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Isolation and evolutionary analysis of Australasian topotype of bluetongue virus serotype 4 from India

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Summary

Bluetongue (BT) is a *Culicoides*-borne disease caused by several serotypes of bluetongue virus (BTV). Similar to other insect-borne viral diseases, distribution of BT is limited to distribution of *Culicoides* species competent to transmit BTV. In the tropics, vector activity is almost year long, and hence, the disease is endemic, with the circulation of several serotypes of BTV, whereas in temperate areas, seasonal incursions of a limited number of serotypes of BTV from neighbouring tropical areas are observed. Although BTV is endemic in all the three major tropical regions (parts of Africa, America and Asia) of the world, the distribution of serotypes is not alike. Apart from serological diversity, geography-based diversity of BTV genome has been observed, and this is the basis for proposal of topotypes. However, evolution of these topotypes is not well understood. In this study, we report the isolation and characterization of several BTV-4 isolates from India. These isolates are distinct from BTV-4 isolates from other geographical regions. Analysis of available BTV seg-2 sequences indicated that the Australasian BTV-4 diverged from African viruses around 3,500 years ago, whereas the American viruses diverged relatively recently (1,684 CE). Unlike Australasia and America, BTV-4 strains of the Mediterranean area evolved through several independent incursions. We speculate that independent evolution of BTV in different geographical areas over long periods of time might have led to the diversity observed in the current virus population.

KEYWORDS
Australasia, bluetongue, bluetongue virus, BTV-4, India, isolation, RT–PCR, sequencing, typing

1 | INTRODUCTION

Bluetongue (BT) is caused by bluetongue virus (BTV), an *Orbivirus* belonging to family *Reoviridae*. The virus is transmitted by midges belonging to different species of the genus *Culicoides*. Similar to other insect-borne diseases, the virus distribution is limited to the distribution of its vector. However, only a limited number of *Culicoides* species are capable of transmission of BTV. BTV transmission-competent *Culicoides* are not present in extreme polar regions (Antarctica), New Zealand, Iceland, Patagonia and Hawaiian islands, and these places are hence free from BT (Mellor, Boorman, & Baylis, 2000). Among the different climatic areas, the tropics are the most suitable for breeding and propagation of *Culicoides*, leading to year-long occurrence of BT (Rao et al., 2017; Sellers, 1980). In general, several serotypes of BTV are in circulation in the tropics due to the congenial conditions for vector sustenance. The tropical regions of three continents, that is, Asia, Africa and America, are separated from each other as well as from other geographical regions either by oceans or by subtropical deserts that are not congenial for *Culicoides* propagation. This segregation of the three major tropical areas may have led to independent evolution of topotypes within different serotypes of BTV (Rao et al., 2017).

Bluetongue virus can broadly be classified into African, American, Australasian and Mediterranean topotypes based on geographical distribution (Rao et al., 2017). However, the distribution of several serotypes is not uniform in different geographical regions, and hence, topotypes within each serotype are not present in all geographical regions. BTV-1, BTV-2 and BTV-3 are exceptions and are found in all the four geographical regions.

Bluetongue virus genome consists of ten double-stranded RNA segments (seg) with each coding for at least one protein. The four major virus proteins (VP), VP-2, VP-3, VP-5 and VP-7 involved in capsid formation are coded by seg-2, seg-3, seg-6 and seg-7 respectively. The three minor viral proteins VP1, VP4 and VP6 are involved in replication of the viral genome and are coded by seg-1, seg-4 and seg-9, respectively. The three major non-structural proteins (NS) NS-1, NS-2 and NS-3 are coded by seg-5, seg-8, and seg-10 respectively (Roy, 1992; Roy, Marshall, & French, 1990). Apart from these proteins, seg-10 codes for NS3a which is produced from a different initiation codon in the same reading frame as that of NS3. Seg-9 and seg-10 code for NS4 and NS5 using a different reading frame than those used for coding VP6 and NS3, respectively (Belhouchet et al., 2011; Ratinier et al., 2011; Stewart et al., 2015). Among the major capsid proteins of BTV, VP2 and VP5 form the outer capsid and determine the serotype (Mertens et al., 1989). VP2 is the major serotype determining protein, whereas VP5 is shared among closely related serotypes (Cowley & Gorman, 1989; Shafiq, Minakshi, Anshul, Koushlesh, & Gaya, 2013; Shaw et al., 2013).

