9/96	Howard	C.crep.	30	pos.	neg.	neg.	neg.
A-96-18	4/9/96	Howard	C.v.	30	neg.	neg.	neg.	neg.
A-96-19	4/9/96	Howard	C.v.	30	neg.	neg.	neg.	neg.
A-96-20	4/9/96	Howard	C.v.	30	neg.	neg.	neg.	neg.
A-96-21	4/9/96	Howard	C.v.	30	neg.	neg.	neg.	neg.
A-96-22	4/9/96	Howard	C.crep.	30	pos.	neg.	neg.	neg.
A-96-23	4/9/96	Howard	C.v.	30	neg.	neg.	neg.	neg.
A-96-24	4/9/96	Howard	C.v.	30	pos.	neg.	neg.	neg.
A-96-25	4/9/96	Howard	C.v.	30	pos.	neg.	neg.	neg.
A-96-26	4/9/96	Howard	C.v.	30	pos.	neg.	neg.	neg.
A-96-27	4/9/96	Howard	C.v.	30	neg.	neg.	neg.	neg.
A-96-28	4/9/96	Howard	C.crep.	30	pos.	neg.	neg.	neg.
A-96-29	4/9/96	Howard	C.v.	30	neg	neg.	neg.	neg.
A-96-30	4/9/96	Howard	C.v.	30	pos.	neg.	neg.	neg.
A-96-31	4/9/96	Howard	C.v.	30	neg.	neg.	neg.	neg.
A-96-32	4/9/96	Howard	C.v.	30	pos.	neg.	neg.	neg.
A-96-33	4/9/96	Howard	C.v.	30	neg.	neg.	neg.	neg.
A-96-34	4/9/96	Howard	C.v.	30	neg.	neg.	neg.	neg.
A-96-35	4/9/96	Howard	C.v.	30	pos.	neg.	neg.	neg.
A-96-36	4/9/96	Howard	C.crep.	30	pos.	neg.	neg.	neg.
A-96-37	4/9/96	Howard	C.stell.	8	pos.	neg.	neg.	neg.
A-96-38	4/9/96	Howard	C.v.	30	pos.	neg.	neg.	neg.
A-96-39	4/9/96	Howard	C.crep.	30	pos.	neg.	neg.	neg.
A-96-40	4/9/96	Howard	C.v.	30	neg.	neg.	neg.	neg.

NUMBER	DATE COLL.	SITE	SPP.	# IN POOL	ELISA	MOS. BHK	ECE. BHK	BHK
A-96-41	4/9/96	Howard	C.v.	30	neg.	neg.	neg.	neg.
A-96-42	4/9/96	Howard	C.crep.	30	pos.	neg.	neg.	neg.
A-96-43	4/9/96	Howard	C.v.	30	neg.	neg.	neg.	neg.
A-96-44	4/9/96	Howard	C.v.	30	neg.	neg.	neg.	neg.
A-96-45	4/9/96	Howard	C.v.	30	pos.	neg.	neg.	neg.
A-96-46	4/9/96	Howard	C.v.	30	pos.	neg.	neg.	neg.
A-96-47	4/9/96	Howard	C.crep.	30	neg.	neg.	neg.	neg.
A-96-48	6/13/96	Fields	C.v.	30	neg.	neg.	neg.	neg.
A-96-49	6/13/96	Fields	C.v.	30	neg.	neg.	neg.	neg.
A-96-50	6/13/96	Fields	C.crep.	10	pos.	neg.	neg.	neg.
A-96-51	6/13/96	Fields	mixed	24	pos.	neg.	neg.	neg.
A-96-52	6/13/96	Fields	Selfia	2	pos.	neg.	neg.	neg.
A-96-53								
A-96-54	6/13/96	Red Barn	C.crep.	1	pos.	neg.	neg.	neg.
A-96-55	6/13/96	Red Barn	Selfia	2	pos.	neg.	neg.	neg.
A-96-56								
A-96-57								
A-96-58	6/13/96	Howard	C.v.	30	neg.	neg.	neg.	neg.
A-96-59	6/13/96	Howard	C.v.	34	pos.	neg.	neg.	neg.
A-96-60	6/13/96	Howard	mixed	13	pos.	neg.	neg.	neg.
A-96-61	6/13/96	Howard	C.crep.	4	pos.	neg.	neg.	neg.
A-96-62	6/13/96	Fields	C.v.	24	pos.	neg.	neg.	neg.
A-96-63	6/13/96	Fields	C.cockerelli	1	pos.	neg.	neg.	neg.
A-96-64								
A-96-65								
A-96-66	6/13/96	Howard	C.v.	30	pos.	neg.	neg.	neg.
A-96-67								
A-96-68	6/13/96	Howard	C.cockerelli	1	neg.	neg.	neg.	neg.
A-96-69								
A-96-70	6/13/96	Fields	C.v.	1	neg.	neg.	neg.	neg.
A-96-71	6/13/96	Fields	mixed	1	neg.	neg.	neg.	neg.
A-96-72	6/13/96	Howard	C.v.	1	pos.	neg.	neg.	neg.
A-96-73	6/26,7/2/96	Howard	C.v.	30	pos.	neg.	neg.	neg.
A-96-74	6/26,7/2/96	Howard	mixed	30	pos.	neg.	neg.	neg.
A-96-75	6/26,7/2/96	Howard	C.v.	30	pos.	neg.	neg.	neg.
A-96-76	6/26,7/2/96	Howard	C.v.	30	pos.	neg.	neg.	neg.
A-96-77	6/26,7/2/96	Howard	C.v.	30	pos.	neg.	neg.	neg.
A-96-78	6/26,7/2/96	Howard	mixed	30	pos.	neg.	neg.	neg.
A-96-79	6/26,7/2/96	Howard	mixed	30+	pos.	neg.	neg.	neg.
A-96-80	6/26,7/2/96	Howard	C.v.	30	pos.	neg.	neg.	neg.
A-96-81	6/26,7/2/96	Howard	C.v.	30	pos.	neg.	neg.	neg.
A-96-82	6/26,7/2/96	Howard	C.v.	30	pos.	neg.	neg.	neg.
A-96-83	6/26,7/2/96	Howard	C.v.	30	pos.	neg.	neg.	neg.
A-96-84	6/26,7/2/96	Howard	C.v.	30	neg.	neg.	neg.	neg.

