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Genomic and phylogenetic characterization of viruses included in the Manzanilla and Oropouche species complexes of the genus *Orthobunyavirus*, family *Bunyaviridae*

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Summary

A thorough characterization of the genetic diversity of viruses present in vector and vertebrate host populations is essential for the early detection of and response to emerging pathogenic viruses, yet genetic characterization of many important viral groups remains incomplete. The Simbu serogroup of the genus *Orthobunyavirus*, family *Bunyaviridae* is an example. The Simbu serogroup currently consists of a highly diverse group of related arboviruses that infect both humans and economically important livestock species. Here, we report complete genome sequences for 11 viruses within this group, with a focus on the large and poorly characterized Manzanilla and Oropouche species complexes. Phylogenetic and pairwise divergence analyses indicate the presence of high-levels of genetic diversity within these two species complexes, on par with that seen among the five other species complexes in the Simbu serogroup. Based on previously reported divergence thresholds between species, the data suggest that these two complexes should actually be divided into at least five species. Together these five species form a distinct phylogenetic clade apart from the rest of the Simbu serogroup. Pairwise sequence divergences among viruses of this clade and viruses in other Simbu serogroup species complexes are similar to levels of divergence among the other orthobunyavirus serogroups. The genetic data also suggest relatively high levels of natural reassortment, with three potential reassortment events present, including two well-supported events involving viruses known to infect humans.

Introduction

Globalization of travel and trade, climate change and ever-growing human population sizes are all contributing to an increase in the emergence of pathogenic viruses (Lipkin, 2013). Many of these viruses are coming from well characterized and expected groups (e.g., influenza, flaviviruses), whereas others belong to groups that have been largely ignored by past surveillance programs. An example of the latter group are members of the genus *Phlebovirus*, which, with the notable exceptions of Rift Valley fever and Toscana viruses were generally thought to be of little current public health importance until the recent emergence of Severe Fever with Thrombocytopenia Syndrome virus (SFTSV) and Heartland virus (HRTV) (McMullan *et al.*, 2012; Yu *et al.*, 2011). Another, even larger, neglected group is the orthobunyaviruses. Due to their abundance and diversity, many orthobunyaviruses have yet to be fully sequenced and there are likely many others that have yet to be detected. However, a thorough understanding of the sequence diversity of such circulating viruses is a critical part of surveillance and preparedness for future disease outbreaks.

Orthobunyavirus is the largest genus in the *Bunyaviridae* family with over 170 named viruses corresponding to 18 different serogroups and 48 species complexes (Elliott & Blakqori, 2011; Nichol *et al.*, 2005). Although the term ‘serogroup’ is not currently utilized by the International Committee on Taxonomy of Viruses (ICTV), the concept of serogroups has played an important historical role in viral taxonomy (Calisher & Karabatsos, 1988); the classification of arthropod-borne viruses (arboviruses) was initially based on antigenic relationships revealed by serological tests. (Casals, 1957). In general, genetic-based classifications are starting to supplant antigenic classifications;

however, due to the lack of genetic information for many named viruses in *Bunyaviridae*, most current taxonomic assignments are still based on serological criteria (Nichol *et al.*, 2005; Plyusnin *et al.*, 2012). Thus in this report, in the interests of continuity and clarity, the term "serogroup" will continue to be used for a group of serologically related viruses and the term "species complex" will be used for ICTV-defined (Nichol *et al.*, 2005) groups of closely related, differently named viruses whose exact taxonomic status remains uncertain because of slight antigenic variation or differences in host range, vector species, geographic distribution and/or pathogenic potential from the designated type species. The purpose of the present report is to explore genetic diversity within the Simbu serogroup of the genus *Orthobunyavirus*, a diverse and geographically widespread group that includes important human and livestock pathogens (Kinney & Calisher, 1981; Saeed *et al.*, 2001a).

The Simbu serogroup currently includes 22 officially recognized viruses that have been grouped into seven different species complexes (Akabane, Manzanilla, Oropouche, Sathuperi, Simbu, Shamonda and Shuni) (Nichol *et al.*, 2005), as well as several other recently described viruses that have yet to be officially assigned to a species (Aguilar *et al.*, 2011; Figueiredo & Da Rosa, 1988; Goller *et al.*, 2012; Plyusnin *et al.*, 2012; Saeed *et al.*, 2001b). Full genomes have recently been obtained for 12 viruses within the Simbu serogroup (Goller *et al.*, 2012); however, these genome sequences are not equally distributed among the seven species complexes. For example, only one representative, Oropouche virus (OROV), has been fully sequenced from the Oropouche species complex, and no complete sequences are available from the Manzanilla species complex (Kinney & Calisher, 1981). Yet, these are two of the largest species complexes within the

Simbu serogroup, and the Oropouche species complex is the only one with members that are known to cause human disease. The lack of complete genomes for all members of these species complexes impacts diagnostic capacity (e.g., the ability to identify conserved/divergent regions for primer design in PCR applications and for recognition with sequence-based diagnostic methods); it also prevents the recognition of reassortants.