Twenty-seven serotypes of BTV have been recognized (Bumbarov, Golender, Erster, & Khinich, 2016; Chaignat et al., 2009; Erasmus, 1990; Hofmann et al., 2008; Maan et al., 2009; Maan, Maan, Nomikou, Batten et al., 2011; Maan, Maan, Nomikou, Veronesi et al., 2011; Savini et al., 2017; Schulz et al., 2016; Sun et al., 2016; Zientara et al., 2014). Although serotyping of BTV is based on the neutralization of virus using homotypic sera, typing based on reverse transcription–PCR and sequencing of seg-2 is gaining popularity. However, nucleic acid sequences of seg-2 of BTV isolated from different geographical areas differ significantly and the primers designed based on the reference viruses generally fail to detect different seg-2 topotypes of the same serotype (Maan et al., 2012, 2016; Mertens et al., 2007; Reddy et al., 2016). Hence, it is important to identify different topotypes and subtopotypes circulating in different geographical regions. In this study, we describe the isolation and partial characterization of BTV-4 from India. We compared seg-2 sequences of the viruses isolated between 2007 and 2013 with those of BTV-4 from other geographical areas. The Indian BTV-4 isolates were distinct from isolates of Africa, Americas and the Mediterranean and may represent an Australasian topotype.

2 | MATERIALS AND METHODS

2.1 | Isolation of bluetongue virus

Blood samples from sheep showing bluetongue-like symptoms were aseptically collected in ethylenediaminetetraacetic acid (EDTA) blood collection tubes and stored at 4°C until processing. Blood cells were pelleted by centrifugation at 1000 × *g* for 15 min at 4°C. The cell pellet was washed twice with phosphate-buffered saline (PBS, pH 7.4) and repelleted. Equal volume of sterile water was added to 500 μl of blood cell pellet to lyse the red blood cells (RBC). To the lysate, 50 μl of sterile 10 × PBS (pH 7.4) was added to make this lysate isotonic. KC cells (Wechsler & McHolland, 1988), maintained in T-25 tissue culture flask, were washed twice with PBS, and one millilitre of blood cell lysate along with 9 ml of Schneider's growth medium was added to the cells and incubated at 28°C (25-30°C) for 10 days. Cells were harvested on day 10 by scraping and resuspension by repeated pipetting. One millilitre of KC cell harvest was used to inoculate BHK-21 cells, incubated at 37°C, and the cells were observed daily for the appearance of cytopathic effect (CPE). BHK-21 cell culture supernatant was passaged further in fresh BHK-21 cells if CPE was not observed by the 10th day.

2.2 | Reverse transcription–polymerase chain reaction

Lysates of BHK-21 cells showing CPE characteristic of BTV infection were pelleted, and total RNA was isolated from the pellet using monophasic acid phenol guanidium thiocyanate method (Gauthami et al., 2015). RNA was tested for *Orbivirus*-like RNA pattern by agarose gel electrophoresis, and cDNA was synthesized from total RNA using RevertAid Reverse transcriptase (Thermofischer, Bengaluru, India) with random hexamers as primers. The isolates were confirmed as BTV by seg-5-specific PCR of the cDNA as described earlier (Katz, Alstad, Gustafson, & Moser, 1993; OIE, 2015). For serotyping, the cDNA was subjected to serotype-specific PCR for eight common serotypes (BTV-1, BTV-2, BTV-9, BTV-12, BTV-16,

BTV-21, BTV-23 and BTV-24) isolated from India during the last decade (Krishnajyothi et al., 2016; Reddy et al., 2016). BTV-4-specific primers for virus typing and seg-2 sequencing were designed based on the next-generation sequencing data of an Indian isolate of BTV-4 isolated in 2008 (Acc. No. KF560418) (Table 1). For typing of BTV isolates, cDNA was subjected to PCR with 40 μmol each of dNTPs, 10 picomoles of each primer, 1.25 units of JumpStart AccuTaq DNA Polymerase (Sigma, Bengaluru, India) in 25 μl of AccuTaq Buffer (Sigma) with 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s and extension at 68°C for 1 min, along with initial denaturation at 94°C for 3 min and final extension for 10 min at 68°C. The PCR amplicons were visualized after electroporation on 1% agarose gel.

