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Genetic Evidence for Rift Valley Fever Outbreaks in Madagascar Resulting from Virus Introductions from the East African Mainland rather than Enzootic Maintenance▽†‡

Serena A. Carroll,[1] Jean-Marc Reynes,[2]§ Marina L. Khristova,[3] Soa Fy Andriamandimby,[2] Pierre E. Rollin,[1]* and Stuart T. Nichol[1]*

Viral Special Pathogens Branch, Division of High Consequence Pathogens and Pathology, Centers for Disease Control and Prevention, 1600 Clifton Road, MS G-14,[1] and Biotechnology Core Facility Branch, Centers for Disease Control and Prevention, 1600 Clifton Road,[3] Atlanta, Georgia 30333, and Virology Unit, Institut Pasteur de Madagascar, BP 1274, Antananarivo 101, Madagascar[2]

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Rift Valley fever virus (RVFV), a mosquito-borne phlebovirus, has been detected in Madagascar since 1979, with occasional outbreaks. In 2008 to 2009, a large RVFV outbreak was detected in Malagasy livestock and humans during two successive rainy seasons. To determine whether cases were due to enzootic maintenance of the virus within Madagascar or to importation from the East African mainland, nine RVFV whole genomic sequences were generated for viruses from the 1991 and 2008 Malagasy outbreaks. Bayesian coalescent analyses of available whole S, M, and L segment sequences were used to estimate the time to the most recent common ancestor for the RVFVs. The 1979 Madagascar isolate shared a common ancestor with strains on the mainland around 1972. The 1991 Madagascar isolates were in a clade distinct from that of the 1979 isolate and shared a common ancestor around 1987. Finally, the 2008 Madagascar viruses were embedded within a large clade of RVFVs from the 2006–2007 outbreak in East Africa and shared a common ancestor around 2003 to 2004. These results suggest that the most recent Madagascar outbreak was caused by a virus likely arriving in the country some time between 2003 and 2008 and that this outbreak may be an extension of the 2006–2007 East African outbreak. Clustering of the Malagasy sequences into subclades indicates that the viruses have continued to evolve during their short-term circulation within the country. These data are consistent with the notion that RVFV outbreaks in Madagascar result not from emergence from enzootic cycles within the country but from recurrent virus introductions from the East African mainland.

Rift Valley fever virus (RVFV) is a mosquito-borne, negative-strand RNA virus in the family *Bunyaviridae*, genus *Phlebovirus* (24). RVFV readily infects livestock, including sheep, goats, and cattle, and causes mortality in young and adult animals, as well as sweeping abortion storms. Although infection in humans is most often self-limiting, 1% to 2% of cases result in more-severe disease, with clinical signs ranging from hepatitis to encephalitis to hemorrhagic syndrome. Transmission can occur as a result of contact with infected livestock or directly from the bite of infected mosquitoes.

RVFV was first isolated in 1931, in Kenya (6, 11), but Bayesian coalescent analyses suggest that the most recent common ancestor (MRCA) of extant RVFVs can be traced back as far as the late 1800s (3, 4). The virus can be found throughout continental Africa, Madagascar, and, more recently, in the Arabian Peninsula. Epizootic/epidemic cycles appear to be periodic and are often associated with climatic variations, such as increased rainfall, which can be used to generate predictive risk maps for RVFV activity in East Africa (2, 7, 15). Elevated rainfall results in expanding mosquito populations, which, in turn, allow for greater RVFV transmission not only within the vector populations but to other susceptible species as well. Recently Bird et al. (3) confirmed that RVFV activity also occurs during interepizootic/interepidemic periods, albeit at much lower levels.

