Molecular characterisation of lineage IV peste des petits ruminants virus using multi gene sequence data

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ABSTRACT

Peste des petits ruminants is responsible for an economically important plague of small ruminants that is endemic across much of the developing world. Here we describe the detection and characterisation of a PPR virus from a recent outbreak in Tamil Nadu, India. We demonstrate the isolation of PPR virus from rectal swab and highlight the potential spread of disease to in-contact animals through faecal materials and use of faecal material as non-invasive method of sampling for susceptible wild ruminants. Finally we have performed a comprehensive 'multi-gene' assessment of lineage IV isolates of PPRV utilising sequence data from our study and publically available partial N, partial F and partial H gene data. We describe the effects of grouping PPRV isolates utilising different gene loci and conclude that the variable part of N gene at C terminus gives the best phylogenetic assessment of PPRV isolates with isolates generally clustering according to geographical isolation. This assessment highlights the importance of careful gene targeting with RT-PCR to enable thorough phylogenetic analysis.

1. Introduction

Peste des petits ruminants virus (PPRV), a member of the morbillivirus genus, causes an acute and devastating viral disease of small ruminants. The morbillivirus genome consists of a single strand of negative sense RNA of approximately 16 kilobases containing six genes, encoding six to 8 proteins. The first clinical description of PPR was made in West Africa in the 1940s although it was not until 1979 that the virus was characterised as a distinct entity (Gibbs et al., 1979). Since then the virus has been reported in many countries across Sub Saharan Africa, the Middle East, the Arabian Peninsula and Asia (Banyard et al., 2010). The virus exists as one serotype, but genetically divides into four distinct lineages.

Within India, the disease was first reported in Southern India (Shaila et al., 1989) before being detected in northern India during 1994 (Nanda et al., 1996) and later across the Indian sub-continent (Dhar et al., 2002; Nanda et al., 1996; Roy et al., 2010; Sreeramulu, 2000; Taylor et al., 2002).

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Recent estimates on the potential impact of PPR have suggested that 63% of the small ruminant populations of Southern Africa, central Asia, South-east Asia, China, Turkey and Southern Europe at risk to PPR as determined by the Food and Agriculture Organisation (Libeau et al., 2014).

The present study describes an outbreak of PPR during 2011 in the Coimbatore District of Tamil Nadu, India. Further to this we have analysed the rectal swabs from the infected goats in the outbreak for the presence of both viral antigen and live virus and hypothesise that live virus may be present in faecal matter that may plays a role for spreading the diseases. Finally we have genetically analysed the sequence derived from the virus of this outbreak and shown it to be a lineage IV virus. With this new genetic data we have then performed an extensive phylogenetic comparison of available data from the GenBank and generated phylogenetic trees for global PPRV isolates (lineage IV) using partial gene sequences of the nucleocapsid (N), fusion (F) and haemagglutinin (H) genes of PPR. Finally we have compared the grouping of Asian virus isolates, focussing on those derived from Indian PPRV outbreaks to investigate differences in phylogenetic grouping of isolates. Previous phylogenetic studies of lineage IV PPR have been limited in the availability of data for comparison (Balamurugan et al., 2010; Dhar et al., 2002; Kerur et al., 2008; Shaila et al., 1996; Nanda et al., 1996). The phylogenetic analyses of the global PPRV sequences presented here benefit from the increased reporting and characterisation of PPR and, comparing multiple genetic loci give a comprehensive overview of the geographic distribution of lineage IV PPRV and the relatedness of isolates across Asia.

2. Material and methods

2.1. Outbreak information and clinical disease

In the Coimbatore District of Tamil Nadu, India, a total of 40 non-descript goats were purchased from a local market at Coimbatore and after one and half months the animals developed clinical illness consistent with infection with PPRV. After the onset of clinical signs specific to PPR (Pope et al., 2013) 18 goats died within a period of six days and another 18 were ailing. A detailed post mortem examination was conducted to assess the cause of death. The gross lesions were recorded and both clinical signs and post mortem lesions were suggestive of PPR. Faecal swabs, ocular swabs, nasal swabs and faeces were collected from sick animals whereas tissues were collected from the spleen and mesenteric lymph nodes from dead animals. All samples were stored in phosphate buffered saline (PBS) and maintained at 4 °C during transport to the laboratory.

2.2. Virus isolation in cell culture

Vero cells were grown in tissue culture flasks (25 cm^2) and on cover slips. For virus isolation from post mortem tissue, homogenate was filtered through a 0.22 μm membrane and inoculated onto a confluent monolayer. For detection of viral material in faecal matter, rectal swabs were transported to the laboratory on ice. The faecal material and rectal swab samples were clarified by centrifugation before being filtered and inoculated onto a confluent monolayer.

For all samples, the inoculated monolayers were incubated at 37 °C and checked regularly for the detection of cytopathic effect (CPE). In the absence of CPE, and where monolayers required splitting, cells were passaged until CPE consistent with morbillivirus infection was seen. Uninoculated Vero cells were treated in the same way and acted as a negative control. The Vero cells propagated on cover slips that developed CPE were fixed and stained using standard techniques (Pope et al., 2013).

2.3. Reverse transcriptase-polymerase chain reaction (RT-PCR) and sequencing

The presence or absence of PPRV nucleic acid in tissue samples, oculo and nasal swabs and faecal swabs was assessed by RT-PCR. RNA was extracted using Trizol (Invitrogen) from all samples collected from the outbreak. Isolated RNA was subjected to RT-PCR using PPRV partial N (Couacy-Hymann et al., 2002) and F (Forsyth and Barrett, 1995) gene primer sets. The partial H gene was amplified by PCR using the forward primer—5′ CCGGCATGGATCTT-TACAACAAC 3′ and the Reverse primer—5′ ATGACGC-CAAGGGAAACACTCTAT 3′ designed using the DNAstar software.