NUMBER	DATE COLL.	SITE	SPP.	# IN POOL	ELISA	MOS. BHK	ECE. BHK	BHK
A-96-85	6/26,7/2/96	Howard	C.v.	30	neg.	neg.	neg.	neg.
A-96-86	6/26,7/2/96	Howard	C.v.	30	pos.	neg.	neg.	neg.
A-96-87	6/26,7/2/96	Howard	mixed	32	neg.	neg.	neg.	neg.
A-96-88	6/26,7/2/96	Howard	C.v.	31	pos.	neg.	neg.	neg.
A-96-89	6/26,7/2/96	Fields	mixed	16	neg.	neg.	neg.	neg.
A-96-90	6/26,7/2/96	Fields	C.stell.	3	pos.	neg.	neg.	neg.
A-96-91	6/26,7/2/96	Fields	C.crep.	5	pos.	neg.	neg.	neg.
A-96-92	6/26,7/2/96	Fields	C.cockerelli	8	pos.	neg.	neg.	neg.
A-96-93	6/26,7/2/96	Fields	C.v.	23	neg.	neg.	neg.	neg.
A-96-94	6/13/96	Red Barn	C.v.	30	pos.	neg.	neg.	neg.
A-96-95	6/13/96	Red Barn	C.cockerelli	17	neg.	neg.	neg.	neg.
A-96-96	6/13/96	Red Barn	mixed	10	neg.	neg.	neg.	neg.
A-96-97	6/13/96	Red Barn	C.v.	30	pos.	neg.	neg.	neg.
A-96-98	6/13/96	Red Barn	C.v.	21	neg.	neg.	neg.	neg.
A-96-99	7/2/96	Howard	C.v.	30	pos.	neg.	neg.	neg.
A-96-100	7/2/96	Howard	C.v.	30	pos.	neg.	neg.	neg.
A-96-101	7/2/96	Howard	C.v.	30	pos.	neg.	neg.	neg.
A-96-102	7/2/96	Howard	C.v.	30	pos.	neg.	neg.	neg.
A-96-103	7/2/96	Howard	C.v.	30	pos.	neg.	neg.	neg.
A-96-104	7/2/96	Howard	C.v.	30	neg.	neg.	neg.	neg.
A-96-105	7/2/96	Howard	mixed	36	pos.	neg.	neg.	neg.
A-96-106	7/2/96	Howard	C.v.	30	pos.	neg.	neg.	neg.
A-96-107	7/2/96	Howard	C.v.	30	pos.	neg.	neg.	neg.
A-96-108	7/2/96	Howard	mixed	30	pos.	neg.	neg.	neg.
A-96-109	7/2/96	Howard	C.v.	30	pos.	neg.	neg.	neg.
A-96-110	7/2/96	Howard	mixed	30	neg.	neg.	neg.	neg.
A-96-111	7/2/96	Howard	mixed	30	pos.	neg.	neg.	neg.
A-96-112	7/2/96	Howard	C.cockerelli	8	neg.	neg.	neg.	neg.
A-96-113	7/2/96	Howard	C.v.	30	pos.	neg.	neg.	neg.
A-96-114	7/2/96	Howard	C.v.	30	pos.	neg.	neg.	neg.
A-96-115	7/2/96	Howard	C.v.	30	neg.	neg.	neg.	neg.
A-96-116	7/2/96	Howard	C.v.	30	neg.	neg.	neg.	neg.
A-96-117	7/2/96	Howard	C.v.	30	neg.	neg.	neg.	neg.
A-96-118	7/2/96	Howard	mixed	30	pos.	neg.	neg.	neg.
A-96-119	7/2/96	Howard	C.v.	30	pos.	neg.	neg.	neg.
A-96-120	7/2/96	Howard	C.v.	30	pos.	neg.	neg.	neg.
A-96-121	7/2/96	Howard	C.v.	5	neg.	neg.	neg.	neg.
A-96-122	7/2/96	Howard	mixed	30	neg.	neg.	neg.	neg.
A-96-123	7/2/96	Howard	mixed	30	neg.	neg.	neg.	neg.
A-96-124	7/2/96	Howard	mixed	23	pos.	neg.	neg.	neg.
A-96-125	7/16/96	Howard	C.v.	30	neg.	neg.	neg.	neg.
A-96-126	7/16/96	Howard	C.crep.	21	neg.	neg.	neg.	neg.
A-96-127	7/16/96	Howard	C.stel.	1	pos.	neg.	neg.	neg.
A-96-128	7/16/96	Howard	C.cockerelli	1	pos.	neg.	neg.	neg.