In Madagascar, RVFV was first isolated in 1979. At that time, the virus was isolated from mosquitoes in the absence of reports of disease in humans or livestock (12, 16). Eleven years later (1990 to 1991), RVFV circulation was detected in the east coast and central highland regions of the country, with reports of human cases and high abortion rates in cattle (18–21). This particular outbreak was thought to have been due to the introduction of livestock from the mainland; however, because import/export records were scarce in Madagascar, this assumption could not be confirmed. Although serological evidence indicated possible RVFV exposure of slaughterhouse workers in the mid-1990s (31), no outbreaks were observed for at least the next 15 years. In 2008, Madagascar experienced its largest RVFV outbreak to date. More than 700 suspected human cases and 26 fatalities were reported during two successive rainy seasons: January through May 2008 and November 2008 through March 2009 (1). Additionally, a large number of livestock, predominantly cattle, were affected. This outbreak came

* Corresponding author. Mailing address: 1600 Clifton Road, MS G-14, Atlanta, GA 30333. Phone for Stuart T. Nichol: (404) 639-1122. Fax: (404) 718-2136. E-mail: snichol@cdc.gov. Phone for Pierre E. Rollin: (404) 639-1124. E-mail: prollin@cdc.gov.

† Supplemental material for this article may be found at http://jvi.asm.org/.

§ Present address: Centre Pasteur du Cameroun, BP 1274, Yaoundé, Cameroon.

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TABLE 1. GenBank accession numbers and collection information for the 9 RVFVs sequenced in this study[a]

SPB no.	ID no.	Species	Sample	Mo	Yr	District	Accession no. for:		
							S segment	M segment	L segment
200803162	0212-08	Human	Isolate (serum)	January	2008	Taolagnaro	JF311386	JF311377	JF311368
200803163	0428-08	Human	Isolate (serum)	February	2008	Anjozorobe	JF311387	JF311378	JF311369
200803164	0619-08	Human	Isolate (serum)	March	2008	Ankazobe	JF311388	JF311379	JF311370
200803165	0846-08	Human	Isolate (serum)	March	2008	Manjakandriana	JF311389	JF311380	JF311371
200803166	S-91-41	Human	Isolate (serum)	February	1991	Antananarivo	JF311390	JF311381	JF311372
200803167	ZF-06	Bovine	Isolate (embryo liver)	February	1991	Antananarivo	JF311391	JF311382	JF311373
200803168	0406-08	Bovine	Embryo liver	February	2008	Miarinarivo	JF311392	JF311383	JF311374
200803169	0413-08	Bovine	Embryo liver	February	2008	Antsirabe II	JF311393	JF311384	JF311375
200803170	0855-08	Bovine	Serum	March	2008	Manjakandriana	JF311394	JF311385	JF311376

[a] SPB, Special Pathogens Branch; ID, identification.

on the heels of a substantial RVFV outbreak in 2006 to 2007 on the East African mainland, predominantly in Kenya, Tanzania, and Somalia (3, 17, 30), and occurred during the time when RVFV cases were reported from Mayotte, the Republic of the Comoros, and South Africa between 2007 and 2009 (27, 28).

Although the predictive model developed for the Horn of Africa failed to show heightened risk for RVFV infection in Madagascar in 2008 (5), a nationwide cross-sectional serological survey showed extensive and widespread RVFV exposure at that time (1). Preliminary phylogenetic analyses using partial sequences showed that Malagasy viruses were similar to those found circulating during the 2006–2007 East African outbreak; however, the initial source of the 2008 Madagascar outbreak remained unclear (1). The purpose of this study, therefore, was to generate whole genomic sequences from 2008 Malagasy outbreak samples to determine whether cases most likely represented enzootic maintenance within Madagascar or epizootic/epidemic spread or importation from East Africa.

MATERIALS AND METHODS

Three clinical specimens (liver or serum) and four RVFV isolates from the 2008 outbreak and two RVFVs isolated from the 1991 outbreak were sent from the Institut Pasteur in Madagascar to the Centers for Disease Control and Prevention (CDC) in Atlanta, GA, for genetic characterization (Table 1). Whole genomic sequences of approximately 11.9 kb were generated for each of the nine samples.