Here we utilize high-throughput sequencing technologies to improve our understanding of the diversity and evolutionary history of these two species complexes by obtaining full genome sequences for 11 previously uncharacterized viruses, including the three remaining members of the Oropouche species complex, four of the five members of the Manzanilla species complex and four other unssigned viruses that have demonstrated genetic and/or antigenic similarities to one of these two species complexes. In order to compare the sequences with previous taxonomic characterizations, serological comparisons were conducted among these 11 uncharacterized viruses and the other fully sequenced members of these two species complexes.

Results & Discussion

Genome Sequences

Genome sequences for 11 viruses (Table 1) were obtained through *de novo* assembly from either 454 (Roche) or Illumina sequences. Each sequenced orthobunyavirus genome consisted of three distinct RNA segments, and the sizes and organization of the open-reading frames (ORFs) were generally consistent with what has been previously described for the genus (Plyusnin *et al.*, 2012) (Table S1). The 3' terminal sequence was obtained for 18 segments (9 different viruses) and the 5' terminal sequence was obtained for 7 segments (5 different viruses). In all cases, the ten most terminal nucleotides were identical to those that have previously been reported for the genus (Plyusnin *et al.*, 2012). Segments without sufficient coverage to assemble the ends were completed using primers targeting these conserved terminal sequences. The largest (L) genome segment of *Orthobunyavirus* contains a single ORF that encodes an RNA polymerase; in our sequences, this ORF ranged in size from 6756-6783 bases. The medium-sized (M) segment also contains a single ORF, which encodes a polyprotein that is co-translationally cleaved into two envelope glycoproteins and a non-structural protein. The M segment ORF of the 11 viruses ranged in size from 4254-4299 bases. The smallest (S) segment typically contains two overlapping ORFs, which code for a nucleocapsid protein (NP) and a non-structural protein (NSs). The NP ORF of the 11 viruses ranged in size from 693-699 bases, while the NSs ORF ranged from 273-288 bases, with two exceptions; Utinga (UTIV) and Utive (UVV) both contained severely truncated, presumably non-functional, versions of the NSs ORF due to multiple nonsense mutations (81 bases, 27 aa). When functional, the NSs proteins of orthobunyaviruses have been

shown to act as interferon antagonists (Elliott & Weber, 2009), and therefore it is reasonable to suggest that loss of the NSs protein in UTIV and UVV may have altered the virulence of these viruses. Similar loss of the NSs has been reported in several other orthobunyavirus serogroups, including Anopheles A and B, Tete and Wyeomyia (Chowdhary *et al.*, 2012; Mohamed *et al.*, 2009); however, to our knowledge this is the first description of a single serogroup with members that both contain and lack a NSs protein.

Genus-level Phylogenetic Relationships

Phylogenetic analyses of the three genome segments confirmed that the 11 sequenced viruses are all genetically closely related to the previously sequenced Simbu serogroup viruses. The Simbu serogroup forms a monophyletic clade in both the M and S segment trees (bootstraps=66.9 and 100, respectively; Figures S1-S2), and in the L segment tree the serogroup is paraphyletic, also including Leanyer virus (LEAV; Figure 1) (Savji *et al.*, 2011). Uncertainty in placing LEAV is the main reason for a low bootstrap in the M segment tree; without LEAV, the Simbu serogroup clade is supported in 90% of bootstrap analyses. Our extended genetic sampling of the Oropouche and Manzanilla species complexes illuminated a deep evolutionary divide within the Simbu serogroup; one phylogenetic clade (Clade A) includes the Manzanilla and Oropouche species complexes and a second clade (Clade B) includes the other five Simbu species complexes (Figures 1, S1-S2) (Kinney & Calisher, 1981). These monophyletic clades are well-supported across all three genomic segments with bootstrap support ranging from 97.5-100%, and there is no evidence of reassortment between these clades.