NUMBER	DATE COLL.	SITE	SPP.	# IN POOL	ELISA	MOS. BHK	ECE. BHK	BHK
A-96-129	7/16/96	Howard	mixed	30	neg.	neg.	neg.	neg.
A-96-130	7/16/96	Howard	C.v.	30	pos.	neg.	neg.	neg.
A-96-131	7/16/96	Howard	mixed	30	pos.	neg.	neg.	neg.
A-96-132	7/16/96	Howard	C.v.	30	pos.	neg.	neg.	neg.
A-96-133	7/16/96	Howard	C.v.	22	pos.	neg.	neg.	neg.
A-96-134	7/16/96	Howard	mixed	28	pos.	neg.	neg.	neg.
A-96-135	7/2/96	Fields	C.v.	22	pos.	neg.	neg.	neg.
A-96-136	7/2/96	Fields	mixed	30	neg.	neg.	neg.	neg.
A-96-137	7/2/96	Fields	mixed	34	neg.	neg.	neg.	neg.
A-96-138	7/2/96	Red Barn	C.v.	30	neg.	neg.	neg.	neg.
A-96-139	7/2/96	Red Barn	C.v.	30	pos.	neg.	neg.	neg.
A-96-140	7/2/96	Red Barn	C.v.	30	neg.	neg.	neg.	neg.
A-96-141	7/2/96	Red Barn	C.v.	30	neg.	neg.	neg.	neg.
A-96-142	7/2/96	Red Barn	C.v.	30	neg.	neg.	neg.	neg.
A-96-143	7/2/96	Red Barn	mixed	30	neg.	neg.	neg.	neg.
A-96-144	7/2/96	Red Barn	mixed	21	neg.	neg.	neg.	neg.
A-96-145	7/2/96	Red Barn	C.v.	30	neg.	neg.	neg.	neg.
A-96-146	7/2/96	Red Barn	C.v.	30	neg.	neg.	neg.	neg.
A-96-147	7/2/96	Red Barn	mixed	11	neg.	neg.	neg.	neg.
A-96-148	7/2/96	Red Barn	C.v.	19	neg.	neg.	neg.	neg.
A-96-149	7/2/96	Howard	C.v.	30	neg.	neg.	neg.	neg.
A-96-150	7/2/96	Howard	C.v.	30	neg.	neg.	neg.	neg.
A-96-151	7/2/96	Howard	C.v.	30	neg.	neg.	neg.	neg.
A-96-152	7/2/96	Howard	C.v.	30	neg.	neg.	neg.	neg.
A-96-153	7/2/96	Howard	mixed	15	pos.	neg.	neg.	neg.
A-96-154	7/2/96	Howard	C.v.	30	neg.	neg.	neg.	neg.
A-96-155	7/2/96	Howard	C.v.	30	neg.	neg.	neg.	neg.
A-96-156	7/2/96	Howard	C.v.	14	neg.	neg.	neg.	neg.
A-96-157	7/2/96	Howard	mixed	17	neg.	neg.	neg.	neg.
A-96-158	7/16/96	Howard	C.v.	24	neg.	neg.	neg.	neg.
A-96-159	7/16/96	Howard	C.v.	30	neg.	neg.	neg.	neg.
A-96-160								
A-96-161	7/16/96	Howard	mixed	32	neg.	neg.	neg.	neg.
A-96-162	7/16/96	Howard	mixed	11	neg.	neg.	neg.	neg.
A-96-163	7/16/96	Howard	C.v.	30	neg.	neg.	neg.	neg.
A-96-164	7/16/96	Howard	C.v.	32	neg.	neg.	neg.	neg.
A-96-165	7/16/96	Howard	C.v.	32	neg.	neg.	neg.	neg.
A-96-166	7/30/96	Howard	C.v.	21	neg.	neg.	neg.	neg.
A-96-167	7/30/96	Howard	C.cockerelli	1	neg.	neg.	neg.	neg.
A-96-168	7/30/96	Howard	mixed	5	neg.	neg.	neg.	neg.
A-96-169	7/30/96	Howard	C.v.	30	neg.	neg.	neg.	neg.
A-96-170	7/30/96	Howard	C.v.	30	neg.	neg.	neg.	neg.
A-96-171	7/30/96	Howard	C.v.	30	neg.	neg.	neg.	neg.
A-96-172	7/30/96	Howard	mixed	30	pos.	neg.	neg.	neg.

NUMBER	DATE COLL.	SITE	SPP.	# IN POOL	ELISA	MOS. BHK	ECE. BHK	BHK
A-96-173	8/6/96	Howard	C.v.	30	pos.	neg.	neg.	neg.
A-96-174	7/30, 8/6-13/1996	Howard	mixed	35	pos.	neg.	neg.	neg.
A-96-175	7/30, 8/6-13/1996	Howard	C.v.	21	pos.	neg.	neg.	neg.
A-96-176	7/30/96	Howard	C.v.	30	neg.	neg.	neg.	neg.
A-96-177	8/6/96	Fields	C.v.	1	neg.	neg.	neg.	neg.
A-96-178	8/6/96	Howard	C.v.	30	neg.	neg.	neg.	neg.
A-96-179	8/6/96	Howard	C.v.	30	neg.	neg.	neg.	neg.
A-96-180	8/6/96	Howard	C.v.	23	neg.	neg.	neg.	neg.
A-96-181	8/6/96	Howard	mixed	17	pos.	neg.	neg.	neg.
A-96-182	8/20/96	Howard	C.v.	30	pos.	neg.	neg.	neg.
A-96-183	8/20/96	Howard	C.v.	30	neg.	neg.	neg.	neg.
A-96-184	8/20/96	Howard	C.cockerelli	1	pos.	neg.	neg.	neg.
A-96-185	8/20/96	Howard	C.v.	30	neg.	neg.	neg.	neg.
A-96-186	8/20/96	Howard	C.v.	30	neg.	neg.	neg.	neg.
A-96-187	8/13/96	Howard	C.cockerelli	2	pos.	neg.	neg.	neg.
A-96-188	8/13/96	Howard	C.v.	30	pos.	neg.	neg.	neg.
A-96-189	8/13/96	Howard	C.v.	30	pos.	neg.	neg.	neg.
A-96-190	8/13/96	Howard	C.v.	30	neg.	neg.	neg.	neg.
A-96-191	8/13/96	Howard	mixed	30	neg.	neg.	neg.	neg.
A-96-192	8/13/96	Howard	C.v.	21	neg.	neg.	neg.	neg.
A-96-193	8/13/96	Howard	mixed	10	neg.	neg.	neg.	neg.
A-96-194	8/20/96	Howard	C.v.	30	neg.	neg.	neg.	neg.
A-96-195	8/20/96	Howard	C.v.	30	neg.	neg.	neg.	neg.
A-96-196	8/20/96	Howard	C.v.	22	pos.	neg.	neg.	neg.
A-96-197	8/20/96	Howard	mixed	5	pos.	neg.	neg.	neg.
A-96-198	8/20/96	Howard	C.cockerelli	1	neg.	neg.	neg.	neg.
A-96-199	8/13/96	Howard	C.v.	30	pos.	neg.	neg.	neg.
A-96-200	8/13/96	Howard	C.v.	30	pos.	neg.	neg.	neg.
A-96-201	8/13/96	Howard	mixed	11	pos.	neg.	neg.	neg.
A-96-202	8/13/96	Howard	C.v.	30	pos.	neg.	neg.	neg.
A-96-203	8/13/96	Howard	C.v.	30	pos.	neg.	neg.	neg.
A-96-204	8/13/96	Howard	C.v.	30	pos.	neg.	neg.	neg.
A-96-205	8/13/96	Howard	C.v.	30	neg.	neg.	neg.	neg.
A-96-206	8/13/96	Howard	mixed	29	neg.	neg.	neg.	neg.
A-96-207	8/13/96	Howard	C.v.	30	pos.	neg.	neg.	neg.
A-96-208	8/13/96	Howard	C.v.	30	pos.	neg.	neg.	neg.
A-96-209	8/13/96	Howard	C.v.	15	pos.	neg.	neg.	neg.
A-96-210	8/13/96	Howard	mixed	11	pos.	neg.	neg.	neg.
A-96-211	8/13/96	Howard	C.v.	30	pos.	neg.	neg.	neg.
A-96-212	8/13/96	Howard	C.v.	30	pos.	neg.	neg.	neg.
A-96-213	8/13/96	Howard	C.v.	30	pos.	neg.	neg.	neg.
A-96-214	8/13/96	Howard	mixed	34	neg.	neg.	neg.	neg.