Alongside this, to provide genetic material for H gene phylogenetic analysis, historical PPRV samples from lineage III isolates (Oman 1983 and UAE 1986) were analysed by RT-PCR to determine H gene data for lineage III. The cDNA was prepared using Revert Aid First strand cDNA synthesis Kit (Fermentas, USA). Briefly, 10 μl of RNA was mixed with 1 μl of random hexanucleotide primers (50 pmol/μl), 1 μl 5× reaction buffer, 1 μl RNAse inhibitor, 1 μl Moloney's Murine Leukaemia Virus (MMLV) Reverse transcriptase and 2 μl 10 mM dNTP mix and the first strand synthesis reaction was carried out as per the manufacturer's instructions. The cDNA was stored at −20 °C until use. PCR reaction mixes were made as follows: 5 μl cDNA, 1 μl forward primer (10 pmol/μl), 1 μl reverse primer (10 pmol/μl), 10 μl Amplicon red dye Master mix (2×) and 5 μl nuclease free water. The PCR reaction was carried out in an Applied Bio system thermo cycler with the following thermal cycling conditions: 95 °C for 3 min for initial denaturation, 94 °C for 30 s, 50 °C for 30 s, 72 °C for 30 s for 30 cycles and final extension at 72 °C for 5 min. The PCR product was analysed on a 2% agarose gel containing ethidium bromide (2.5 μg/ml) at 100 mV for 45 min in 1× TAE buffer along with 100 bp DNA marker. The amplicons were viewed under a UV transilluminator.

The resulting PCR products were purified using gel extraction kit (RBC Real Biotech, USA) according to the manufacturer's instructions. The eluted DNA was stored at −20 °C until use. The purified products were then sequenced using Big Dye-Terminator® v3.1 Cycle Sequencing Reaction Kit on ABI-3730 DNA Analyzer (Applied Biosystems) following the manufacturer's instructions.

2.4. Sequence data sets

The PPRV (Lineage IV) N, F and H gene nucleotide sequence data available at GenBank were selected based on the criteria of the information available regarding the location and the year of isolation. The retrieved data were aligned separately using the Clustal-W algorithm incorporated in BioEdit software version 7.0.5.3 and MEGA 5.2. Phylogenetic analyses was carried out using the neighbour joining method with nucleotides 1253 to 1470 of the N gene, nucleotides 1372 to 1670 of the H gene and nucleotides 256 to 575 of F gene (all regions numbered according to the start codon in each gene) following the Kimura 2-parameter nucleotide substitution model. Sequences that did not fit these parameters or containing gaps were removed from the data set. In addition partial H gene sequences of two historic isolates from Oman (1983) and UAE (1986) and a new isolate from a 2011 outbreak in Tamil Nadu, India were included in the analysis. Neighbour-joining trees were then constructed using the distance matrices in MEGA 5.2 with 1000 bootstrap replicates to test the robustness of the tree topology.

3. Result and discussion

3.1. The 2011 Tamil Nadu PPR outbreak virus

The molecular characterisation of the outbreak material confirmed that the virus involved displayed similar properties similar to previously reported outbreaks of PPRV (Dhar et al., 2002; Kulkarni et al., 1996). Despite the high morbidity (90%) and mortality (45%) rates, infection appeared to be less severe than earlier reports of infection in Tellichery goats (Roy et al., 2010). Variability in mortality and morbidity seen in PPRV outbreaks may depend on numerous factors such as breed susceptibility, the genetic composition of the infecting virus, the presence of pre-existing parasitic pathogens and the immunological status of the animals infected (Banyard et al., 2010). Samples from the outbreak were investigated using RT-PCR targeting the nucleocapsid (N), fusion (F) and haemagglutinin (H) genes. Both post mortem tissues and faecal swabs were investigated.

Recently, the detection of PPRV antigen in faecal matter has suggested the possibility of transmission via this route (Abubakar et al., 2012). In the present study, live virus was recovered from rectal swabs. Whilst this demonstrates that live virus is present in the bowel of clinically sick animals, it does not confirm excretion of virus in faecal material. However, as severe PPRV leads to diarrhoea with the potential for sloughing of virus laden necrotic tissue and blood stained faecal matter (FAO, 2008) then this mode of transmission is plausible, if inefficient. Certainly, with animals kept in close contact the potential for transmission where soft watery faecal matter stains the hind quarters of clinically sick animals may represent a potential transmission route. The detection of live virus from rectal swabs in this study extends the previous findings of PPRV antigen in faecal matter (Abubakar et al., 2012; Ezeibe et al., 2008; Singh et al., 2004a,b) and requires further investigation. The detection of viral antigen in

faecal samples suggests this sampling method as a potential mechanism to sample fresh faecal materials from small wild ruminants, providing a non-invasive method of sampling that overcomes the requirement to capture suspected wild animals. This hypothesis also suggests that biosecurity measures, including the proper disinfection of all secretions and excretions from infected animals, may be necessary to contain the disease.

3.2. Genetic and phylogenetic assessment of PPRV

Irrespective of the length of the genome fragments used in our analyses, all the phylogenetic trees constructed using the partial H (Fig. 1a and b), N (Fig. 2a and b) or F (Fig. 3a and b) gene sequences clearly distinguished the four different lineages of PPRV with high bootstrap support values.