158 In 1981, Kinney and Calisher (Kinney & Calisher, 1981) used a combination of
159 serological analyses to provide finer levels of classification within the Simbu serogroup;
160 the two genetic clades seen in our analysis are consistent with the serocomplexes they
161 identified. Clade B corresponds completely to their original Simbu serocomplex, whereas
162 the Oropouche and Manzanilla species complexes (clade A) each correspond to unique
163 serocomplexes. Pairwise genetic similarities at the amino acid-level between clades A
164 and B ranged from 56.6-61.5%, 32.4-39%, and 59.7-71.9% for the L, M and S segments,
165 respectively, while similarities within the clades ranged from 65.9-99.7%, 34.4-89.1%
166 and 69.8-100%, respectively (Figure 2). In our previous evaluation of LEAV (Savji *et al.*,
167 2011), minimum percent amino acid similarities were proposed as criteria for inclusion of
168 viruses within the same group, where the term "group" refers to the taxonomic division
169 currently occupied by serogroups. Clear distinctions were found between intra- and inter-
170 group genetic similarities at the L and S segments, and cutoffs of 59 and 60%,
171 respectively, were proposed (Savji *et al.*, 2011). With currently available data, we see
172 similarly clear distinctions in comparisons of L segment sequences, even when Simbu
173 serogroup clades A and B are treated separately. In fact, ~77% (92/120) of the pairwise
174 comparisons between these two clades fall at or below the proposed 59% similarity cutoff,
175 and 90.8% (109/120) of the pairwise comparisons are below a similarity cutoff of 60%,
176 while all intra-clade comparisons are well above these cutoffs. In general, we found
177 levels of intra- and inter-serogroup genetic similarity to be less distinct when comparing
178 the S segments and no pairwise comparisons between clades A and B met the previously
179 proposed cutoff for different serogroups. However, 76.9% (120/156) of pairwise
180 comparisons between clades A and B exhibit <69% similarity, while all comparisons

within the two clades are above this threshold. Furthermore, this degree of S segment similarity is on par with pairwise comparisons between members of the Wyeomyia and Bunyamwera serogroups (Figure 2); the former was not included in our previous analysis (Savji *et al.*, 2011). Therefore, we argue that the level of evolutionary divergence between these two Simbu serogroup clades is more consistent with levels of divergence seen among the other orthobunyavirus serogroups than that seen within serogroups.

One of the hallmarks of the Simbu serogroup is its extensive geographic distribution, and this has been suggested to be one of the major factors behind the high-level of genetic diversity found within this group (Saeed *et al.*, 2001a). Even if the two Simbu serogroup clades are considered as distinct serogroups, both still exhibit extensive geographic distributions (Figure S3), which may explain the high levels of genetic diversity within each of these two groups as compared to many other orthobunyavirus serogroups (Figure 2). However, clade A (Manzanilla and Oropouche species complex viruses) is unique in being found within the Americas. To date, 77% (10/13) of Manzanilla and Oropouche species viruses have been isolated from North and/or South America (including Inini virus (Saeed *et al.*, 2001a)), but no clade B viruses have been found yet in this region. However, Clade A viruses are not restricted only to the Americas, as isolates of some representatives have been obtained from Australia, South Africa and Vietnam (Bryant *et al.*, 2005; Doherty *et al.*, 1979; McIntosh *et al.*, 1965), and serological evidence exists for the presence of Ingwavuma virus (INGV) in Nigeria, the Central African Republic, India, Thailand and Cyprus (http://wwwn.cdc.gov/arbocat/). These differences in distribution between the two genetic clades may relate to differences in vector and/or host range, which can facilitate or restrict the geographic spread of

viruses. More effort on the surveillance and identification of arthropod vectors of viruses in this group is necessary to better understand the forces that have shaped and maintain these distinct distributions.

Species-level Phylogenetic Relationships

Within clade A, two species complexes have been proposed (Nichol *et al.*, 2005); however, the genetic data presented here suggests the presence of at least five different lineages, which should likely be considered distinct species (Figures 1, S1-S2). Based on a limited number of available sequences, it has been observed that distinct *Orthobunyavirus* species tend to differ by at least 10% when comparing nucleoprotein (N) amino acid sequences (S-segment). Utilizing this criterion, clade A should be divided into five different species with Buttonwillow (BUTV) forming its own species apart from the other Manzanilla species complex viruses and with the Oropouche species complex being split into three distinct species. Together, UTIV and UVV form one of the new species within the current Oropouche species complex; the name Utinga is suggested for this species since this is the first of its members to be described (Shope *et al.*, 1967). Facey's Paddock virus (FPV), which is a phylogenetic outlier and highly divergent from all of the other clade A viruses (minimum N protein divergence=26%), represents the other new species previously attributed to the Oropouche species complex.

These species divisions are phylogenetically consistent in both the S-segment and L-segment trees with between species amino acid divergences of at least 14.1% and 20.9%, respectively, and maximum within species divergences of 7.3% and 17.3%, respectively. The M segment phylogeny is also generally consistent with these species,

with the exception of the Oropouche species, which is complicated by several potential reassortment events (see below). Excluding Oropouche species viruses, all M-segment, amino acid divergences within species are ≤21.4% while all divergences between species are ≥32.8%. These species are also consistent with serological comparisons (Tables 2-3), and in certain cases, correspond with available phenotypic information. For example, mosquitos are the primary vectors for most of the Manzanilla species complex viruses, whereas BUTV has only been associated with *Culicoides* midges (http://wwwn.cdc.gov/arbocat/). Throughout the rest of the manuscript, we utilize these five species designations without the use of the term 'complex,' in order to distinguish them from the species previously identified by the ICTV (Nichol *et al.*, 2005).