2.3 | Sequencing and sequence analysis

To sequence seg-2 of BTV-4, cDNA of positive isolates was subjected to PCR to produce overlapping PCR amplicons (Table 1). The PCR conditions for amplification were similar to that of typing as described above, except that the 20 pmols of each primer was added in a 50 μl reaction with primer extension of 90 s at 68°C. PCR products were sequenced by conventional method using the same primers used for PCR amplification, and the sequences were assembled using SeqMan programme (DNA Star Inc, USA). Indian BTV sequences were analysed against BTV-4 sequences from China, Africa, the Mediterranean, Europe and the Americas. To classify Indian isolates into serotype and topotype, phylogenetic tree was constructed using maximum-likelihood (ML) method along with selected isolates from different geographical areas (Africa, America, Mediterranean and Australasia) (Data S1 and Table S1). For estimation of nucleotide substitution rate and time to most recent common ancestor (tMRCA) of seg-2 of BTV-4, Markov chain Monte Carlo (MCMC) method was used using BEAUti and BEAST Programme (Drummond, Suchard, Xie, & Rambaut, 2012). As estimation of the substitution rates can be seriously affected by inclusion of isolates derived from modified live virus (MLV) vaccine strains (Hicks & Duffy, 2012), virus sequences that are nearly identical with MLVs or viruses used as MLVs were excluded during estimation of substitution rates. Sequences were subjected to GARD programme (Pond, Posada, Gravenor, Woelk, & Frost, 2006) using Datamonkey Suite (Delport, Poon, Frost, & Kosakovsky Pond, 2010) to eliminate sequences suspected to have undergone recombination. Selected sequences (Data S1 and Table S2) were aligned using ClustalW (Larkin et al., 2007), followed by selection of best

TABLE 1 Primers used for RT–PCR-based typing and sequencing of seg-2 of BTV-4

Primer designation	Primer sequence (5′-3′)	Position in the genome		Reference/sequence
BTV—group-specific primers				
BTVNS1A	GTTCTCTAGTTGGCAACCACC	10	30	Katz et al., 1993
BTVNS1B	AAGCCAGACTGTTTCCCGAT	283	264	
RT–PCR typing Primers (BTV-4 eastern)				
BTV04/644-663F	GTTGGATCTGAGAAATGGGT	644	663	Current study KF560418
BTV04/1108-1089R	AAGACACGGATAAGGATTCG	1,108	1,089	
Sequencing primers (BTV-4 eastern)				
BTV04 F1	GTTAAAAGAGTGTTCCATCAT	1	21	Current study KF560418
BTV04 R1	AAGGATTCGTTCTCCAAACT	1,078	1,097	
BTV04 F2	GCATTACGCCGTGAACCATC	391	410	
BTV04 R2	AAGGCGTCCTTCTCGAAGTC	1,499	1,518	
BTV04 F3	GTGCAGCTTACGATCGGAGT	816	835	
BTV04 R3	TTAATGCCAGGGCTACTCGC	1,879	1,898	
BTV04 F4	AATGCGAGTTGGGTGACGTA	1,131	1,150	
BTV04 R4	TCACGTACTTGCTGCACCTT	2,189	2,208	
BTV04 F5	TGGACGAGCCGATAGAGACA	1,308	1,327	
BTV04 R5	TGAGTCACCGGGGTGAAAAC	2,385	2,404	
BTV04 F6	CACGGAGAGATCGGTACAGC	1,607	1,626	
BTV04 R6	CTTCTTTGGATGCGCGACAG	2,592	2,611	
BTV04 F7	GTCAGCAGGAGGACTTTCCA	1,791	1,810	
BTV04 R7	GTAAGTGTAAGAGGCCACCGA	2,906	2,926	
BTV04 3′F	TCAGGGCTTGCCGATTCTAT	2,558	2,577	
BTV04 5′R	CGCCTTACTTTCCACCGGAT	474	493	

substitution model with lowest Bayesian information criterion (BIC) score using MEGA 6.0 (Tamura, Stecher, Peterson, Filipski, & Kumar, 2013). Initially, using MCMC methods, nucleic acid substitution rate excluding MLV- and MLV-like strains was estimated. Later, estimated nucleic acid substitution rate was used to estimate tMRCA of different topotypes of global isolates including MLV- and MLV-like viruses using MCMC methods. During estimation of nucleic acid substitution rates as well as tMRCA, chain length of 100 million was used to achieve estimated sample size (ESS) of >200. Input file (.xml file) with details of parameters used for MCMC analysis by BEAST 1.8 for estimation of nucleic acid substitution rates (Data S2) and tMRCA (Data S3) are shown as supplementary data.