RNA isolation, RT-PCR, and complete genome sequencing. Total RNA was obtained directly from clinical specimens or after virus isolation on Vero E6 cells, using the ABI 6100 nucleic acid PrepStation (Applied Biosystems, Foster City, CA) and associated reagents, as described previously (3, 4). Reverse transcriptase PCR (RT-PCR) was performed to amplify the three single-stranded genomic RNA segments (small [S], medium [M], and large [L]) using the SuperScript III one-step RT-PCR system with Platinum *Taq* HiFi (Invitrogen, San Diego, CA). The primers and thermal profiles were as described by Bird et al. (3, 4), with annealing temperature adjustments (46 to 56°C) for some clinical samples. RT-PCR products were purified using ExoSAP-IT (USB Corporation, Cleveland, OH), and sequences were generated on an ABI 3730XL automated DNA sequencer using BigDye chemistry (version 3.1; Applied Biosystems, Foster City, CA). A total of 21, 52, and 50 sequencing primers were used for the S, M, and L segments, respectively; additional strain-specific primers were employed for difficult-to-sequence regions. For each genome, approximately 140 to 150 reads were obtained, with an average 6-fold redundancy at each nucleotide position.

Phylogenetic and coalescent analyses. In addition to the nine Madagascar samples described here, all available RVFV whole S, M, and L segment sequences (with dates of collection) in GenBank were used for the phylogenetic and coalescent analyses. A total of 120 S segment sequences, 77 M segment sequences, and 74 L segment sequences were examined (see Table S1 in the supplemental material). Multiple sequence alignments were generated in SeaView (13) using the MAFFT function (14), and nucleotide substitution models were selected based on Akaike's information criterion (AIC) in ModelTest, version 3.7 (22). The general time-reversible model with gamma distribution (GTR+G) was employed for all three segments.

Bayesian coalescent analyses were performed in the BEAST (version 1.4.8) and Tracer (version 1.4.1) software packages (10). For each segment, preliminary analyses (10,000,000 generations each) were conducted to determine the most appropriate molecular clock (strict versus relaxed uncorrelated lognormal versus relaxed uncorrelated exponential) and demographic (constant versus Bayesian skyline population size) models. The relaxed uncorrelated exponential clock (9) and Bayesian skyline population size models were chosen for the S, M, and L segments on the basis of an analysis of marginal likelihoods (29). Final runs consisted of 100,000,000 to 175,000,000 generations to ensure effective sample sizes (ESSs) of at least 200. Maximum clade credibility trees were summarized with TreeAnnotator and were depicted using FigTree (10).

Nucleotide sequence accession numbers. The S, M, and L sequences of the 9 RVFVs sequenced in this study have been deposited in GenBank under the accession numbers given in Table 1.

RESULTS

Phylogeny. Tree topologies based on all of the available RVFV S, M, and L sequences are shown in Fig. S1, S2, and S3 in the supplemental material, respectively. For the sake of clarity, abbreviated trees, including relevant S (Fig. 1), M (Fig. 2), and L (Fig. 3) segment sequences generated for this study, are displayed here. Tree topologies were consistent with those generated for all previously available RVFV genomic RNA segments (3, 4). The 1979 Madagascar virus is most closely associated with viruses from the mid- to late 1970s from Zimbabwe and falls within the clade containing samples from the large Egyptian outbreak of that time. The two 1991 Madagascar isolates are members of a different clade, along with three additional 1991 Madagascar RVFVs and viruses from Kenya (1997 to 1998), Somalia (1998), South Africa (1999 to 2000), Saudi Arabia (2000), and East Africa (2006 to 2007). All seven of the 2008 Madagascar RVFVs, and two viruses from the 2008 outbreak in South Africa, were embedded within the larger 2006-2007 East African clade, specifically within the lineage previously termed Kenya-I (3). None of the viruses sampled from Madagascar or South Africa in 2008 fell within the Kenya-II lineage. Instead, most formed subclades with other 2008 viruses (i.e., Mdg3164 and Mdg3168; Mdg3165, and Mdg3170) within the larger Kenya-I lineage.