Unassigned viruses

Four of the viruses sequenced here have not been officially assigned to a species complex by the ICTV. However, using a combination of genetic (Figures 1, S1-S2) and serological data (Tables 2-3) it is clear that one of these viruses (VN04-2108) belongs to the Manzanilla species, while the other three (Iquitos (IQTV), Jatobal (JATV) and FMD1303) belong to the Oropouche species. Virus strain VN 04-2108 was originally reported to be Oya virus (OYAV), based on indirect immunofluorescence assays and its high nucleotide similarity to OYAV, based on a partial S segment sequence (Bryant *et al.*, 2005). Our S genome segment is identical to the sequence from the original characterization of VN 04-2108; in fact, this sequence is characteristic of all the "Oya" isolates obtained in Vietnam during that study (Bryant *et al.*, 2005). However, when compared now to all of our newly available sequence data, VN 04-2108 exhibits similar

levels of divergence to four named viruses in the Manzanilla species complex: the original OYAV isolate (4.1% aa, 9.6% nt), Manzanilla virus (MANV, 2.5% aa, 13.1% nt), INGV (5.7% aa, 11.2% nt) and Mermet virus (MERV, 4.1% aa , 14.5% nt). The prototype OYAV virus was isolated from a sick pig in Malaysia during a Nipah virus outbreak and was not available for this study, so all of these divergences are based only on the published portion of the S segment available for the original OYAV isolate (AB075611). No genus-wide framework has been proposed for genetically determining which orthobunyaviruses should be uniquely named; however, based on the levels of genetic divergence among currently named viruses in this group, VN 04-2108 should likely be given its own unique name, in which case, Cat Que (CQV) is suggested as this is the name of the community in Vietnam where the infected mosquitoes were collected (Bryant *et al.*, 2005). Alternatively, given the overall genetic and phylogenetic similarity of VN 04-2108, MANV, MERV and INGV across all three genome segments (Figures 1, S1-S2), it may be more prudent to simplify the nomenclature by referring to all four simply as distinct strains of a single named virus, utilizing the name of the species to which they all belong (e.g., Manzanilla virus VN 04-2108, Manzanilla virus AV 782). UTIV and UVV are also strong candidates for synonymization under a single virus name, as they exhibit even higher levels of sequence similarity than those within the Manzanilla species. High levels of genetic similarity are also seen among named members of the Oropouche species at particular segments; however, relationships are complicated by patterns of reassortment (see below).

FMD1303 is a previously uncharacterized orthobunyavirus that was isolated from a febrile human in the Madre de Dios region of Peru. Serologically, FMD1303 is broadly

reactive with OROV, IQTV and JATV, which suggests that it is a member of the Oropouche species, and this is consistent with the available genetic data. The S segment of FMD1303 is identical at the amino acid-level to both OROV and IQTV. This is consistent with what is known about the epidemiology of FMD1303, as OROV and IQTV are the only other two Simbu serogroup viruses that have been shown to cause disease in humans (Aguilar *et al.*, 2011; Anderson *et al.*, 1961). However, based on the nucleotide sequence of the S segment, FMD1303 falls outside of the range of diversity currently described for OROV/IQTV (Figure S4). The L segment phylogeny is also consistent with this finding, though for this segment many fewer sequences are available for comparison, while the patterns on the M segment are complicated by reassortment (see below). Nonetheless, this virus expands the known diversity of Oropouche species viruses that infect humans. Due to its distinctiveness, we suggest that this virus be given its own name; we propose the name Madre de Dios virus (MDDV), based on the collection locality.

Partial genome sequences from JATV have previously demonstrated an association with the Oropouche species (Saeed *et al.*, 2001a; Saeed *et al.*, 2001b), and our genetic and serological analyses confirm this assignment. However, the complete genome sequence of Jatobal (JATV) (JQ675601- JQ675603) differs from the partial sequences previously reported for the same strain (AF312380 & AF312382) (Saeed *et al.*, 2001a; Saeed *et al.*, 2001b). These results were confirmed at the Center for Technological Innovation, Genomic core, Evandro Chagas Institute, by obtaining a second full-length sequence of a lower-passage of JATV strain BeAn 423380 (original seed). Sequences of the original seed and the passaged virus from the World Reference Center for Emerging

Viruses and Arboviruses (WRCEVA) collection are identical. The largest discrepancy between the JATV sequences described here and those that were previously reported lies on the S segment where the two sequences are only 83.4% identical at the nucleotide-level (90.4% aa), with polymorphisms present throughout the ~700 nucleotide bases. The M segment sequences, on the other hand, are essentially identical across the 570 bases covered by the partial sequence, except for several differences at the ends of the partial M sequence. We cannot account for these differences in the S segment sequences, as we could not detect the previously published sequence in either of our JATV samples, but we are confident in the quality of the genome sequence reported here.