3 | RESULTS

Blood samples from sheep showing BT-like symptoms were collected from several outbreaks from different parts of Andhra Pradesh and Telangana states in South India, and virus isolation was accomplished by passaging the RBC lysates in KC cells followed by BHK-21 cells. All the isolates (n = 108) were tested by RT–PCR with primers against eight serotypes of BTV (BTV-1, BTV-2, BTV-9, BTV-12, BTV-16, BTV-21, BTV-23, BTV-24) circulating in India during the last decade (Krishnajyothi et al., 2016; Reddy et al., 2016). Sequence generated from one of the previously untyped virus isolates using next-generation sequencing platform revealed that the isolate was related to BTV-4 (Acc. No. KF560418). These sequence data were used to design a set of primers (Table 1) which were used to type the 108 isolates, of which, 45 were positive with the newly designed BTV-4 primers. Apart from BTV-4-like isolates, BTV-1, BTV-2, BTV-9, BTV-12, BTV-16, and BTV-24 were also isolated. Some of the samples (n = 13) which yielded virus contained more than one serotype, suggesting mixed infections (Data S1 and Table S3, Figure S1). The isolates were also positive with real-time PCR developed based on a Chinese BTV-4-like isolate (Maan et al., 2016). Seg-2 of ten of our BTV-4-like isolates was amplified and sequenced by conventional sequencing (Acc.No. KY947342-51), aligned and subjected to phylogenetic analysis.

Seg-2 sequences of the Indian isolates were closely related to the BTV-4-like isolates of China (>90% nucleotide [nt] similarity). The Indian and Chinese isolates were distantly related to BTV-4 isolates of South Africa, the Mediterranean and the Americas (72% nt similarity) and to BTV-17, BTV-20 (71% nt similarity), BTV-10 and BTV-11 (69% nucleotide similarity). The translated seg-2 sequence of the Indian BTV-4 isolates had >98% sequence similarity with the Chinese isolates, 79%-81% similarity with the African, Mediterranean and American isolates of BTV-4 and 71%-76% similarity with BTV-10, BTV-11, BTV-17 and BTV-20. Phylogenetic analysis also indicated that the Indian viruses analysed in this study grouped together with the Chinese isolates, whereas the BTV-4 isolates of African, Mediterranean and American origin grouped separately (Figure 1). Based on the sequence similarities and phylogenetic analysis, the Indian and Chinese isolates could be grouped under the Australasian topotype of BTV-4 (Table 2).

To estimate the nucleotide substitution rates for seg-2 of BTV-4, sequences of BTV-4 isolated from different parts of the world were analysed using the MCMC method. South African reference virus, MLV vaccine strains and some of the European isolates similar to MLV were not included for this analysis. The estimated nucleotide substitution rate was 2.0×10^{-4}/nucleotide/year (Table 3), similar to those estimated for different genomic segments of BTV earlier (Boyle et al., 2014).

Using the estimated nucleotide substitution rate, all BTV-4 isolates including South African reference strains, vaccine viruses and related vaccine-like viruses were also analysed by MCMC method, and tMRCA was estimated for sub-Saharan African, Australasian, American and Mediterranean isolates (Table 4) (Figure 2). All the estimations are based on the date of isolation of the latest BTV isolate used for analysis (2014). As per the tMRCA estimation, Australasian viruses diverged from the rest of BTV-4 about 3,531 years (1517 [2218-851 BCE]) ago. However, Australasian viruses for which sequence data are available have a most common recent ancestor circulating about 277 years (1735 CE [1699-1775 CE]) ago. The American viruses formed a single cluster with an estimated tMRCA of about 162 years (1852 CE [1828-1875 CE]). The Mediterranean and European viruses clustered into several groups which were distinct from the South African reference virus and related viruses used as MLV in the Mediterranean and Europe.

4 | DISCUSSION

Tropical regions of America, Africa and Asia are the three major endemic areas of BTV, where year-long activity of the virus and its vector is observed. Apart from these hot spots, the Mediterranean region forms another important endemic area owing to its proximity to Asian and African tropics and European temperate areas. These BT endemic tropical areas are physically separated by tropical deserts (Asia and Africa) and oceans (America with Asia and Africa). The physical separation may prevent free movement of BTV among these major endemic areas, potentially resulting in independent evolution of BTV strains in these episystems, and may lead to independent evolution of geo-specific topotypes (Rao et al., 2017).

Bluetongue virus is broadly classified into eastern and western topotypes, and the topotype variation is observed for all the ten genome segments of the virus (Maan et al., 2008). Variations in seg-2, which codes for the major serotype determining protein VP2, are used for topotype classification of various serotypes of BTV. Among the known 27 serotypes, BTV-1, BTV-2, BTV-3, BTV-9, BTV-15 and BTV-16 have been reported to have evolved as eastern and western topotypes, and the rest of the serotypes are classified either as eastern (BTV-20, BTV-21, BTV-23) or as western (BTV-4, BTV-5, BTV-6, BTV-7, BTV-8, BTV-10, BTV-11, BTV-12, BTV-13, BTV-14, BTV-17, BTV-18, BTV-19, BTV-22, BTV-24, BTV-25, BTV-26, BTV-27) topotypes (Maan et al., 2009). Here, we report that BTV-4 isolates from