3.3. Phylogenetic analysis of PPRV using partial H gene data

The genetic data generated confirmed that the clinical disease observed in goats was caused by PPRV and the virus belonged to lineage IV. The H gene sequence data (accession number KJ867534) for the present study is described as 'India Tamil Nadu Goat 2011' in Fig. 1a and b. Conventionally, phylogenetic analyses of PPRV using H gene sequence data is not performed due to limited submission of partial H gene sequences to the GenBank, the majority of the H gene data available are from Indian isolates with the exception of five isolates from China (2007 and 2008) and one isolate from each of Morocco (2008), Iraq (2011) and Turkey (2000). As H gene sequence data for lineage III PPRV isolates was not available, we also generated partial H gene data for two PPRV isolates from Oman 1983 (accession number KJ867532) and UAE 1986 (accession number KJ867533) known to be of lineage III based on their N gene phylogenetic analysis. These two viruses formed a distinct branch of the phylogenetic tree representing lineage III. Indeed, the amplification of H gene material was based on studies by Kaul (2004) who demonstrated that the H gene of PPRV is more variable than the F gene and that the phylogenetic relatedness of isolates is more appropriately resolved with respect to geographical isolation than when comparing the F gene data (Kaul, 2004). Further to this, PPRV isolates circulating in sheep and goats in North India are more closely related to each other than the viruses circulating in sheep and goats in South India, possibly because of the close sheep–goat interaction in North India (Balamurugan et al., 2010). In this study the PPR outbreak was caused by a lineage IV isolate (India Tamil Nadu Goat 2011) that was closely related to a South Indian isolate (India Tamil Nadu-G Goat-2002, AY602964) and was phylogenetically grouped within lineage IV, sub-clade 5 (SC-5) (Fig. 1a). The phylogenetic analysis of the global H gene data shows that all the Indian isolates along with three Chinese (2007 and 2008) and one Moroccan (2008) isolate forms a major clade (C1) whilst two isolates, Turkey (2000) and Iraq (2011), form a second clade (C2) (Fig. 1a). Within C1, several sub clusters exist with the only African isolate for which H gene data exists representing a single sub cluster (SC4) whilst the remaining SCs are made up of Asian PPRV

strains, with the vast majority being of Indian origin (Fig. 1a).

The phylogenetic analysis of the H gene data set exclusively from India showed three major clades C1, C2 and C3 (Fig. 1b). C1 comprises of three sub clusters (SC-I, SC-II and SC-III). Clades 2 and 3 (C2 and C3) are represented by

one isolate each (India Tamil Nadu-G Goat-2002, AY602964 and India Tamil Nadu Goat 2011, respectively) and are from the southernmost state of India (Tamil Nadu) suggesting that these two isolates are significantly distant to each other (Fig. 1b) despite their apparent relatedness (SC5) in the global H gene tree (Fig. 1a). This difference may reflect

Fig. 1. Midpoint-rooted neighbour-joining tree based on the partial PPRV H gene sequences showing the relationships between the global lineage IV PPRV isolates (a) and between lineage IV isolates from India (b). Scale bar indicates nucleotide substitutions per site. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The isolates sequenced in this study are indicated by arrows.

b

Fig. 1. (*Continued*).

evolutionary changes present within the 2011 virus isolate following maintenance of transmission in the sheep and goat population of the Tamil Nadu area over the 9 year period between isolations. Previously, Balamurugan et al. (2010) also identified 3 sub clusters using a 612 bp region of the H gene (1174–1785) which encompassed the region used in the analysis presented here. However, the division of viruses into sub clusters 2 and 3 containing one isolate each from Rajasthan (west) and West Bengal (east) of India, does not correlate with our analysis. Due to the limited number of the H gene sequences of the PPRV isolates it is difficult to confirm if these clusters are evolutionarily distinct groups that circulate in restricted geographic areas.