Reassortment

Reassortment in multi-segmented viruses is a form of genetic exchange that has the potential to provide many of the benefits of sexual exchange, for example, the rapid introduction of novel variation within a lineage, the uncoupling of beneficial mutations from detrimental changes and the ability to combine multiple beneficial mutations that originated in different lineages (Simon-Loriere & Holmes, 2011). In fact, reassortment has been implicated in multiple instances of host/vector range shifts and changes in pathogenicity (Briese *et al.*, 2006; Idris *et al.*, 2008; Le Nouen *et al.*, 2006; Nelson & Holmes, 2007; Parrish & Kawaoka, 2005; Schrauwen *et al.*, 2011). Laboratory experiments have demonstrated that reassortment is common between many bunyaviruses, *in vitro* (Gentsch & Bishop, 1976; Gentsch *et al.*, 1977; Gentsch *et al.*, 1980; Iroegbu & Pringle, 1981; Pringle & Iroegbu, 1982; Reese *et al.*, 2008), and a number of recent genomic analyses have suggested that reassortment also plays an

important role in viral evolution in natural populations (Aguilar *et al.*, 2011; Briese *et al.*, 2013; Briese *et al.*, 2006; Kobayashi *et al.*, 2007; Nunes *et al.*, 2005; Reese *et al.*, 2008; Yanase *et al.*, 2010; Yanase *et al.*, 2006). These examples include multiple viruses within the *Orthobunyavirus* genus. Given the potential evolutionary implications, it is important to monitor for the prevalence of viral reassortment events, especially in virus groups known to infect mammals.

To look for reassortment events, nucleotide-level phylogenetic analyses were conducted, which included only fully sequenced members of the five clade A species (Figure 3). Phylogenetic discordance, representing potential reassortment, is evident among the phylogenetic trees built from the three different genome segments. More specifically, while the S and L trees exhibit nearly identical branching patterns, the M segment supports different relationships among several of the sequenced viruses. In total, there are three discrepancies between the M segment tree and the S/L segment trees. The two best supported discrepancies (bootstraps ≥75.9 in all trees) involve viruses in the Oropouche species. The first involves OROV, IQTV and MDDV. OROV and IQTV are sister taxa in the S/L trees, while IQTV and MDDV are sister taxa in the M tree (Figure 3). Support for the relationships of these three taxa is extremely high in all trees (≥99 bootstrap). Sliding window analyses in RDP confirm that these disparate patterns of divergence are consistent throughout each genomic segment, as expected with a reassortment event along one of these three lineages.

IQTV was recently described as a reassortant between OROV and an unknown Simbu serogroup virus based on partial sequences from the S and M segments (Aguilar *et al.*, 2011). Our results are consistent with this finding; furthermore, we have been able to

identify MDDV as a potential source for the M segment of IQTV. Natural reassortment between MDDV and OROV is certainly plausible given their documented geographic distributions; both viruses have been isolated from the Madre de Dios region of Peru, and the S segment of IQTV is most similar to the S segments from clade II of OROV, which is the only clade, thus far, that has been found in Peru (Aguilar *et al.*, 2011; Saeed *et al.*, 2000). Interestingly, the level of amino acid divergence between OROV and both IQTV and MDDV (41-42%) is on par with levels of divergence seen between species. Whether this reassortment event has resulted in any changes in virulence, vectors or range has yet to be determined. It is also important to keep in mind that with the current available data it is impossible to know for certain which of these three viruses represents the true reassortant (Briese *et al.*, 2013). The addition of more complete genome sequences from each of these viral lineages should clarify relationships.

The second potential case of reassortment involves JATV, the Utinga species (i.e., UTIV/UVV) and the lineage leading to the three human viruses (OROV, IQTV and MDDV). In the L and S trees, JATV forms a clade with the three human viruses, whereas in the M segment tree JATV forms a well-supported clade with the Utinga species (Figure 3). The RDP analyses demonstrate that these discordant relationships are consistent throughout the entirety of each genome segment (Figures S5-S6). Based on the high-levels of sequence divergence among all three groups of viruses (>45%), this reassortment event is likely to have occurred many generations ago or to have involved parental viruses that have yet to be isolated and/or sequenced.