Evolutionary rates and time to MRCA. The addition of the Madagascar RVFV sequences resulted in mean molecular evolutionary rates (and 95% highest posterior density [HPD] intervals) similar to those previously generated by Bird et al. (3,

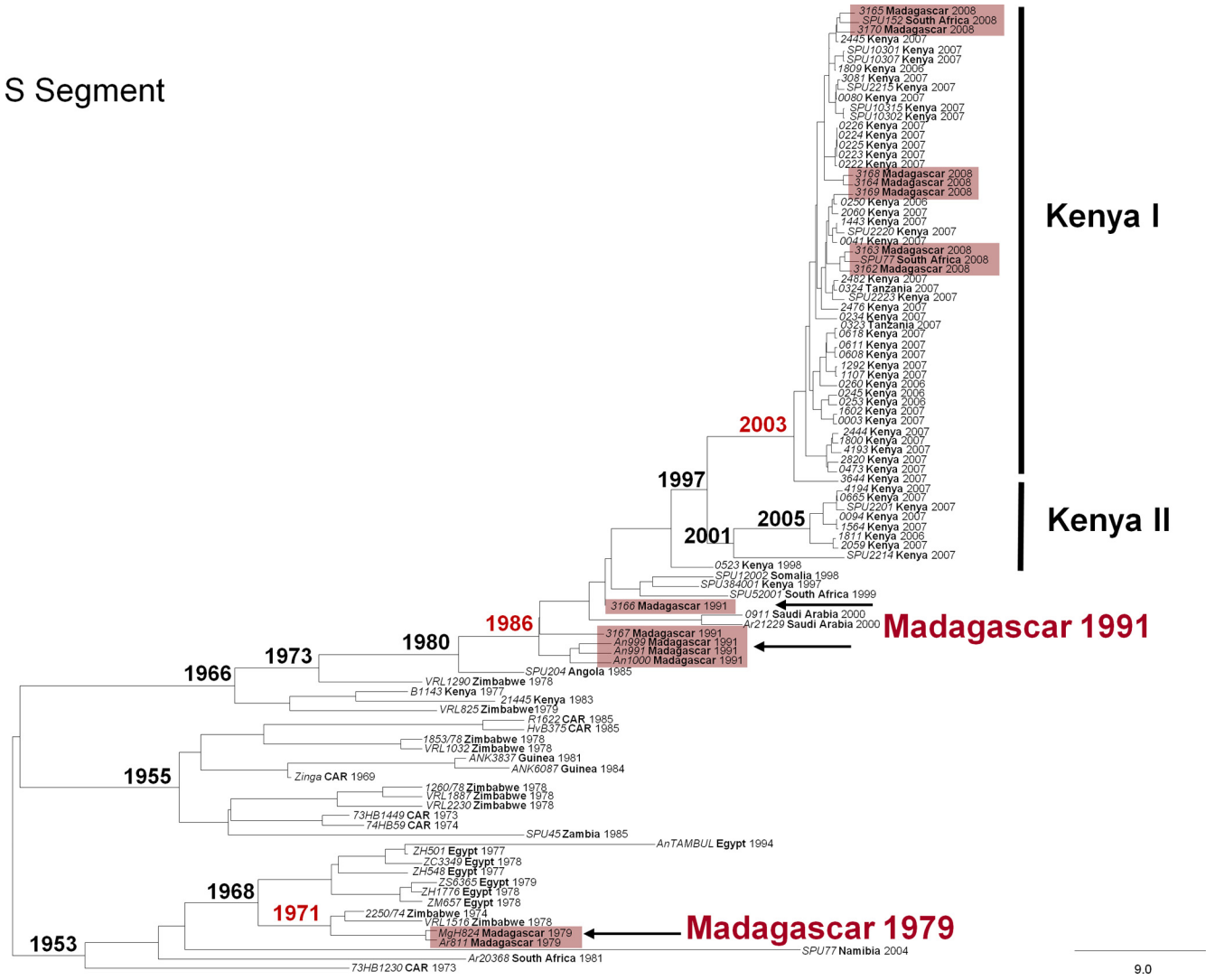

FIG. 1. Bayesian coalescent analysis of RVFVs based on the S segment. The maximum clade credibility tree is shown with the year of the MRCA, in boldface, at each node. Viruses from Madagascar and nodes containing associated MRCA estimates are shown in red. Lineages from the 2006–2007 East African outbreak are designated Kenya I and Kenya II.

4): for the S segment, 4.16×10^{-4} (95% HPD interval, 2.78×10^{-4} to 5.47×10^{-4}) nucleotide substitutions/site/year; for the M segment, 5.09×10^{-4} (95% HPD interval, 3.38×10^{-4} to 6.75×10^{-4}) nucleotide substitutions/site/year; and for the L segment, 4.33×10^{-4} (95% HPD interval, 2.73×10^{-4} to 5.98×10^{-4}) nucleotide substitutions/site/year.

The 1979 Madagascar RVFV shares a MRCA with viruses from Zimbabwe around 1971 to 1972 (Fig. 1 to 3). All RVFVs detected since the early 1980s in East Africa, Madagascar, and the Middle East are contained in a single clade and share a MRCA in the 1960s (S segment) (Fig. 1) or 1970s (M and L segments) (Fig. 2 and 3). The MRCA of the clade containing the 1991 Madagascar RVFVs occurred between 1986 and 1988 (Fig. 1 to 3), whereas viruses in the 2006–2008 East Africa/Kenya-I lineage, including the 2008 Madagascar and South Africa RVFV samples, share a MRCA approximately 3 to 5 years before the 2008 Madagascar outbreak (2003 to 2005). The subclades containing solely Madagascar sequences appear to be more recent, having arisen less than a year prior to their detection.

DISCUSSION

Genomic findings from this study revealed that the viruses circulating in Madagascar in the late 1970s did not give rise to the 1991 or 2008 Malagasy RVFV outbreaks, eliminating the role of this first isolate in enzootic maintenance and the successive outbreaks. Three distinct importation events, from the East African mainland to Madagascar, appear likely: one in the early 1970s, another in the mid- to late 1980s, and the most recent between 2003 and 2008. In all three instances, considerable RVFV activity was detected on the African mainland prior to the detection of these viruses in Madagascar. This pattern is particularly evident with the large sweeping outbreak in Egypt (1978 to 1979) prior to the Madagascar 1979 virus