3.4. Phylogenetic analysis of PPRV using partial N gene sequence data

The phylogenetic tree constructed using the available N gene data in GenBank and N gene data from the virus isolated from India Tamil Nadu Goat 2011′ (accession number KM105872) demonstrated that two major clades of lineage IV PPRV exist (Fig. 2a). Clade-1 (C1) consists of viruses from south Asia (India and Pakistan), middle-east (Saudi Arabia and Israel) and Africa (Cameroon, Central African Republic, Egypt, Eritrea, Morocco, Nigeria and Sudan). Clade 2 (C2) consists of viruses from South Asia (India, Bangladesh and Pakistan), China and UAE. Each of the major clades has a number of sub clusters within them. Although the bootstrap values were very low to support such clusters, a distinct geographic clustering was noticed among the viruses. Two sub clusters (SC-1 and SC-2) of viruses were identified within clade-1. SC-1 comprises of viruses from India 1994 to 2012. SC-2 comprises of viruses from Pakistan (2011 to 2012) and from Africa (1997 to 2012). Two virus sequences from the Middle East, Saudi Arabia (1999) and Israel (1998) also clustered within SC-2. The African isolates under SC-2 form two distinct groups based on geographical location. The first group comprises
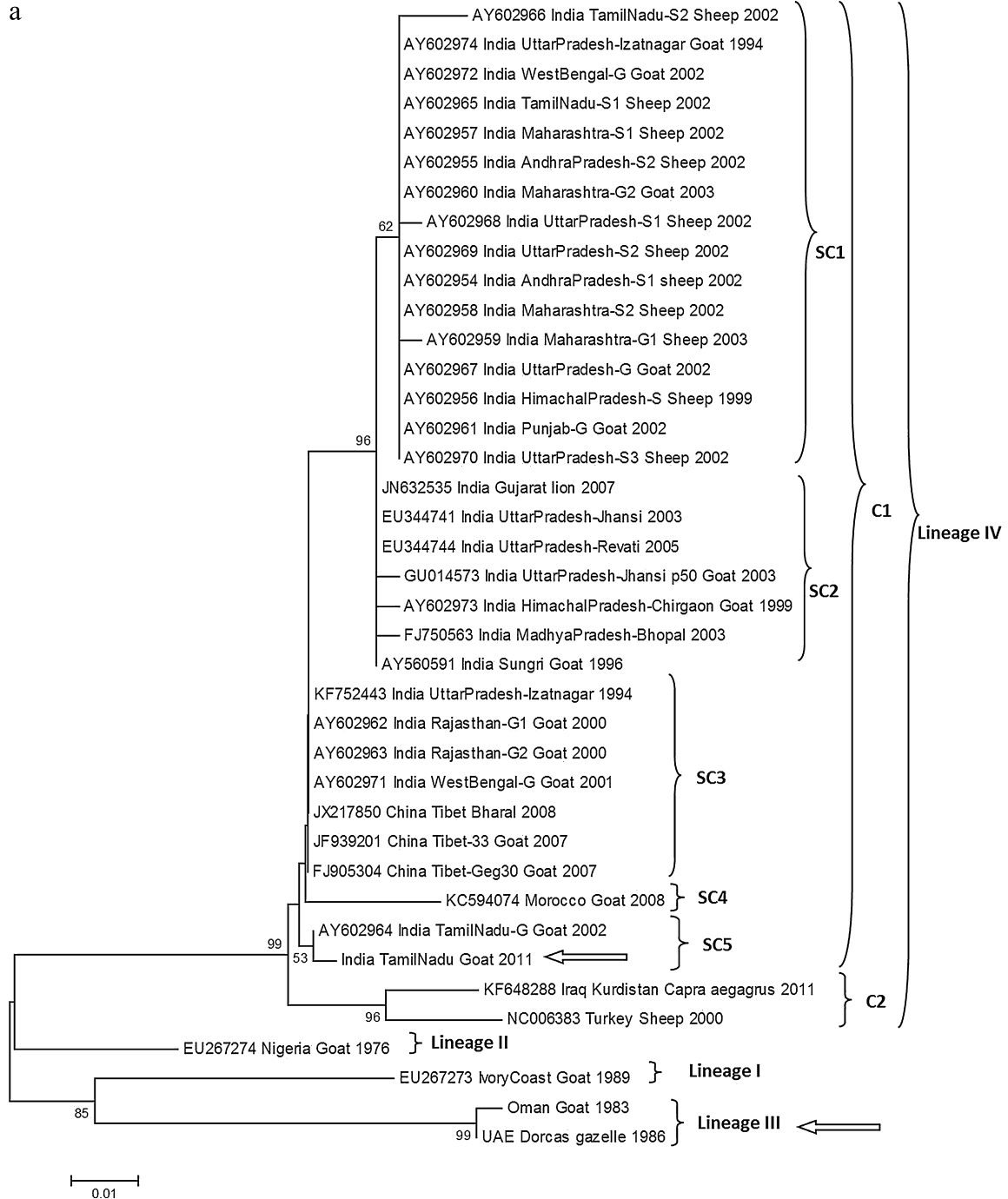
Fig. 2. Midpoint-rooted neighbour-joining tree based on the partial PPRV N gene sequences showing the relationships between the global lineage IV PPRV isolates (a) and Indian lineage IV PPRV isolates (b). Scale bar indicates nucleotide substitutions per site. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The isolate sequenced in this study are indicated by arrow.

b

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        95 ┌ KC200262_India_Nanakpur_Goat_2012
      61 ┌─┤
         │  └── EF641263_India_Mukteshwar_Cattle_2007
         └── AY560591_India_Sungri/96_Vaccine_strain
      62 ┌ DQ267191_India_BskGuj_Goat_2005
        ├── DQ267188_India_AnaGuj_Sheep_2005
        └── DQ267192_India_RjkGuj_Sheep_2005
      FJ750560_India_Bhopal_2003
      ┌ JN632530_India_Gujarat_Lion_2007
   63 ┤
      └── DQ267189_India_PtnGuj_Sheep_2005
   99 ┤── DQ267190_India_MehGuj_Goat_2005
      GU014571_India_Jhansi_Goat_2003
      FJ750559_India_Revati_2005
      EU344738_India_Jhansi_2003
      JN647694_India_Pradesh95_1995
      JN647700_INDIA_1994
      DQ840176_India_isolate94
      DQ840178_India_Pradesh1__1995
  72 ── DQ840177_India_Calcutta_1995
      DQ840182_India_Tamilnadu21_1995
  87 ┌ DQ176751_India_2TNVero_2004
     India_TamilNadu_Goat_2011  ◄━━━━━
  99 ┤ DQ176748_India_Tamilnadu_1TNVero_2004
  70 ┤ DQ176754_India_Tamilnadu_3TNVero_2004
     DQ176755_India_3TNswab_2004
67 ── DQ840180_India_Thatagullan1_1994
      ┌── DQ185592_India_Pondswab_2004
  87 ┌┤── DQ176749_India_1TNswab_2004
     │ └───── DQ185591_India_4TNswab_2003
     └── DQ176752_India_2TNswab_2004
      ┌ GQ122187_India_Dindori_Goat_2008
   75 │ GQ122189_India_Katni_Goat_2008
91 ┤ 98 │ GQ122188_India_Balaghat_Goat_2008
     │  GQ122186_India_Jabalpur_Goat_2008
     DQ185577_India_2MPswab_2005
  50 │ DQ185578_India_3MPswab_2004
     DQ185579_India_4MPswab_2004
  70 │ DQ185576_India_1MPswab_2005
     DQ185580_India_MPswab_2004
     └── DQ185581_India_Chswab_2005
96 ┤
53 ┤
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Lineage IV

```
      ┌── DQ840160_Nigeria_1_1975
 100 ┤
      └── DQ840164_Nigeria_1_Goat_1976   } Lineage II
      ┌── DQ840199_IvoryCoast_Goat_1989
  99 ┤                                    } Lineage I
      └── DQ840170_Guinea_1988
     ┌ DQ840168_Oman_Ibri_Goat_1983
 100 ┤                                    } Lineage III
     └ DQ840169_UAE_Dorcas_gazelle_1986
```