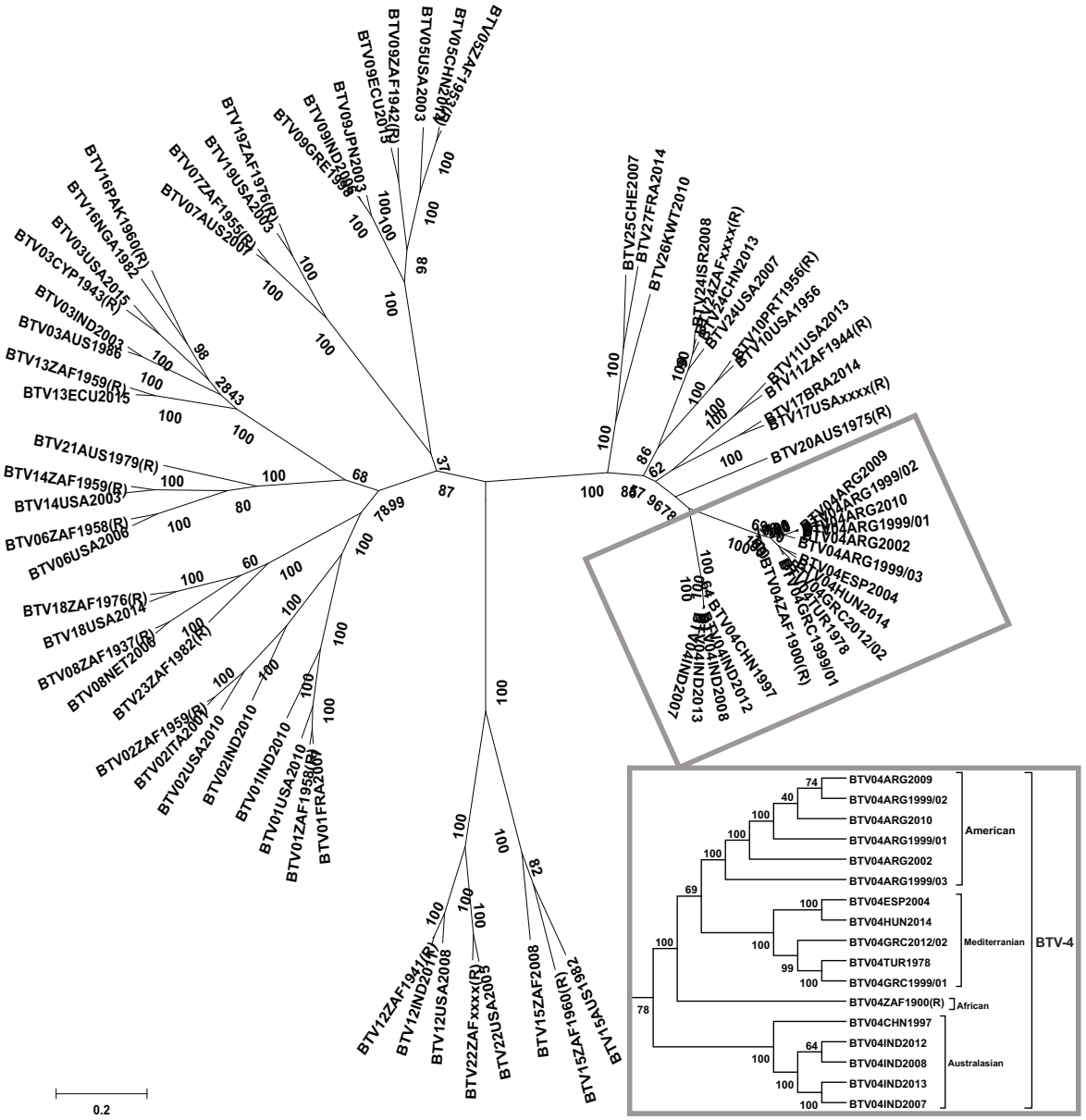

FIGURE 1 **Maximum-likelihood tree of different serotypes and topotypes of BTV-4 in relation to other serotypes.** Maximum-likelihood tree for nucleic acid sequences of seg-2 of BTV were constructed using MEGA 6.0. Phylogenetic relations of BTV-4 from different geographical regions (Africa, America, Mediterranean and Australasia) depicting their topotypes are shown (Box)

TABLE 2 Sequence similarities (%) seg-2 of BTV-4 isolated from different geographical areas

	Sub-Saharan African	Mediterranean	American	Australasian
Sub-Saharan African	99.8-99.9	88.3-90.6	89.1	72.2-72.6
Mediterranean		92.5-100	88.8-91.4	71.3-73
American			92.5-100	71.3-72.3
Australasian				91.2-99.8

India show up to 27% nucleotide sequence variation in seg-2 with their western counterparts (sub-Saharan African, Mediterranean and American isolates). The phylogenetic analysis indicates that the isolates can be classified as Australasian (eastern) topotype of BTV-4. With this report, BTV-4 has now been reported from all the four BTV episystems and would be a good model to study the evolution of BTV topotypes.