NUMBER	DATE COLL.	SITE	SPP.	# IN POOL	ELISA	MOS. BHK	ECE. BHK	BHK
A-96-215	8/13/96	Howard	C.v.	30	pos.	neg.	neg.	neg.
A-96-216	8/13/96	Howard	C.v.	30	neg.	neg.	neg.	neg.
A-96-217	8/13/96	Howard	C.v.	30	pos.	neg.	neg.	neg.
A-96-218	8/13/96	Howard	C.v.	30	pos.	neg.	neg.	neg.
A-96-219	8/13/96	Howard	C.v.	30	pos.	neg.	neg.	neg.
A-96-220	8/13/96	Howard	C.v.	30	pos.	neg.	neg.	neg.
A-96-221	8/13/96	Howard	C.cockerelli	1	pos.	neg.	neg.	neg.
A-96-222	8/13/96	Howard	mixed	31	pos.	neg.	neg.	neg.
A-96-223	8/13/96	Howard	C.stell.	2	pos.	neg.	neg.	neg.
A-96-224	8/13/96	Howard	C.v.	22	pos.	neg.	neg.	neg.
A-96-225	8/20/96	Howard	C.v.	30	neg.	neg.	neg.	neg.
A-96-226	8/20/96	Howard	C.v.	30	neg.	neg.	neg.	neg.
A-96-227	8/20/96	Howard	mixed	27	neg.	neg.	neg.	neg.
A-96-228	8/20/96	Howard	C.v.	30	neg.	neg.	neg.	neg.
A-96-229	8/20/96	Howard	C.v.	30	neg.	neg.	neg.	neg.
A-96-230	8/20/96	Howard	C.stell.	1	neg.	neg.	neg.	neg.
A-96-231	8/20/96	Howard	C.v.	30	neg.	neg.	neg.	neg.
A-96-232	8/20/96	Howard	C.v.	30	neg.	neg.	neg.	neg.
A-96-233	8/20/96	Howard	C.v.	30	neg.	neg.	neg.	neg.
A-96-234	8/20/96	Howard	C.v.	30	neg.	neg.	neg.	neg.
A-96-235	8/20/96	Howard	C.v.	30	neg.	neg.	neg.	neg.
A-96-236	8/20/96	Howard	C.v.	30	neg.	neg.	neg.	neg.
A-96-237	8/20/96	Howard	mixed	27	pos.	neg.	neg.	neg.
A-96-238	8/20/96	Howard	C.v.	30	pos.	neg.	neg.	neg.
A-96-239	8/20/96	Howard	C.stell.	2	neg.	neg.	neg.	neg.
A-96-240	8/20/96	Howard	C.v.	30	neg.	neg.	neg.	neg.
A-96-241	8/20/96	Howard	C.v.	30	neg.	neg.	neg.	neg.
A-96-242	8/20/96	Howard	C.v.	30	neg.	neg.	neg.	neg.
A-96-243	8/20/96	Howard	C.v.	30	neg.	neg.	neg.	neg.
A-96-244	8/20/96	Howard	mixed	16	neg.	neg.	neg.	neg.
A-96-245	8/20/96	Howard	C.v.	30	pos.	neg.	neg.	neg.
A-96-246	8/20/96	Howard	C.v.	30	pos.	neg.	neg.	neg.
A-96-247	8/20/96	Howard	C.v.	23	pos.	neg.	neg.	neg.
A-96-248	8/13/96	Howard	C.v.	30	pos.	neg.	neg.	neg.
A-96-249	8/13/96	Howard	C.v.	30	pos.	neg.	neg.	neg.
A-96-250	8/13/96	Howard	mixed	33	pos.	neg.	neg.	neg.
A-96-251	8/13/96	Howard	C.v.	30	neg.	neg.	neg.	neg.
A-96-252	8/13/96	Howard	C.v.	25	neg.	neg.	neg.	neg.
A-96-253	8/20/96	Howard	C.v.	30	neg.	neg.	neg.	neg.
A-96-254	8/20/96	Howard	C.v.	30	neg.	neg.	neg.	neg.
A-96-255	8/20/96	Howard	C.v.	30	neg.	neg.	neg.	neg.
A-96-256	8/20/96	Howard	C.v.	30	neg.	neg.	neg.	neg.
A-96-257	8/20/96	Howard	C.v.	30	pos.	neg.	neg.	neg.
A-96-258	8/20/96	Howard	C.v.	30	pos.	neg.	neg.	neg.