⊢———⊣
0.02
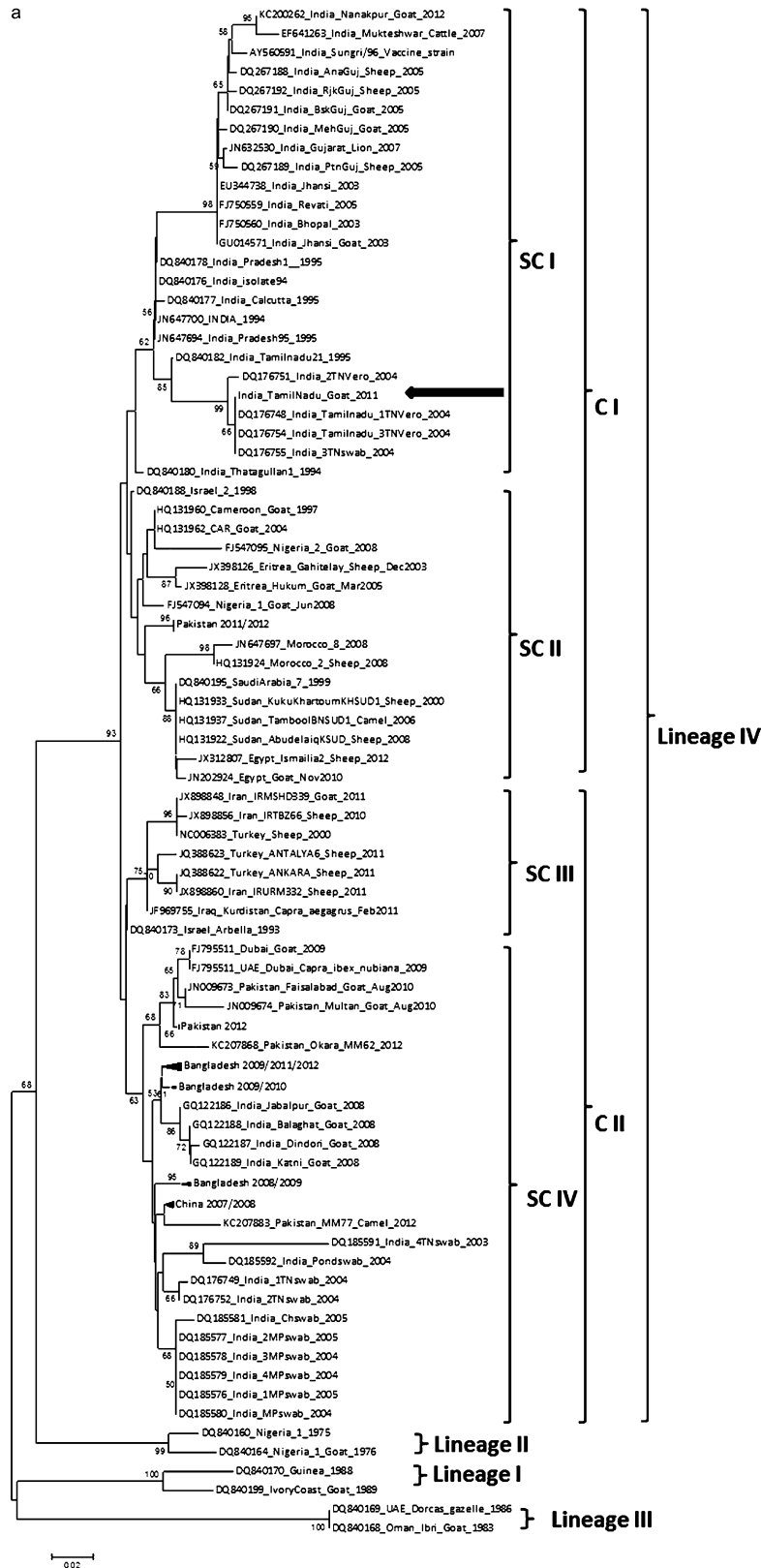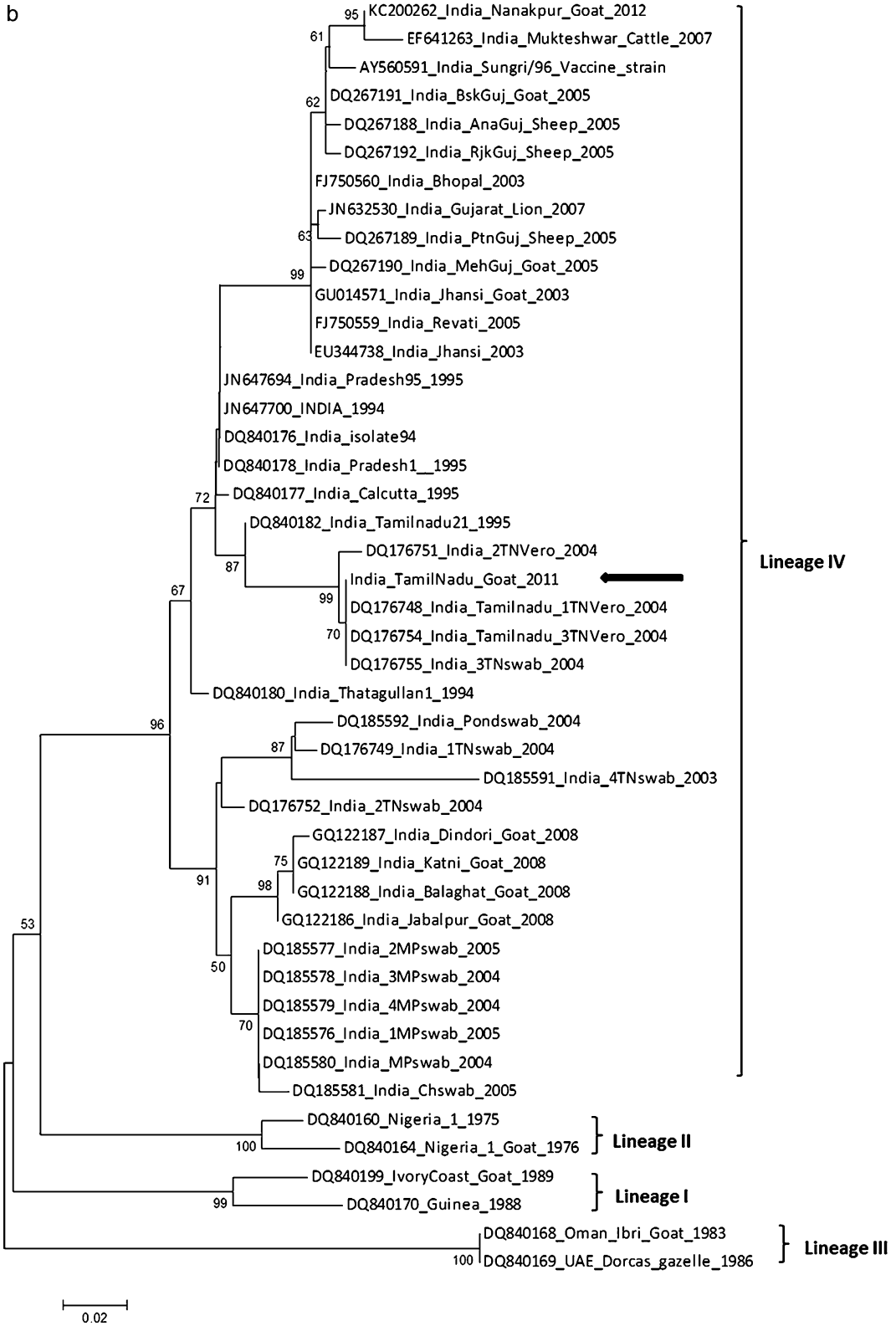