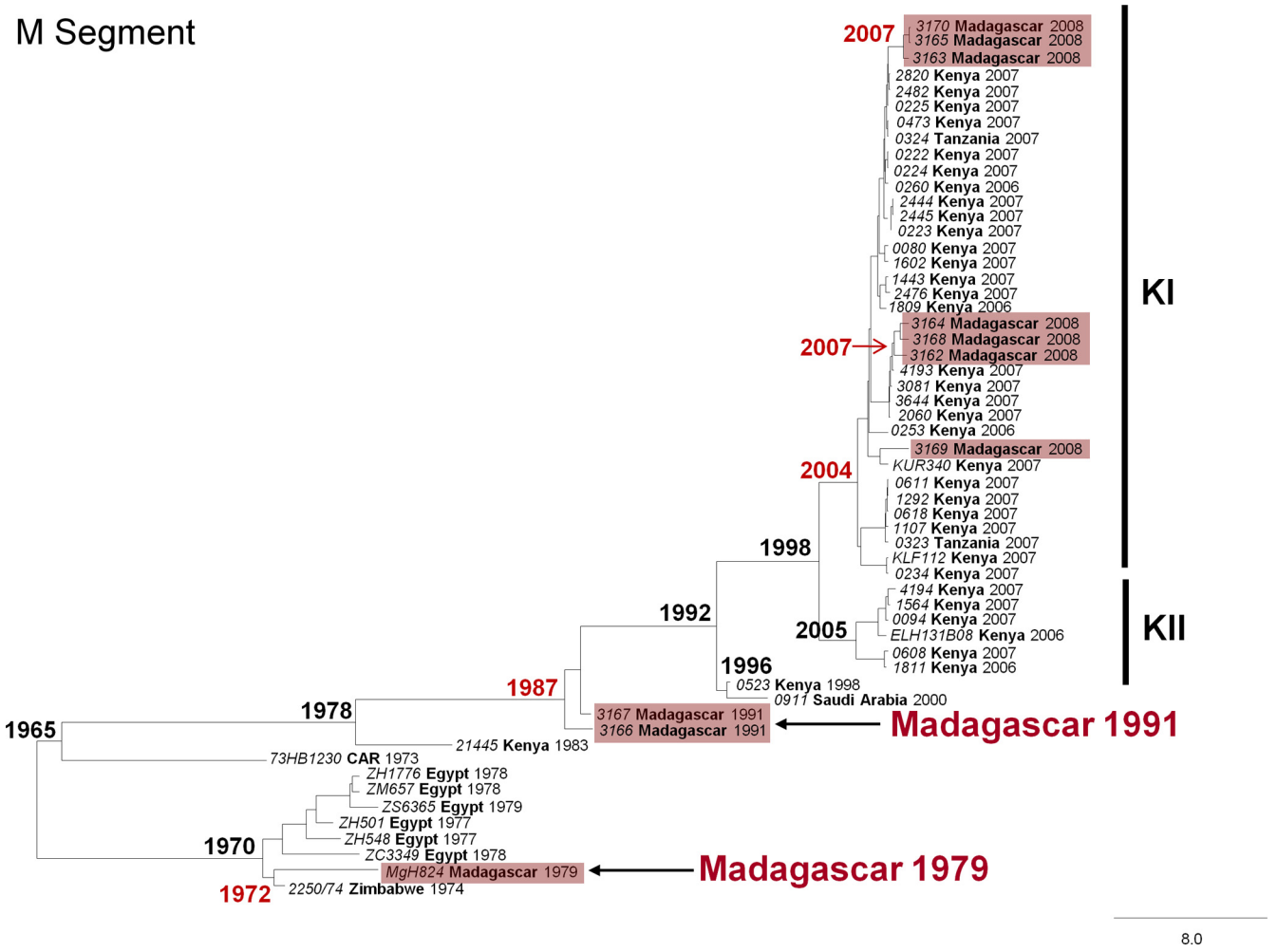

FIG. 2. Bayesian coalescent analysis of RVFVs based on the M segment. The maximum clade credibility tree is shown with the year of the MRCA, in boldface, at each node. Viruses from Madagascar and nodes containing associated MRCA estimates are shown in red. KI, Kenya-I lineage; KII, Kenya-II lineage.

isolation and the extensive East African outbreak (2006 to 2007) that preceded the 2008–2009 activity in Madagascar.

The timing of the events associated with the recent outbreak in Madagascar also supports RVFV importation from East Africa, possibly even during the 2006–2007 outbreak. Although most cases were reported from January through April 2008 (1), epidemiologic evidence has linked the 2008–2009 Malagasy outbreak to that occurring on the mainland a few years earlier. Specifically, livestock abortion was observed in one Malagasy district (Toliara II) beginning in early 2007, and a retrospective investigation revealed that RVFV had been circulating in the livestock population at least since December 2007 (1). The same retrospective investigation demonstrated widespread exposure to RVFV among Malagasy slaughterhouse workers since at least 2007, as evidenced by the presence of antibodies in serum samples (1). The combination of the genetic and serological data would be consistent with the view that in Madagascar, large outbreaks of RVF are associated with viruses introduced from the mainland, but the virus activity likely ramps up over the preceding year or two and likely persists at decreasing levels for a year or two following the large outbreak. Unfortunately, disease surveillance is insufficiently sensitive to detect these pre- and postoutbreak periods of virus activity. Increased surveillance and genetic characterization of interepizootic virus isolates would shed light on this issue. RVFV activity during an interepizootic/interepidemic period is not surprising, especially in the years immediately preceding (3) or following (31) an outbreak. Even when the virus fails to become established in the long term, it is likely that there is a temporary enzootic state with short-term circulation for a couple of years surrounding a RVFV outbreak.