NUMBER	DATE COLL.	SITE	SPP.	# IN POOL	ELISA	MOS. BHK	ECE. BHK	BHK
A-96-259	8/20/96	Howard	C.v.	30	neg.	neg.	neg.	neg.
A-96-260	8/20/96	Howard	mixed	35	neg.	neg.	neg.	neg.
A-96-261	8/20/96	Howard	C.v.	30	neg.	neg.	neg.	neg.
A-96-262	8/20/96	Howard	mixed	12	neg.	neg.	neg.	neg.
A-96-263	8/20/96	Howard	C.v.	26	pos.	neg.	neg.	neg.
A-96-264	9/6/96	Howard	C.v.	28	pos.	neg.	neg.	neg.
A-96-265	9/6/96	Howard	mixed	12	neg.	neg.	neg.	neg.
A-96-266	9/12/96	Howard	C.v.	17	pos.	neg.	neg.	neg.
A-96-267	9/12/96	Howard	mixed	8	pos.	neg.	neg.	neg.
A-96-268	9/17/96	Howard	C.v.	26	neg.	neg.	neg.	neg.
A-96-269	9/17/96	Howard	mixed	8	neg.	neg.	neg.	neg.
A-96-270	8/13/96	Howard	mixed	16	neg.	neg.	neg.	neg.
A-96-271	8/13/96	Howard	C.v.	30	pos.	neg.	neg.	neg.
A-96-272	8/13/96	Howard	C.crep.	13	pos.	neg.	neg.	neg.
A-96-273	8/13/96	Howard	mixed	30	pos.	neg.	neg.	neg.
A-96-274	8/13/96	Howard	C.v.	30	neg.	neg.	neg.	neg.
A-96-275	8/13/96	Howard	C.v.	30	pos.	neg.	neg.	neg.
A-96-276	8/13/96	Howard	C.v.	30	neg.	neg.	neg.	neg.
A-96-277	8/13/96	Howard	C.v.	13	pos.	neg.	neg.	neg.
A-96-278	8/20/96	Howard	C.v.	30	pos.	neg.	neg.	neg.
A-96-279	8/20/96	Howard	C.v.	30	pos.	neg.	neg.	neg.
A-96-280	8/20/96	Howard	C.v.	30	pos.	neg.	neg.	neg.
A-96-281	8/20/96	Howard	C.v.	30	pos.	neg.	neg.	neg.
A-96-282	8/20/96	Howard	C.v.	30	pos.	neg.	neg.	neg.
A-96-283	8/20/96	Howard	C.v.	32	pos.	neg.	neg.	neg.
A-96-284	8/20/96	Howard	C.v.	30	pos.	neg.	neg.	neg.
A-96-285	8/20/96	Howard	C.v.	30	pos.	neg.	neg.	neg.
A-96-286	8/20/96	Howard	C.v.	12	neg.	neg.	neg.	neg.
A-96-287	8/20/96	Howard	mixed	32	pos.	neg.	neg.	neg.
A-96-288	8/20/96	Howard	mixed	9	pos.	neg.	neg.	neg.
A-96-289	9/12/96	Howard	C.v.	25	pos.	neg.	neg.	neg.
A-96-290	9/12/96	Howard	mixed	5	pos.	neg.	neg.	neg.
A-96-291	9/17/96	Howard	C.v.	30	pos.	neg.	neg.	neg.
A-96-292	9/17/96	Howard	mixed	10	pos.	neg.	neg.	neg.
A-96-293	9/17/96	Howard	C.v.	15	pos.	neg.	neg.	neg.
A-96-294	8/13/96	Howard	C.v.	30	neg.	neg.	neg.	neg.
A-96-295	8/13/96	Howard	C.v.	30	neg.	neg.	neg.	neg.
A-96-296	8/13/96	Howard	mixed	30	neg.	neg.	neg.	neg.
A-96-297	8/13/96	Howard	C.v.	30	neg.	neg.	neg.	neg.
A-96-298	8/20/96	Howard	C.v.	30	neg.	neg.	neg.	neg.
A-96-299	8/20/96	Howard	C.v.	30	neg.	neg.	neg.	neg.
A-96-300	8/20/96	Howard	mixed	30	neg.	neg.	neg.	neg.
A-96-301	8/20/96	Howard	C.v.	30	neg.	neg.	neg.	neg.
A-96-302	9/12/96	Howard	C.v.	14	neg.	neg.	neg.	neg.

NUMBER	DATE COLL.	SITE	SPP.	# IN POOL	ELISA	MOS. BHK	ECE. BHK	BHK
A-96-303	9/17/96	Howard	C.v.	13	neg.	neg.	neg.	neg.
A-96-304	8/13/96	Howard	C.v.	30	neg.	neg.	neg.	neg.
A-96-305	8/13/96	Howard	C.v.	9	neg.	neg.	neg.	neg.
A-96-306	8/13/96	Howard	mixed	10	neg.	neg.	neg.	neg.
A-96-307	8/20/96	Howard	C.v.	28	neg.	neg.	neg.	neg.
A-96-308	8/20/96	Howard	C.v.	30	neg.	neg.	neg.	neg.
A-96-309	8/20/96	Howard	C.v.	19	neg.	neg.	neg.	neg.
A-96-310	8/20/96	Howard	mixed	27	neg.	neg.	neg.	neg.
A-96-311	8/13/96	Howard	C.v.	30	neg.	neg.	neg.	neg.
A-96-312	8/13/96	Howard	C.v.	30	neg.	neg.	neg.	neg.
A-96-313	8/13/96	Howard	C.v.	12	neg.	neg.	neg.	neg.
A-96-314	8/13/96	Howard	mixed	12	neg.	neg.	neg.	neg.
A-96-315	10/23/96	Howard	C.v.	30	neg.	neg.	neg.	neg.
A-96-316	10/23/96	Howard	mixed	16	neg.	neg.	neg.	neg.
A-96-317	11/12/96	Howard	mixed	19	neg.	neg.	neg.	Neg.
A-96-318	11/12/96	Howard	C.v. & cock.	30	neg.	neg.	neg.	Neg.
A-96-319	11/12/96	Howard	C.v. & cock.	14	neg.	neg.	neg.	Neg.

Footnotes for Appendix G:

Number = Pool number as described in the flow diagram, Chapter 2.

Date Coll. = Date of the mud collection (NOT the date of pupal collection from the mud)

Site = Name of the site (Red Barn is less than 1 mile from the Howard dairy)

Spp. = Specie of the reared adults as determined by wing vein pattern.

Mos. BHK = One passage by intrathoracic inoculation of *Aedes triseriatus* mosquitoes, then three blind passages on BHK-21 cells.

ECE. BHK = One passage by intravenous inoculation of embryonated chicken eggs, then three blind passages on BHK-21 cells.

BHK = Three blind passages on BHK-21 cells.

Missing data are pools that were lost for any of several reasons during processing (freezer failure being the most common).

APPENDIX H: Cell culture maintenance and propagation protocols.

General Information for Baby Hamster Kidney Clone 21 cells:

BHK-21 cells grow rapidly, therefore they are routinely passed every 3-5 days. They will grow at a variety of temperatures: 33, 37, or 40°C. Normally, they are grown at 37°C.

When the cells are confluent, they may be passed or placed on maintenance medium.