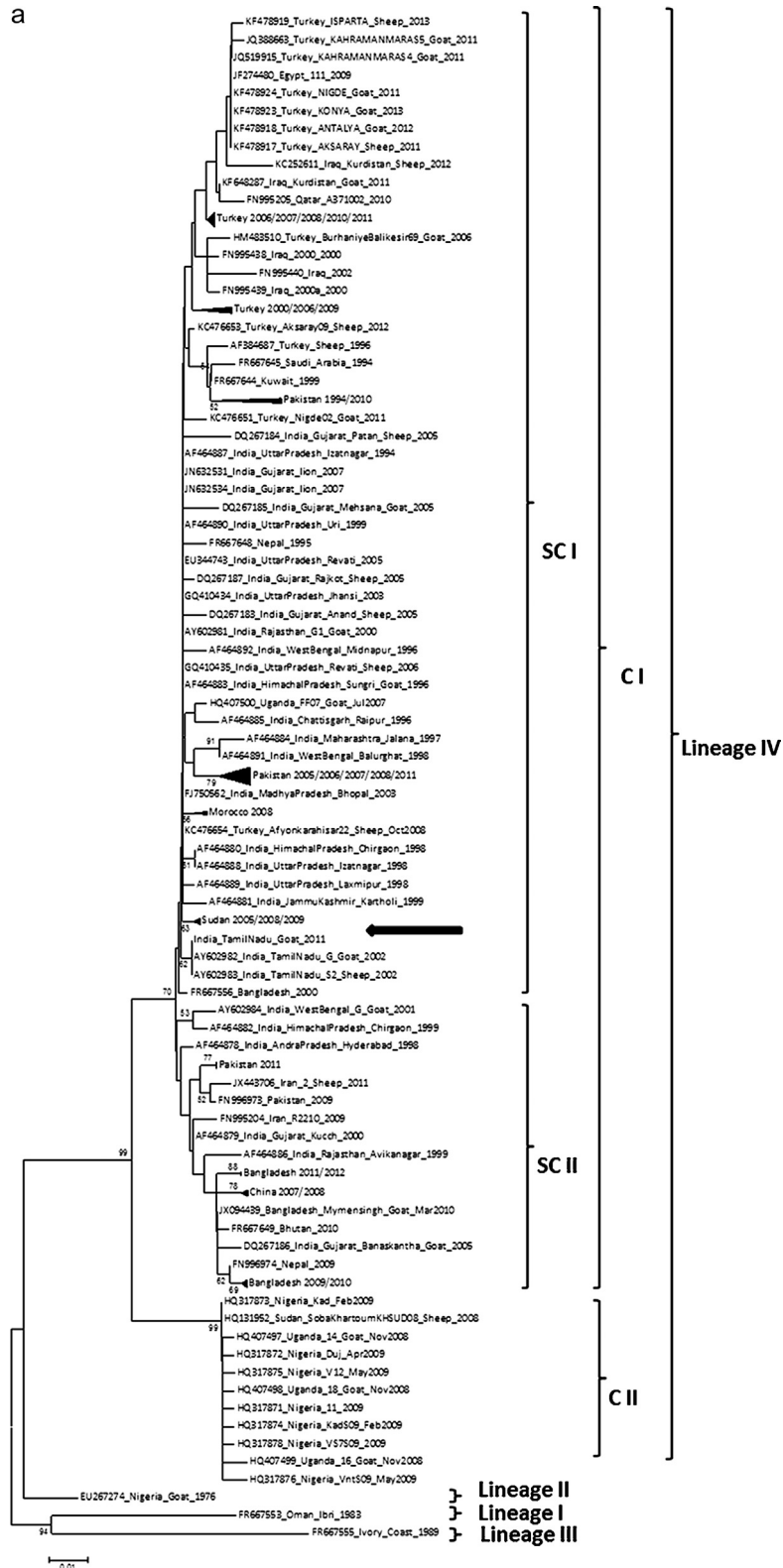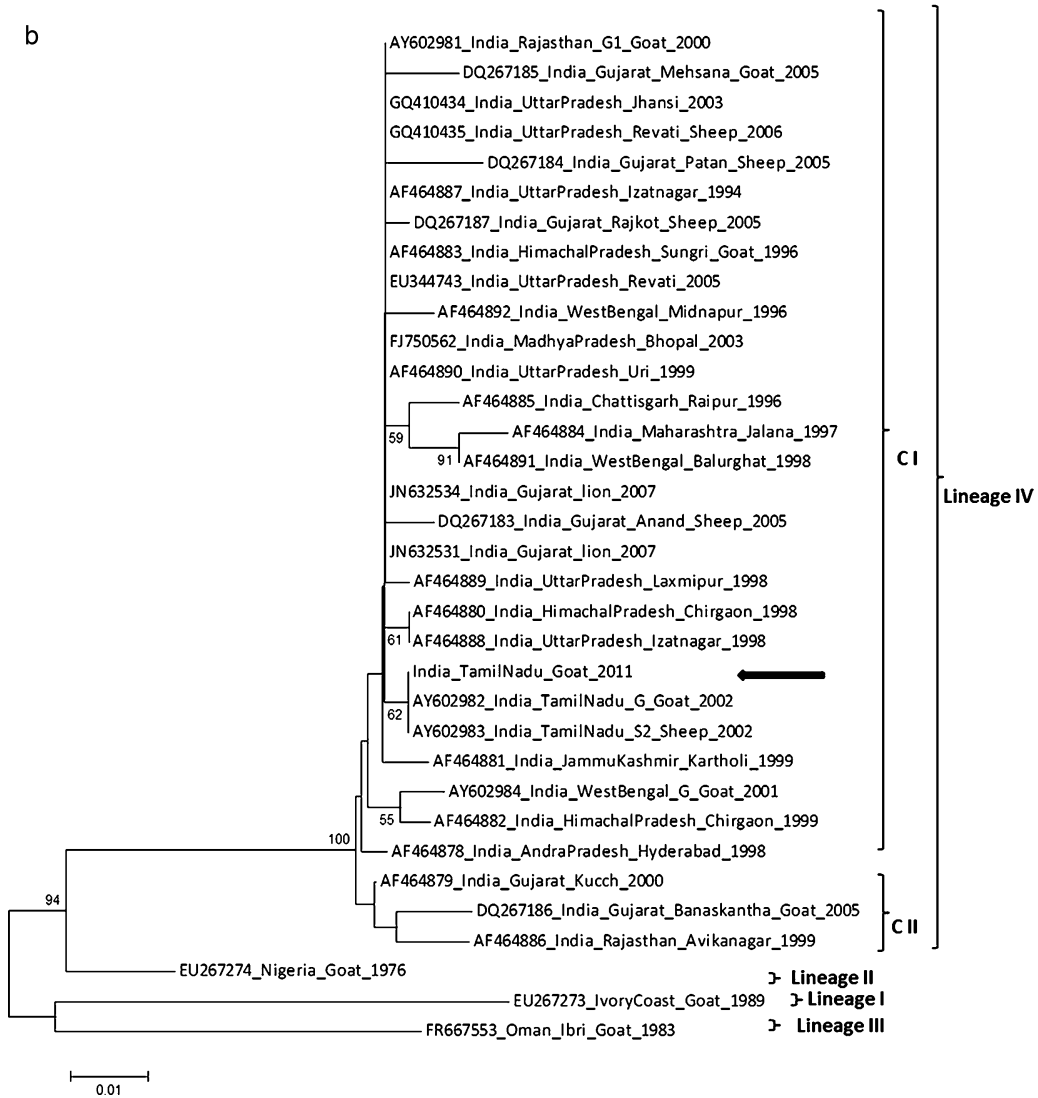
Fig. 2. (*Continued*).