BTV-4 is the first BTV to be identified in South Africa in 1,900 (Theiler/79043) and probably the oldest BTV isolate available (Coetzee, Stokstad, Venter, Myrmel, & Van Vuuren, 2012). This strain was passaged in sheep, and infected sheep blood was used as a

TABLE 3 Bayesian estimates of nucleotide substitution rate of seg-2 of BTV-4

Parameter	Value
Mean	2.0595E-04
Standard error of mean	1.1162E-06
Standard deviation	3.5027E-05
Variance	1.2269E-09
Median	2.0474E-04
Geometric mean	2.0294E-04
95% HPD interval	1.3754E-4 to 2.7341E-4
Autocorrelation time (ACT)	91399.7776
Effective sample size (ESS)	984.70

vaccine for several decades in South Africa. Later, this strain was adopted, further attenuated by passaging in embryonated chicken eggs and used for MLV vaccine production in mammalian cells (Oya et al., 2009; Verwoerd, 2012). BTV-4 is endemic in South Africa and is reported to be one of the common serotypes encountered, and an MLV vaccine is in use as part of a multivalent vaccine along with other common serotypes present in the country (Coetzee et al., 2012; Van den Bergh, Coetzee, Guthrie, le Grange, & Venter, 2016). Apart from this virus, only one sequence of BTV-4 isolated in 2011 from South Africa is available (Van den Bergh et al., 2016) from this region.

Apart from sub-Saharan Africa, BTV-4 is the oldest and the most important circulating serotype recognized in the Mediterranean episystem, causing severe outbreaks in the Mediterranean, the Middle East and southern Europe (Breard et al., 2007; Gomez-Tejedor, 2004; Mellor, Carpenter, Harrup, Baylis, & Mertens, 2008; Mellor & Wittmann, 2002; Saegerman, Berkvens, & Mellor, 2008). Although anti-BTV-4 antibodies were reported from Cyprus as early as 1967 (Sellers, 1975), the first BTV-4-associated outbreak was reported in 1969 (Sellers, 1975). Later, this serotype was implicated in outbreaks during 1979, got established in the Mediterranean and invaded southern Europe on several occasions (Mellor et al., 2008; Nomikou, Mangana-Vougiouka, & Panagiotatos, 2004), the latest invasion being since 2014 (Hornyak et al., 2015). South African MLV vaccine was used in Israel and parts of Europe to control this serotype (Savini, MacLachlan, Sanchez-Vizcaino, & Zientara, 2008), and some of the isolates from this region are suspected to have evolved from MLV.

The available data indicate that several BTV-4 lineages have been circulating in the Mediterranean during 1979-2014 (Barros et al., 2007; Breard et al., 2007; Hornyak et al., 2015; Lorusso et al., 2013; Saegerman et al., 2008). Although some of the isolates from the Mediterranean and Europe are similar to South African reference or vaccine strains, the phylogenetic analysis indicates that the MLV vaccine strain is not the source for most of the lineages of BTV-4 which evolved in this region. The available data indicate that the Mediterranean BTV-4 isolates are different from the historic BTV-4 reference strain isolated from South Africa in the year 1900 (Acc no JX272580). These Mediterranean lineages could have evolved from incursions of BTV-4 from sub-Saharan Africa at different time points. The estimated tMRCA of the Mediterranean isolates indicates that ancestors of the Mediterranean lineages could have entered this region in the years 1959 (1950-1967), 1990 (1986-1994) and 2007 (2005-2010) CE. Interestingly, the recent Hungarian isolate (Hornyak et al., 2015) (ACC. No. KP268815) is different from the hitherto circulating BTV-4 lineage viruses of the Mediterranean. The Hungarian isolate is more similar to a field isolate of 2011 from South Africa (Acc. No. KT317666) and Sudanese isolates (Acc. No. AJ585166, KP821071) rather than to the strains of BTV-4 which have been circulating in the Mediterranean, and this Hungarian isolate may represent a new incursion.