I. Procedure for passing cells:

1. Pour off old medium into a sterile beaker.
2. Rinse the cell sheet with Saline-A.
3. Pour off the Saline A into the same beaker.
4. Add an appropriate amount of 1X trypsin. For a T-25 flask, none (you must scrape to remove the cells in a T25). For a T-75, 2.5-3ml. For a T-150, 5-6ml. For a glass flask, 11ml. Place the flask in a 37°C incubator for 5-10 minutes. Observe the cells after 5 minutes. If they have not completely detached, put back in the incubator for a few more minutes.
5. Add new growth medium to the flask. Use an amount equal to the amount of trypsin used in step number 4.
6. Place the appropriate amount of growth medium into your new flasks.
   - For T-25, 3ml total
   - For T-75, 10ml total (add a full 5ml pipette)
   - For T-150, 20ml total (add full 10ml pipette)
   - For glass, 60-75ml (enough to cover the bottom)
7. Now add cells according to your intended use. Usually, BHK-21 cells are split 1:10. This means you can make 10 new flasks from one old flask of the same size. We usually do not need to do this. Therefore we divide the cells into 10 parts, and use only one part to seed the new flask.
   - Please note that if you are stepping up to a larger flask, you will have to add an appropriate amount of cells to make up for the larger growth area. Generally, it is best to only step up one size at a time, for eg. from T-75 to T-150, not from T-25 to T-150.
8. Write the passage number, complete date, and your name on the flask.
9. Incubate your cells in the appropriate incubator until they are ready to split again. If you split cells at 1:10, they will be confluent in 3-5 days

Growth medium = L-15 base with 5% fetal bovine serum
Trypsin = Trypsin-EDTA (Sigma, others) diluted in Saline A to 1X concentration
Saline A:

- 8grams NaCl
- 0.4g KCl
- 1.0g Dextrose
- 0.35g $NaHCO_3$
- 0.005g Phenol Red
- Q.S. to 1 liter with ddH_20

- Filter sterilize with .22μm cellulose nitrate filter

II. Putting cells on maintenance medium.

If you do not want to split your cells when they are confluent, they may be placed on maintenance medium. This will slow down their growth. It is best also to incubate them at a lower temperature after they have been put on maintenance medium, either room temperature or 33°C will be adequate. It is also possible to leave the flask at room temperature for 1-3 days without changing the medium to maintenance medium.

To put cells on maintenance medium, simply pour off the old medium into a sterile beaker, or use a pipette (depending in the size of the flask) and add the appropriate amount of maintenance medium to the flask.

Maintenance medium = L-15 base with 2% fetal bovine serum

III. More general information for working with cell cultures.

- The culture medium should always be clear. If medium looks cloudy, it is probably contaminated.
- Phenol red is added to tissue culture medium as a pH indicator. This indicator is pink at high pH (7.6 and up), salmon at 7.2-7.6 and yellow-orange at 6.8-7.2. Below 6.8 it is bright yellow.
- The culture medium should always be maintained in a pH range of 7.0-7.6. If the pH drops below 7.0, the cells will probably die.
- When the cells are first seeded into the medium, the pH of the medium will be about 7.6. Thus the medium will be pink in color. As the cells grow, the pH will drop gradually. The color of the medium is a good indicator of how much your cells have grown.
- Avoid touching or passing your hand over an open medium bottle or tissue culture flask.
- Clean the hood with alcohol (70%EtOH) or Roccal before starting and after finishing.
- Wash your hands thoroughly.

General Information for C6/36 (*Aedes albopictus*) cells:

C6/36 cells grow fairly slowly, therefore they are routinely passed every 7-10 days. They will grow best at a constant (room) temperature which emulates the body temperature of the insect (usually 28°C).

When the cells are confluent, they may be passed or placed on maintenance medium.

I. Procedure for splitting cells:

1. Pour off old medium into a sterile beaker (leave some in to scrape the cells into).
2. Scrape cells gently off the surface of the flask with a sterile cell scraper..
3. Place the appropriate amount of growth medium into your new flasks (if needed).

- For T-25, 3ml total

- For T-75, 10ml total (add a full 5ml pipette)
- For T-150, 20ml total (add full 10ml pipette)
- For glass, 60-75ml (enough to cover the bottom)

4. Now add cells according to your intended use. Usually, C6/36 cells are split 1:5 to 1:10. This means you can (ideally) make 10 new flasks from one old flask of the same size. To be safe, however, a split level of 1:5 is preferred. Therefore we divide the cells into 5 parts, and use only one part to seed the new flask.

- Please note that if you are stepping up to a larger flask, you will have to add an appropriate amount of cells to make up for the larger growth area. Generally, it is best to only step up one size at a time, for eg. from T-75 to T-150, not from T-25 to T-150.

5. Write the passage number, complete date, and your name on the flask.
6. Incubate your cells in the appropriate incubator until they are ready to split again. If you split cells at 1:10, they will be confluent in 7-10 days

Growth Medium = L-15 base with 10% fetal bovine serum

II. Putting cells on maintenance medium.

If you do not want to split your cells when they are confluent, they may be placed on maintenance medium. This will slow down their growth.

To put cells on maintenance medium, simply pour off the old medium into a sterile beaker, or use a pipette (depending in the size of the flask) and add the appropriate amount of maintenance medium to the flask.

Maintenance Medium = L-15 base with 5% fetal bovine serum

General Information for CuVaW3 (*Culicoides sonorensis*) cells:

CuVaW3 cells grow fairly slowly, therefore they are routinely passed every 7-14 days. They will grow best at a constant (room) temperature which emulates the body temperature of the insect (usually 28-30^{o}C).

I. Procedure for splitting cells:

1. Pour off old medium into a sterile beaker (leave some in to scrape the cells into).
2. Scrape cells gently off the surface of the flask with a sterile cell scraper..
3. Place the appropriate amount of growth media into your new flasks (if needed).

- For T-25, 3ml total
- For T-75, 10ml total (add a full 5ml pipette)
- For T-150, 25ml total (add full 10ml pipette)

4. Now add cells according to your intended use. Usually, CuVaW3 cells are split 1:3. This means you can (ideally) make 3 new flasks from one old flask of the same size. Therefore we divide the cells into 3 parts, and use only one part to seed the new flask.

- Please note that if you are stepping up to a larger flask, you will have to add an appropriate amount of cells to make up for the larger growth area. Generally, it is

best to only step up one size at a time, for eg. from T-75 to T-150, not from T-25 to T-150.