a

Fig. 3. Midpoint-rooted neighbour-joining tree based on the partial PPRV F gene sequences showing the relationships between the global lineage IV PPRV isolates (a) and Indian lineage IV PPRV isolates (b). Scale bar indicates nucleotide substitutions per site. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The isolate sequenced in this study are indicated by arrow.

b

Fig. 3. (*Continued*).

of viruses from Central and West Africa (Nigeria, Cameroon, Central African Republic and Eritrea) between the years 1997 and 2008. The second group comprises of viruses from North and East Africa (Morocco, Egypt and Sudan) between the years 2000 and 2012. This study including earlier reports (Banyard et al., 2010; Kwiatek et al., 2011) predicts the presence of lineage IV PPRV in Africa during the latter half of the 20th century although it presence was only reported following the extensive Moroccan outbreak in 2008. Kwiatek et al. (2011) predicted that a virulent lineage IV strain might have been introduced in to Africa during the 1990s, resulting in outbreaks in both in camels and small ruminants. In their study of lineage IV PPRV isolates in Sudan between 2000 and 2009, Kwiatek et al. (2011) observed that the lineage IV PPRV progressively replaced the lineage III PPRV found in Sudan during the previous decade. In addition, they also suggested that a constant rise of disease incidence has been associated with this lineage, which might indicate an increased virulence of this lineage.

Within clade 2, there are two sub clusters SC-3 and SC-4. SC-3 consists of viruses from the Middle East (Iran, Iraq and Turkey) isolated between 2010 and 2011 and an Israeli isolate from 1993. It is likely that PPRV in Middle East has either not been genetically characterised or not been made publically available. SC-4 includes viruses from Pakistan, Bangladesh, China and India isolated and characterised between 2004 and 2012. SC-4 also contains two isolates from Dubai and UAE (2009) that suggest the transmission of virus from these South Asian countries to Arabian countries mainly through animal trade.