Similar patterns of disease spread have been observed in other areas. For instance, in Mayotte, RVFV cases in humans and cattle in 2007 and 2008 also appeared to be an expansion of the East African outbreak (27). In addition, outbreaks of other pathogens, including the 2003–2004 East Coast fever outbreak in Comoros and the 2005–2006 Chikungunya virus outbreak in the islands of Mayotte, Réunion, Seychelles, Mauritius, and Madagascar are thought to have originated on the East African mainland, specifically in Kenya and Tanzania (8, 25, 26). These cases provide additional illustrations of the potential for infectious agents to be imported from coastal

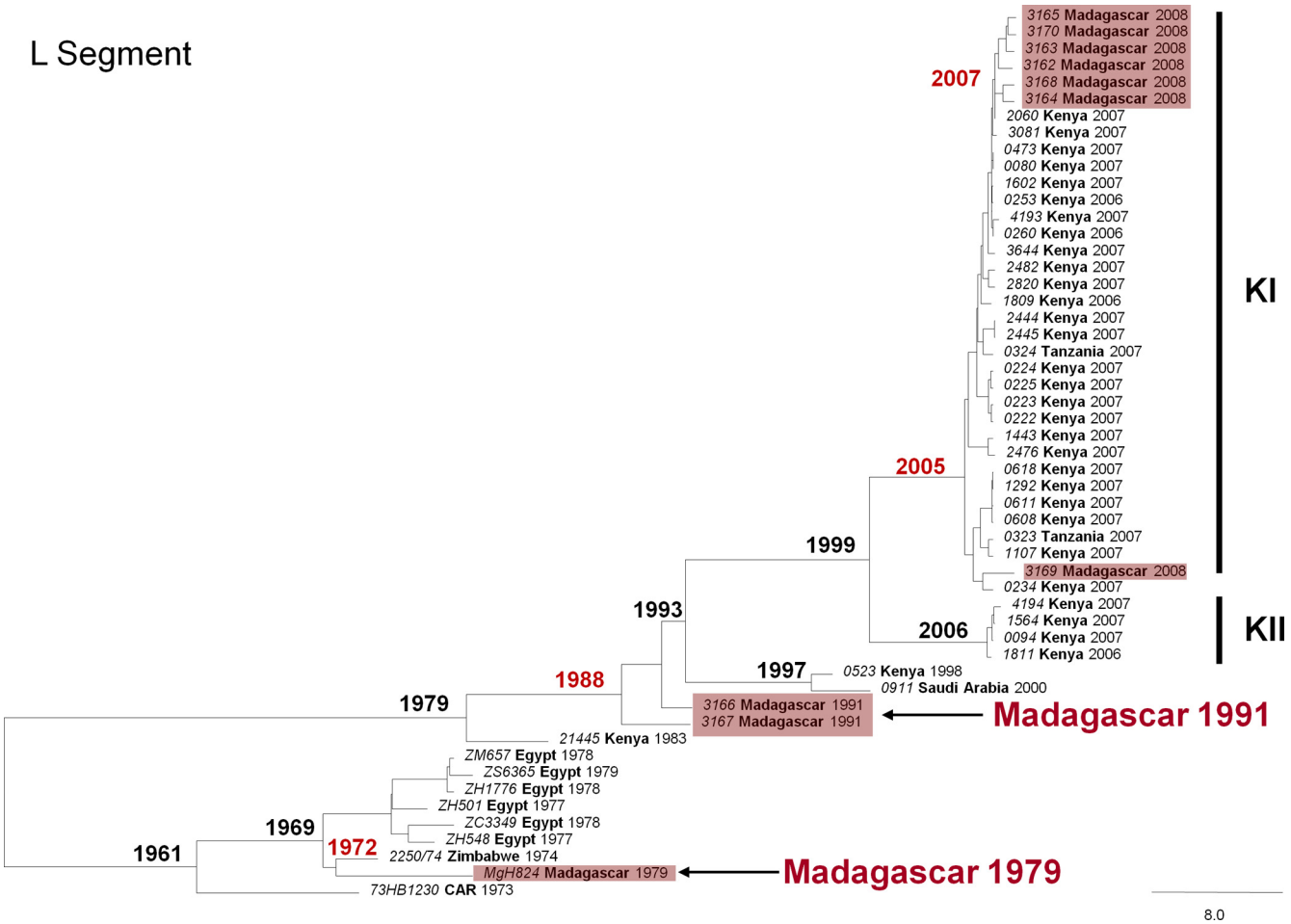

FIG. 3. Bayesian coalescent analysis of RVFVs based on the L segment. The maximum clade credibility tree is shown with the year of the MRCA, in boldface, at each node. Viruses from Madagascar and nodes containing associated MRCA estimates are shown in red. KI, Kenya-I lineage; KII, Kenya-II lineage.

countries in Africa to islands located off the African continent and highlight the risk of subsequent establishment and circulation in a new environment. This is especially important given that replication-competent vectors of several viruses can already be found in these areas (12, 23, 27).

While infected mosquitoes have certainly contributed to the widespread dispersal of RVFV, the role of mosquitoes in the importation of RVFV into Madagascar is unknown. Given the 400 km or more of open water between Madagascar and the African mainland, it is highly improbable, though not impossible, that wind-borne mosquitoes could be the origin of the imported virus. More likely, virus-infected mosquitoes could have been unwittingly transported in air or sea shipments to Madagascar. However, the most likely route remains importation of virus-infected cattle or other livestock. In nearby Comoros, the outbreak of East Coast fever in 2003 and 2004 was attributed to