5. Write the passage number, complete date, and your name on the flask.
6. Incubate your cells in the appropriate incubator until they are ready to split again. If you split cells at 1:3, they will be confluent in 7-10 days

Growth Med. = Schneider's Drosophila medium with 15% Fetal Bovine Serum and:
4.5mU/ml Bovine Insulin
6mg/ml Reduced Glutathione
30 mg/ml L-asparagine
5ml/L 7.5% sodium bicarbonate solution
2mM L-glutamine
Adjust to pH=6.7-6.8 with sterile 1N HCl

APPENDIX I - DNA VACCINATION FOR BT DISEASE

INTRODUCTION

DNA vaccination is a promising new approach utilizing genes that encode antigens from pathogenic agents to vaccinate against infectious diseases. The method has many advantages over other vaccination methods because it induces both humoral and cell-mediated immunity without using a potentially infectious agent. Cell mediated immunity is important because cytotoxic T-cells detect and destroy cells infected with viruses. Humoral immunity is essentially the production of antibodies that neutralize extracellular virus(9), but can also include antibody-dependent cellular cytotoxicity (ADCC) and complement (C') mediated cytotoxicity. Once a DNA vaccine has been injected, host cells take up the foreign DNA, express the viral gene, and make the encoded viral proteins within the cell(9). Since these encoded proteins are produced within the cytoplasm of the cells, they can be processed through MHC Class I presentation pathway and presented on the surface of the cell. These events begin priming the host immune response against the encoded antigen.

There are several factors that must be considered before producing a DNA vaccine construct. One of the most important factors is the choice of antigen encoded by the DNA that will be used as the gene of interest (GOI). Generally, those proteins present on the outer surface of the virus elicit both cytotoxic lymphocyte (CTL) and neutralizing antibody responses in the host. Proteins encapsidating the viral nucleic acid (nucleoproteins) and others present on the interior of the virion, elicit CTL's and non-neutralizing antibody that may have the potential to protect across different (but related) virus serotypes.

A second important factor is the method for inoculation of plasmid DNA. The initial design considerations for the DNA vaccine construct below were based on the intradermal (ID) route of injection. Many studies have used intramuscular (IM) or gene gun injection(12, 13), but we have chosen ID inoculation for a variety of reasons. Intradermal injections may have an advantage because of the high concentration of Langerhan's cells under the surface of the skin. Langerhan's cells are very similar to dendritic cells in their antigen presenting abilities(11). Direct inoculation of antigen presenting cells (APC) has been demonstrated to be the most efficient method in inducing immune responses(3, 4). In addition, ID injections have the fewest limitations in "field" situations because they are easier to administer.

Sindbis Virus Replicon System

While conventional DNA vaccines have yielded good immunological results, there are a few areas that need improvement. The minimum concentration of DNA used in these experiments is 50μg per injection, and occasionally multiple injections are required. One disadvantage of DNA vaccines is the possible production of anti-DNA antibodies, with a larger inoculum size obviously increasing this risk. In an effort to minimize this risk, our DNA vaccine will be modeled after one constructed by Hariharan et al.(7). This protocol uses the Sindbis virus replicon system to enhance cytoplasmic expression of the gene of interest (and the production of its encoded protein) above the level achieved by the CMV promoter alone. This would allow for a large increase in the amount of GOI mRNA produced in an infected cell, and thereby decrease the concentration of DNA that is necessary to one injection of 10ng of DNA.

The Sindbis virus genome is single stranded, positive sense RNA. Sindbis is a

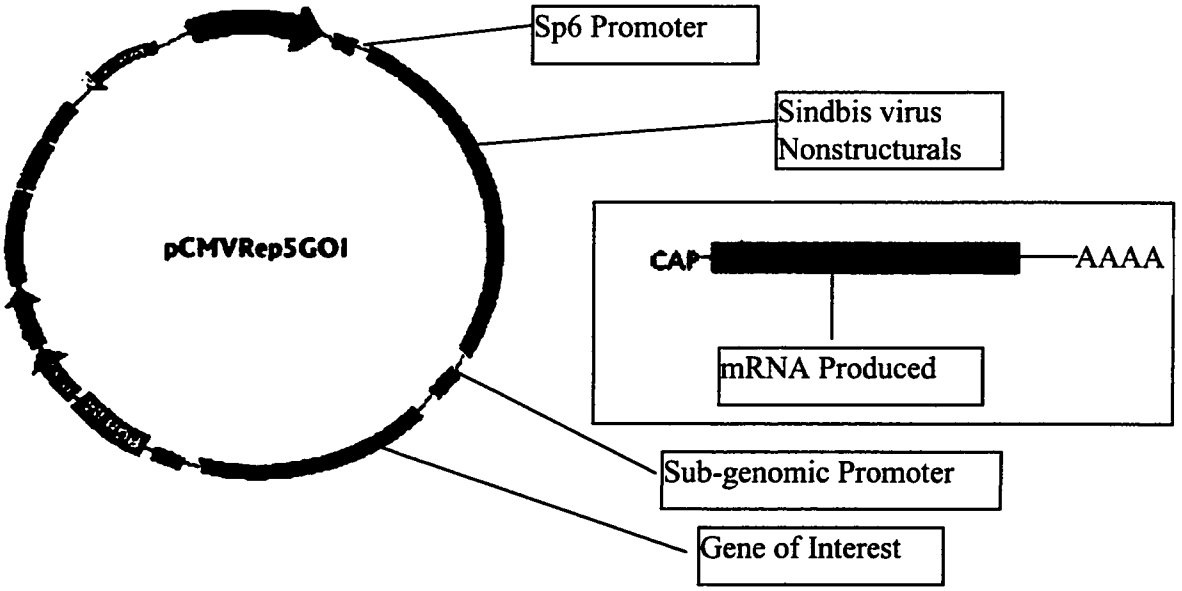

Figure I.1: pCMVRep5GOI. The Sp6 promoter is from the replicon backbone, and is used in the *in vitro* transcription of RNA species from the construct. As discussed in the text, the full length RNA species (Sindbis virus non-structural region + sub-genomic promoter + Gene of Interest) will be transcribed from the CMV promoter in eukaryotic cells, allowing translation and assembly of the Sindbis virus replication complex in the cytoplasm of the cell, and increased transcription of the GOI from the Sindbis sub-genomic promoter (relative to the amount of transcription from the CMV promoter alone). Genes of Interest for this work are to be the 426bp fragment of L3 (see Table 3.1), the 620bp fragment of S7 (see Table 3.1), but probably not a combination of the two. GOI's will be inserted using the strategy outlined in Materials and Methods.

member of the genus *Alphavirus* in the family Togaviridae(1). Scientists at AIDL have used this system extensively to study virus gene expression and viral interference in mosquitoes(10). The replicon construct diagrammed (Figure 4.1) above has a gene of interest in place of the genes encoding the structural proteins, therefore making the construct incapable of a productive infection.