JATV has been previously reported to be a reassortant with an S segment from OROV and an M segment from an uncharacterized virus (Saeed *et al.*, 2001b). While our

findings are generally consistent with the overall conclusion of reassortment, our S segment sequence is not consistent with the previously reported S segment (Saeed *et al.*, 2001b). The S segment in the previous publication fell within the OROV clade, whereas our S sequence is a phylogenetic outgroup to the S segments of OROV, IQTV and MDDV (Figure 3). Our L segment is also consistent with this placement, demonstrating that this is an S,L vs. M segment reassortment event. Also, based on the available data, it is again impossible to distinguish which of the viral lineages involved represents the true reassortant.

The third potential reassortment event involves Manzanilla species viruses. CQV and INGV are sister taxa in the L/S trees, whereas CQV forms a clade with MANV and MERV in the M segment tree (Figure 3). The bootstrap support for these different relationships, however, is lower than that seen in the other discrepancies and the RDP analyses demonstrate that the divergence signals are not consistent across the genome segments (Figures S5-S6). Therefore, it is unclear whether this third discrepancy is due to reassortment or simply the result of ambiguity in the patterns of divergence.

Conclusion

The addition of 11 fully sequenced genomes for viruses in the Manzanilla and Oropouche species complexes has highlighted a deep evolutionary divide between these two species complexes and the rest of the Simbu serogroup. With sequence data from all three genome segments, we find compelling evidence to divide these two species complexes into five distinct species, and we have also been able to identify three potential reassortment events among viruses in these species. Two of these involve viruses that infect humans, and levels of sequence divergence on the reassorted segment

388 are on par with divergences seen between species. Future work is needed to determine
389 whether any of these reassortments have affected virulence.
390

Materials and Methods

Virus Isolates

All virus stocks used in this study were obtained from the World Reference Center for Emerging Viruses and Arboviruses (WRCEVA) at the University of Texas Medical Branch (UTMB). The JATV original seed was provided by the World Health Organization Reference Center for Arboviruses at the Department of Arbovirology and Hemorrhagic Fevers, Instituto Evandro Chagas, Brazilian Ministry of Health. Virus strain FMD1303 was originally isolated at the U.S. Naval Medical Research Unit No. 6 (NAMRU-6) in Lima from a blood sample obtained from a febrile human in Madre de Dios Department, Peru on March 22, 2007. The histories of the other isolates sequenced in this study have been previously published, see Table 1.

Serological characterization

All of the sequenced viruses were compared with each other and with OROV for serological similarity. Methods used to prepare antigens for the complement-fixation (CF) tests and for the preparation of immune ascitic fluids have been described previously (Beaty *et al.*, 1989; Travassos da Rosa *et al.*, 1983; Xu *et al.*, 2007). Both antigens and antibodies were produced in mice. CF tests were performed by the microtiter technique (Beaty *et al.*, 1989; Xu *et al.*, 2007), using two units of guinea-pig complement with overnight incubation of the antigen and antibody at 4^{o}C. CF titers were recorded as the highest dilutions giving 3+ or 4+ fixation of complement. Titers of 1:8 or greater were considered positive. Hemagglutination-inhibition (HI) testing was performed in microtiter plates, as described previously (Travassos da Rosa *et al.*, 1983).

HI tests were performed with four hemagglutination units of virus at the optimal pH (5.75) against serial two-fold antiserum dilutions, starting at 1:20. HI titers of 1:20 or greater were considered positive. CF and HI tests were performed at the University of Texas Medical Branch, Galveston.

Genome sequencing

The BeAn 423380 (JATV) and VN 04-2108 (CQV) strains were sequenced and assembled at the Center for Infection and Immunity, Columbia University. The JATV original seed was sequenced and assembled at the Center for Technological Innovation, Genomic and Bioinformatic Cores, Evandro Chagas Institute, Brazil. For these strains, total RNA was first extracted from viral supernatant preserved in TRIzol LS (Invitrogen, Carlsbad, CA, USA) and then treated with DNase I (DNA-Free, Ambion, Austin, TX, USA). cDNA was generated using the Superscript II system (Invitrogen) with random hexamers linked to an arbitrary 17-mer primer sequence (Cox-Foster *et al.*, 2007). The resulting cDNA was treated with RNase H and then amplified by random PCR (Cox-Foster *et al.*, 2007). Products greater than 70 bp long were selected by column purification (MinElute, Qiagen, Hilden, Germany) and ligated to specific adapters for sequencing on the 454 Genome Sequencer FLX (454 Life Sciences, Branford, CT, USA) without fragmentation of the cDNA (Cox-Foster *et al.*, 2007; Margulies *et al.*, 2005; Palacios *et al.*, 2008). Software programs accessible through the analysis applications at the GreenePortal website (http://tako.cpmc.columbia.edu/portal/) were used for removal of primer sequences, redundancy filtering, and sequence assembly. These genomes were completely confirmed using dye-labeled, dideoxynucleotide sequencing.