Phylogenetic analysis of N gene sequence data of the Indian isolates alone between the years 1994 and 2012 demonstrated the presence of two major clades (Fig. 2b). Both of these clades contained PPRV isolates from the southern and northern regions of India, indicating no geographic restriction of PPRV within the country. However, the viruses from southern India appear to form distinct clusters under both clade 1 and clade 2. Although

this clustering has a high bootstrap support, the limited number of sequences and the unavailability of recent isolates make it difficult to speculate if such a distinct evolutionary group is still in circulation. The recent isolate (India Tamilnadu Goat 2011; Genbank accession no: KM105872) grouped together with the earlier PPRV isolates of Tamilnadu from 2004. Interestingly, reports from India have suggested that PPRV outbreaks are more common in goats than in sheep in Northern India (Sinha, 1998) but more common in sheep than goats in Southern India (Raghavendra et al., 2008). Further, the above observation is supported by a high prevalence of PPRV antibodies in sheep than goats in South India in comparison to North India (Balamurugan et al., 2012; Singh et al., 2004a,b) although interpretation of serological data must be made with caution as uncontrolled vaccination in areas cannot be ruled out as a potential skew of the data. Balamurugan et al. (2012) suggested that these differences in disease prevalence among sheep and goats may not be significant and could be due to the smaller sample size used for analysis. The phylogeny using the N gene data set suggests that there are no species specific lineages or evolutionary groups amongst Indian isolates of PPRV. The observation may be the reflection of a larger population of sheep in South India and larger population of goats in North India

3.5. Phylogenetic analysis of lineage IV PPRV using partial F gene data

The phylogenetic tree constructed using the available F gene data in GenBank and F gene data from the virus isolated from India Tamil Nadu Goat 2011′ (accession number KM105873) grouped the lineage IV PPR isolates into two major clades (C1 and C2) (Fig. 3a). Within clade 1 two sub clusters (SC1 and SC2) were identified with a high bootstrap support values. SC1 comprises of PPRV sequences from Asia (Bangladesh, India, Nepal and Pakistan), the Middle East (Iraq, Kuwait, Qatar, Saudi Arabia and Turkey) and Africa (Egypt, Morocco, Sudan and Uganda). SC2 comprises of PPRV sequences mainly from Asia (Bangladesh, Bhutan, China, India, Nepal and Pakistan) and two Iranian viruses. Clade 2 consists of PPRV isolates from Africa (Nigeria, Uganda and Sudan) between the years 2005 and 2009. Unlike the N gene-based phylogenetic tree, the F gene-based phylogenetic analysis did not form distinct geographical sub-clusters under the major clades.

Phylogenetic analysis of the F gene sequence of the PPRV isolates from India between the years 1994 and 2007, grouped them into two clades (C1 and C2) within the lineage IV (Fig. 3b). The recent isolate (India Tamilnadu Goat 2011; Genbank accession no: KM105873) grouped together with the earlier PPRV isolates of Tamilnadu from 2002 similar to that of the H gene phylogeny. However, due to the limited availability of F gene sequences for PPRV circulating recently, the major clades could not be divided further to classify the sub-clusters.

4. Conclusions

Overall, the phylogenetic trees using the partial regions of N, F or H genes clearly distinguish the four different lineages of PPRV. However, phylogenetic analysis using the global and N, F and H gene sequence data shows that the region encompassing nucleotides 1253 to 1470 of the N gene (amino acids 86 to 191) is more variable than nucleotides 1372 to 1670 of the H gene (amino acids 458 to 556) and nucleotides 256 to 575 of the F gene (amino acids 419 to 490). In addition, the PPRV sequences appear to show a distinct geographic clustering when the variable part of N gene is used for phylogenetic analysis. In contrast, the phylogenetic trees using the F gene data formed a limited number of clusters that although supported by bootstrap support, did not divide isolates according to geographical isolation. Although the H gene partial sequence data are scarce compared to N and F gene partial sequence data, phylogenetic analysis using these data demonstrates the formation of distinct clades that are divided into sub-clusters. Both the N and F gene base phylogenetic trees grouped the isolates into two major clades. However, these major clades from both the trees were different. For example, clade 1 of the F gene phylogenetic tree comprises of almost all the isolates including isolates from Africa (Sudan–2005 to 2009, Egypt 2009 and Morocco, 2008) whilst only a few isolates from Nigeria (2009), Sudan (2008) and Uganda (2008) formed clade 2. In contrast the N gene phylogeny shows a different clustering pattern, where all the isolates of African origin were grouped within SC-2 of clade-1. Clade 2 of the N gene based tree contains isolates from Middle East, Pakistan, Bangladesh, India and China. The PPRV H gene data is limited to only five countries (India, China, Iraq, Turkey and Morocco) which makes comparison with the defined F and N gene clades and sub-clusters problematic. Whilst these analyses have identified clustering of isolates that differ according to geographical isolation and the target gene, it is likely that clustering of sequences will change further with the generation of new genetic data for this important viral pathogen. Importantly, the study reiterates the observation that the N gene based phylogeny gives the greatest clarity with respect to the geographical clustering of isolates. Alongside the molecular fact that wherever PPRV infection occurs more N messenger RNA is produced than for any other gene and thus N gene based PCR assays must be the most sensitive, the current study supports the prioritisation of this gene as a principle target for PCR based assays and phylogenetic assessment. Furthermore this analysis has highlighted the importance of maximising availability of gene data by targeting areas that enable phylogenetic assessment with a maximal number of existing data.

For PPRV, lineage I was first reported in 1942 in West Africa whilst lineage IV isolates were initially detected in Southern India (1987) and in North India and Pakistan by 1994. However, the genetic data for partial N gene (Fig. 3a) suggests that lineage IV was present in Africa and the Middle East during a similar time frame (Cameroon (1997), Israel (1993), Iran (1998), Saudi Arabia (1999)). From the recent outbreaks across Asia, the Middle East and Africa (China (2007), Morocco (2008), Algeria (2011), Tanzania (2012), DRC (2012), Angola (2012) and Comoros (2012)), it is clear that the distribution of lineage IV virus has extended to new regions (Libeau et al., 2014). As a

consequence of this, it is very difficult to predict the origin and spread of lineage IV PPR virus. A molecular phylogenetic assessment of the origin of different lineages of PPRV is beyond the scope of this study and may be more appropriately resolved by evolutionary analysis of complete genomes of PPRV for each lineage where available (Libeau et al., 2014).

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