the importation of cattle from Tanzania (8). Only limited information is available regarding the cattle trade to and from Madagascar, with some recent records documenting exportation of ruminants to the Comoros archipelago (1) and importation of live cattle from New Zealand (United Nations Commodity Trade Statistics Database, 2010 [http://comtrade.un.org/db/]). No official records are available to substantiate the importation of cattle from the African mainland to Madagascar; however, the legal and illegal importation of livestock and goods is a common practice throughout the region and has already been implicated as the possible cause of a case of RVFV in Comoros in 2007 (27). Once infected livestock are introduced into an area, the disease spreads rapidly, particularly in countries, such as Madagascar, where the large-scale movement of cattle is common.

Detailed examination of the phylogenetic trees indicates considerable movement of virus-infected livestock or mosquitoes on the African mainland. It is obvious that during 2006 to 2008, the Kenya-I lineage (Fig. 1; see also Fig. S1 in the supplemental material) was distributed across the large distances separating Kenya, Tanzania, Madagascar, and South Africa. In addition, some of the Central African viruses are dispersed in different RVFV lineages, as are Zimbabwe lineages and South African viruses. Clearly, distant movements of RVFVs are not infrequent events.

In conclusion, the generation of whole genomic sequences and advances in Bayesian coalescent methods enabled us to conclude that the reported RVFV events in Madagascar can

be attributed to importation or expansion from mainland Africa. These findings underscore the importance of maintaining accurate and complete import/export records and remaining vigilant for the signs and symptoms of RVFV infection, including unexpected deaths and/or abortions, in livestock and humans.

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