After injection and uptake of the DNA by eukaryotic cells, the full-length RNA is transcribed from the human cytomegalovirus (CMV) promoter in the nucleus. The CMV promoter was chosen based on its use in other studies(7). Then, that mRNA is translated in the cytoplasm of the cell. The non-structural proteins from the Sindbis virus (colored in blue) then assemble to form the replication complex of the virus. This complex can initiate transcription at the sub-genomic promoter (present in the original, full-length mRNA) to increase expression of the gene of interest (colored in red) above the level that would be transcribed from the CMV promoter alone. As such, the GOI is expressed from the CMV promoter as in a normal DNA vaccine construct, but it then undergoes a cytoplasmic amplification due to the inclusion of the Sindbis virus replication complex. This increases the amount of mRNA in the cell greatly, and consequently should increase the amount of antigen produced. Consequently, less DNA is required in the initial injection, and the safety of the vaccine is increased without sacrificing its efficacy.

Vaccine Development Strategy

In the development of a BTV vaccine, the L3 genome segment was targeted, which encodes the "scaffolding" protein for viral assembly, and the S7 genome segment, which encodes the other major inner core protein, and that has a loop at the N terminus which is expressed on the surface of the virion (see Table 1.1). Both proteins (and

nucleic acid sequences) have been shown to be highly conserved both among serotypes and over time, and are good candidates for a DNA-based vaccination strategy. As was shown in Chapter 2 (see Figure 2.6), the nucleotide sequence of the 5' end of segment S7 varies between BTV serotypes, and as such may not represent an optimal vaccine target. However, it must be remembered that the N-terminus of VP7 is accessible from the surface of the virion, and as such can be accessed by antibodies directed against it. In fact, VP7 is considered a group-specific antigen(2); hence, when considered on the level of the encoded protein, it was felt that it would make an excellent protein for vaccination of vertebrate animals.

The use of gene fragments (instead of the whole coding sequence) as immunogens was shown to hold promise by Ciernik et al.(5). These researchers found that appropriately chosen fragments were as immunogenic and efficacious as whole genes, and size constraints (loss of stability of the integrated sequence above 1Kb – J. Rayner, personal communication) in our proposed vaccination strategy made this a desirable approach. Both gene fragments were cloned into a shuttle vector downstream of in-frame start codons. However, problems with RNA expression after transfection into BHK-21 cells negated completion of the construct. This material is presented here only to document the work done to date.

MATERIALS AND METHODS

The genes of interest from BTV11 Station Strain described above were amplified using RT-PCR and TA-cloned separately into pCR 2.1 (Invitrogen) following protocols outlined in Chapter 2. The L3 gene fragment had a start codon engineered into the forward primer for eventual protein fragment production; the S7 forward primer includes

the naturally occurring start codon. A shuttle plasmid was constructed by Dr. Kirsten Limesand (CSU-AIDL), to facilitate the ease of cloning into the final vaccine construct as well as to facilitate that cloning process for other GOI's in the future. There, she inserted the multiple cloning site (MCS) of the Sindbis virus replicon construct (based on the Proteus 1 plasmid originally isolated from *Proteus* vulgaris) into pBluescript (Stratagene). The gene fragments of both the L3 and S7 genomic segments of BTV11 have been inserted into this plasmid using the XbaI and SpeI sites at either end of the pCR2.1 multiple cloning site, and the XbaI site in the shuttle vector (XbaI and SpeI are isoschizomers). Since the gene fragments are now in the MCS of the replicon, transferring them to the final DNA vaccine construct (which is based on the replicon) could use any of the enzymes for which there are sites.

The following work was performed by Dr. Limesand before she left AIDL: the bovine growth hormone termination (poly A) sequence was cloned by PCR amplification with primers containing Not I sites and subcloned downstream from the gene of interest site in the construct. The CMV promoter was cloned by the use of primers with Sac I sites and inserted upstream of the replicon nonstructural genes and the Sp6 promoter (see Figure 4.1). The gene encoding green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* had previously been inserted into the GOI location on the construct(8). DNA transfection analysis was performed to confirm functionality of the CMV promoter, and was negative for GFP production. This work was then abandoned..

FUTURE WORK

Any future work would involve sequencing the replicon construct to look for obvious errors (inserted stop codons, insertions causing frame shift mutations, etc.) as well as studies quantifying RNA transcription and translation levels. Once activity of CMV promoter function has been confirmed, the GOI's could then be subcloned from the shuttle vector into the pRep5 construct using the strategy outlined in Materials and Methods. The following are the constructs that need to be completed for vaccinations:

- pCMVRep5VP3: CMV promoter driving the Sindbis non-structural genes with the L3 gene fragment from BTV11 downstream of the Sindbis subgenomic promoter.
- pCMVRep5VP7: Similar to above with the S7 gene fragment from BTV11 in place of the L3 gene fragment.

In vitro Analyses

Once the constructs are completed and tested, large quantities of high quality plasmid would be generated in transformed bacteria. Transfection of BHK-21 cells with a DNA plasmid would follow the protocol in Hariharan et al.(7) utilizing Lipofectamine (Gibco-BRL). There, cells would be fixed in acetone forty-eight hours post-transfection for ten minutes. Fluorescent antibody staining of transfected cells, following the protocol outlined in Chapter 3, would verify the expression of the inserted gene fragment. The polyclonal anti-BTV serum generated by el Hussein(6) would be excellent for this purpose, as it would be likely to pick up even the protein fragments that are expected. However, this would also be useful as an initial screening for the biological relevance of the protein fragment – if it was not assuming a native conformation recognizable by the antibody, there would be no use in further testing. Further verification of expression

from the DNA constructs would occur by Northern blot analysis. For this procedure, total RNA would be collected from the transfected cells and detection analysis would be performed using non-isotopic probes made from the original PCR primers of the BTV gene fragments. Then, successful candidate constructs would be available for later *in vivo* studies.

PLANS FOR FUTURE RESEARCH

Development of a Sindbis replicon based expression system for robust expression of BTV gene sequences would provide the critical preliminary information for submission of a grant proposal for vaccine development to USDA. It is important to note that development of this technology can also be exploited to construct DNA vaccines for other important pathogens affecting animals, such as vesicular stomatitis (VS) virus. For example, VS nucleoprotein and glycoprotein sequences, which could confer protective immunity, could quickly be incorporated into the DNA vaccine backbone and characterized. Such DNA vaccine approaches may provide an exciting new technology for efficacious immunization of animals.

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