In the Americas, BTV-4 was first reported from a quarantine station in Florida, USA, from Zebu cattle imported from Brazil in 1980 (Groocock & Campbell, 1982). Later, this serotype was isolated from Puerto Rico and the Dominican Republic in 1990, from Argentina in 1999-2010 and from Brazil in 2014 (Balaro et al., 2014; Lager et al., 2004; Legisa, Gonzalez, De Stefano, Pereda, & Dus Santos, 2013; Legisa, Gonzalez, & Dus Santos, 2014; Mo et al., 1994). Serological survey of Central and South American countries also indicated the prevalence of antibodies against this serotype in some of the countries (Trinidad and Tobago, Barbados, Puerto Rico, Dominican Republic, Argentina, Brazil) in this region (Thompson, Mo, Oviedo, & Homan, 1992). tMRCA analysis of the American strains of BTV-4 suggests that they have evolved from a common ancestor circulating in the year 1852 (1828-1875 CE), indicating that BTV probably entered the Americas almost hundred years before it was reported first from the United States in the year 1952 (Price & Hardy, 1954). The American topotypes diverged from the sub-Saharan african 2 and Mediterranean topotypes as early as 1684 (1650-1717) CE. In Australasia, BTV-4 has been reported from India in the years 1973 and 1975 (Prasad, Jain, & Gupta, 1992; Rao et al., 2016) and from China in the year 1996-97 (Yang et al., 2012). During the current study, we isolated several Australasian topotype strains of BTV-4 during BT outbreaks in 2007 to 2013 from the states of Andhra Pradesh and Telangana, India. Although the virus was isolated sporadically up to 2012, it appeared to have caused widespread outbreaks during the year 2013 in most parts of Telangana and Andhra Pradesh, as well as in the neighbouring state of Tamil Nadu. The estimated tMRCA of Asian (India and China) isolates indicates that they evolved from a common ancestor circulating in the year 1735 (1699-1775 CE). Estimated tMRCA of the BTV-4 circulating in southern India indicates that these viruses probably evolved from a common ancestor circulating in the year 1977 (1968-1985). Interestingly, this reflects the initial reports of BTV-4-associated outbreaks from India occurring during 1973 and 1975 (Prasad et al., 1992; Rao et al., 2016).

To conclude, a new eastern topotype (Australasian) of BTV-4 has been identified in India and viruses belonging to this topotype are also circulating in China. The data indicate that the Asian viruses diverged from African viruses more than 3,500 years ago, whereas the American viruses diverged from African viruses relatively recently (1684 [1650-1717] CE). Unlike Australasian and American

TABLE 4 Bayesian estimates of tMRCA for seg-2 of BTV-4

		Australasian (Eastern)		Western						
Topotype	ALL BTV4	All Australasian	Indian	All Western	American	Mediterranean 1 (1978-2004)	Mediterranean 2 (2003-2010)	Mediterranean 3 (2011-2012)	SS Africa 1	SS Africa 2
Mean Years to 2014 (calendar year)	3,531 (1,517 BCE)	277 (1,735 CE)	37 (1,977 CE)	403 (1,611 CE)	162 (1,852 CE)	55 (1,959 CE)	24 (1,990 CE)	7 (2,007 CE)	115 (1,899 CE)	119 (1,895 CE)
Standard error of mean	5.07	0.11	0.03	0.12	0.05	0.03	0.03	0.01	0.01	0.0651
Standard deviation	354	19	4	19	12	4	2	1	1	8.4858
Variance	1,25000	371	19	347	144	20	5	2	1	72.008
Median Years to 2014 (calendar year)	3,503 (1,489 BCE)	276 (1,738 CE)	37 (1,977 CED)	402 (1,612 CE)	162 (1,852 CE)	55 (1,959 CE)	24 (1,990 CE)	6 (2,008 CE)	115 (1,899 CE)	119 (1,895 CE)
Geometric mean Years to 2014 (calendar year)	3,513 (1,499 BCE)	276 (1,738 CE)	37 (1,977 CE)	403 (1,611 CE)	162 (1,852 CE)	55(1,959 CE)	24(1,990 CE)	6(2,008 CE)	115 (1,899 CE)	119 (1,895 CE)
95% HPD Interval Years to 2014 (calendar year)	2,865- 4,232 (2,218-851 BCE)	239- 315 (1,699-1,775 CE)	29- 46 (1,968-1,985 CE)	367- 439 (1,575-1,647 CE)	139-186 (1,828-1,875 CE)	47- 64 (1,950-1,967 CE)	20-28 (1,986-1,994 CE)	4-9 (2,005-2,010 CE)	114-117 (1,897-1,900 CE)	103-137 (1,877-1,911 CE)
Autocorrelation time (ACT)	18,461	3,026	3,266	3,806	1,867	5,113	20,165	10,363	4,851	5303.7936
Effective sample size (ESS)	4,875	29,743	2,7556	23,645	48,204	17,602	4,463	8,685	18,554	16969.1747