All other strains were processed at the Center for Genome Sciences, USAMRIID, Ft. Detrick. For these strains, total RNA was extracted from viral supernatant preserved in TRIzol LS and was amplified using sequence independent single primer amplification (SISPA) as previously described (Djikeng *et al.*, 2008). Amplicons were sheared to ~400 bp and used as starting material for Illumina TRU-seq DNA libraries. Sequencing was performed on a HiSeq 2500. Primers were trimmed from the sequencing reads using Cutadapt (Martin, 2011), quality filtering was conducted with Prinseq-lite (Schmieder & Edwards, 2011) and then genomes were assembled using Ray Meta (Boisvert *et al.*, 2012) in combination with custom scripts. When necessary, terminal sequences were completed through PCR and dideoxynucleotide sequencing using a universal orthobunyavirus primer targeting the conserved viral termini (5'- AGT AGT GTR C-3') in combination with specific primers designed from the sequences generated from the *de novo* assembly. In addition, four genomes were confirmed with dideoxynucleotide sequencing (BUTV, FPV, UTIV and UVV). These include the six genome segments with the lowest levels of sequence coverage (30-767x). These sequences confirmed the high-quality of assemblies achieved through these methods.

Phylogenetic analysis

Separate phylogenetic analyses were conducted for each of the three genome segments using only the protein coding portions of the genome. Orthobunyavirus sequences from GenBank were included to provide a representative picture of the entire genus; many of the sequences included cover only a portion of the coding region. Sequences were aligned using the CLUSTAL algorithm, which was implemented at the

amino-acid (aa) level in MEGA v5.1 (Tamura *et al.*, 2011) with additional manual editing to ensure the highest possible alignment quality. Neighbor-joining analyses using p-distance at the amino-acid level were performed. The statistical significance of the tree topology was evaluated by 1000 replications of bootstrap re-sampling. Phylogenetic analyses were performed using MEGA v5.1 (Tamura *et al.*, 2011).

Reassortment Analysis

To identify potential reassortment events, the data was mined for evidence of phylogenetic discordance. For this analysis, additional phylogenetic trees were constructed, which included only fully sequenced members of the Oropouche and Manzanilla species complexes. These trees were constructed from the same alignments used above; however, to provide additional power, these trees were conducted using a maximum-likelihood framework at the nucleotide-level (implemented in MEGA v5.1 (Tamura *et al.*, 2011) with the Tamura-Nei substitution model, partial deletion, uniform rates among sites and 1000 bootstrap replications). Potential reassortment events were then verified using the manual BOOTSCAN (Martin *et al.*, 2005) and distance plot methods in RDP4 (Martin *et al.*, 2010).

Pairwise Sequence Analysis

Pairwise sequence divergences were calculated among each of our 11 viruses and all of the other orthobunyaviruses with complete genome segment sequences using MEGA v5.1 with pairwise deletions (Tamura *et al.*, 2011). For the comparisons of divergence within and between serogroups, only one representative of each named

483 species was utilized. This downsampling was done to avoid bias due to intensive
484 sampling of certain viruses.
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Figure 1. Phylogenetic tree of *Orthobunyavirus* based on the protein-coding portion of the L segment. The tree was built using translated amino acid sequences in Mega v5.1 (Tamura *et al.*, 2011) using the Neighbor-joining algorithm and a p-distance matrix. The tree is unrooted and the node labels represent bootstrap support values after 1000 resampling events. Black circles indicate the genomes that were sequenced in this study. Species designations (left brackets) are based on the genetic data presented in this manuscript. Clade labels on the far right correspond to serogroups.

Figure 2. Pairwise genetic similarities (1 – amino acid p-distance) among viruses within and between serogroups of orthobunyaviruses based on the a) L segment and b) S segment. For the two Simbu serogroup clades, an extra category is presented that includes only the pairwise similarities between these two groups; this is a subset of the inter-group distances for both Clade A and Clade B. See supplementary tables S2-S3 for the list of sequences used in these analyses.

Figure 3. Nucleotide-level phylogenetic trees including only the fully sequenced members of the Oropouche and Manzanilla species complexes. All trees were built in Mega v5.1 (Tamura *et al.*, 2011) using the maximum-likelihood framework with partial deletions. The trees are unrooted. Node labels represent bootstrap support values after 1000 resampling events.