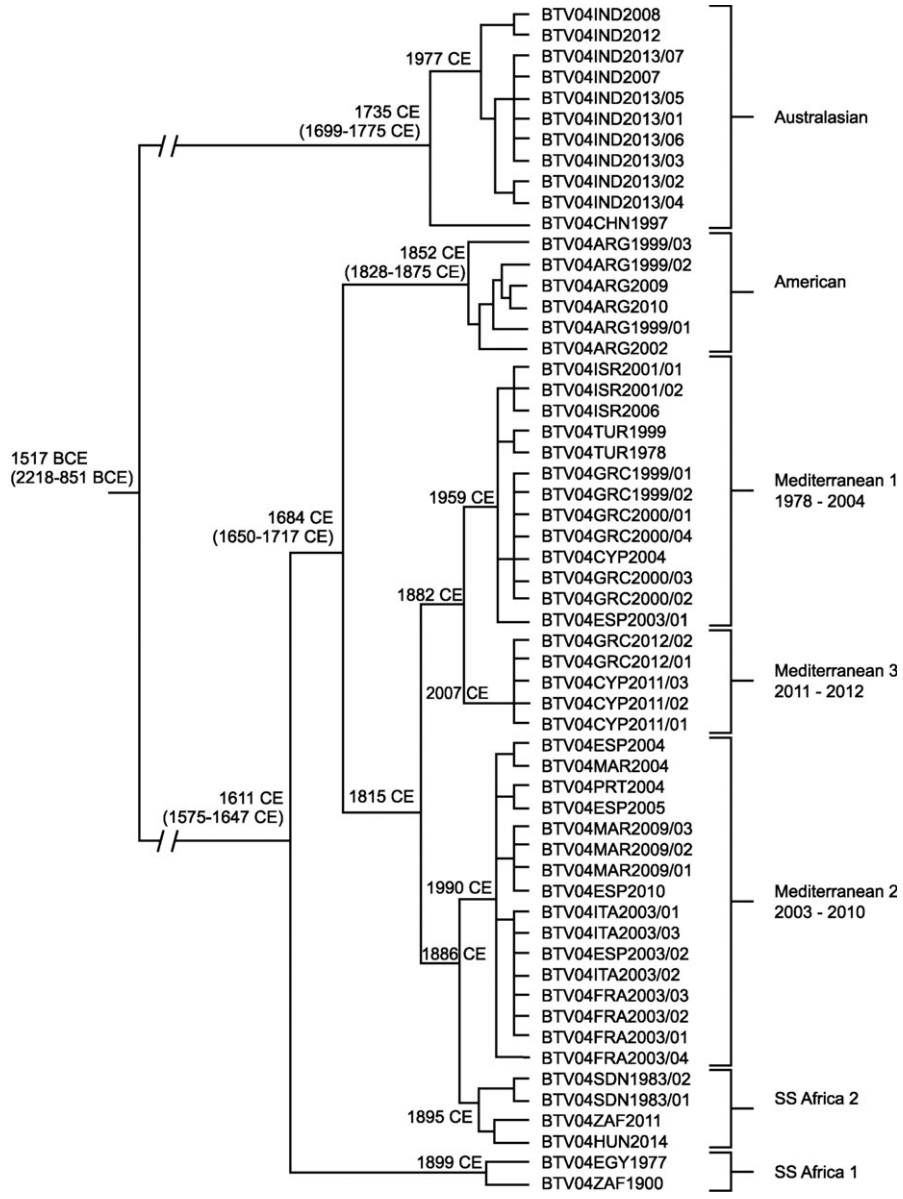

FIGURE 2 **Phylogenetic analysis of seg-2 sequences of BTV-4 from different geographical regions.** tMRCA of seg-2 sequence of BTV-4 isolates from different geographical regions were analyzed by Markov chain Monte Carlo (MCMC) methods using BEAST 1.8. tMRCA is converted to calendar years using year of isolation of latest isolate analyzed (2014). 95% HPD interval is provided in the brackets. Analysis details are provided in Table 4

BTV-4, the Mediterranean BTV-4 strains appeared to have evolved through several independent incursions. Apart from BTV-4, presence of geo-specific serotypes of BTV in Australasia (BTV-1, BTV-2, BTV-3, BTV-4, BTV-9, BTV-16, BTV-21, BTV-23), and Africa and the Mediterranean (including Europe and the Middle East) (BTV-25, BTV-26 and BTV-27) and their absence in the Americas, one can speculate that BTV is present in Africa and Asia (old world) since thousands of years and entry of the virus into the Americas is recent and probably happened after the European invasion of the Americas.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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