732 Table 1. Virus isolates sequenced in this study.

Virus	Strain	Source	Locality	Year	Species	Accession #s	Ref
Manzanilla (MANV)	TRVL 3587	*Alouatta seniculus* (Red Howler Monkey)	Trinidad	1954	Manzanilla	KF697148-50	(Anderson *et al.*, 1960)
Ingwavuma (INGV)	SA An 4165	*Hyphanturgus ocularis* (Spectacled Weaver)	South Africa	1959	Manzanilla	KF697139-41	(McIntosh *et al.*, 1965)
Mermet (MERV)	AV 782	*Progne subis* (Purple Martin)	USA	1964	Manzanilla	KF697151-53	(Calisher *et al.*, 1969)
Cat Que*	VN 04-2108	*Culex* sp. (Mosquitoes)	Vietnam	2004	Manzanilla	JQ675598-600	(Bryant *et al.*, 2005)
Buttonwillow (BUTV)	BFS 5002	*Culicoides* sp. (Biting Midges)	USA	1964	Buttonwillow	KF697160-62	(Reeves *et al.*, 1970)
Facey's Paddock (FPV)	Aus Ch 16129	*Mosquitoes*	Australia	1974	Facey's Paddock	KF697136-38	(Doherty *et al.*, 1979)
Utinga (UTIV)	Be An 84785	*Bradypus tridactylus* (Pale-throated sloth)	Brazil	1965	Utinga	KF697154-56	(Shope *et al.*, 1967)
Utive (UVV)	Pan An 48878	*Bradypus variegatus* (Brown-throated sloth)	Panama	1975	Utinga	KF697157-59	(Seymour *et al.*, 1983)
Jatobal (JATV)	BeAn 423380	*Nasua nasua* (South American Coati)	Brazil	1984	Oropouche	JQ675601-03	(Figueiredo & Da Rosa, 1988)
Iquitos (IQTV)	IQT9924	*Homo sapiens* (Human)	Peru	1999	Oropouche	KF697142-44	(Aguilar *et al.*, 2011)
Madre de Dios^	FMD 1303	*Homo sapiens* (Human)	Peru	2007	Oropouche	KF697145-47	NA

733 Species designations are based on the genetic data presented in this manuscript.
734 ^ "Madre de Dios" is an unofficial name proposed here to refer to isolate FMD 1303.
735 * Previously described as an isolate of Oya virus (OYAV). "Cat Que" is an unofficial name
736 proposed here to refer to isolate VN 04-2108.
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Table 2. Complement fixation results.

Antigen	Complement Fixation test Antibody											
	ORO	**IQT**	**MDD**	**JAT**	**UTI**	**UV**	**BUT**	**CQ**	**ING**	**MER**	**MAN**	**FP**
ORO	**512** **≥32**	512 ≥8	512 ≥8	128 ≥8	8 ≥32	32 ≥32	0	32 ≥8	0	16 ≥8	0	16 ≥8
IQT	512 ≥Φ	**512** **≥Φ**	1024 ≥Φ	256 ≥Φ	0	64 ≥Φ	0	64 ≥Φ	8 ≥Φ	32 ≥Φ	0	16 ≥Φ
MDD^	512 ≥Φ	512 ≥Φ	**1024** **≥Φ**	256 ≥Φ	16 ≥Φ	64 ≥Φ	0	64 ≥Φ	8 ≥Φ	0	0	32 ≥Φ
JAT	512 ≥Φ	512 ≥Φ	1024 ≥8	**256** **≥Φ**	0	32 ≥Φ	0	64 ≥Φ	0	16 ≥Φ	0	8 ≥8
UTI	32 ≥32	64 ≥8	32 ≥8	16 ≥8	**32** **≥32**	256 ≥32	0	32 ≥8	0	8 ≥8	0	32 ≥8
UV	32 ≥32	64 ≥8	64 ≥8	16 ≥8	32 ≥32	**256** **≥32**	0	32 ≥8	0	8 ≥8	0	16 ≥8
BUT	64 ≥8	64 ≥8	64 ≥8	32 ≥8	0	8 ≥8	**32** **≥8**	32 ≥8	8 ≥8	8 ≥8	0	8 ≥8
CQ*	64 ≥Φ	64 ≥Φ	64 ≥8	32 ≥Φ	0	8 ≥Φ	0	**512** **≥Φ**	64 ≥Φ	512 ≥Φ	16 ≥Φ	0
ING	32 ≥8	64 ≥8	64 ≥8	32 ≥8	0	16 ≥8	0	512 ≥8	**64** **≥8**	512 ≥8	16 ≥8	0
MER	8 ≥8	32 ≥8	64 ≥8	16 ≥8	0	0	0	256 ≥8	32 ≥8	**512** **≥8**	**8** **≥8**	0
MAN	64 ≥Φ	64 ≥8	32 ≥8	32 ≥8	0	0	0	512 ≥Φ	64 ≥Φ	512 ≥Φ	**128** ≥Φ	0
FP	64 ≥Φ	32 ≥Φ	64 ≥Φ	0	8 ≥Φ	32 ≥Φ	0	32 ≥Φ	8 ≥Φ	0	0	**512** **≥2**
Normal	0	0	0	0	0	0	0	0	0	0	0	0

Values are displayed as levels of dilution for antibody/antigen. Φ = undiluted. Within the *Bunyaviridae* family, **complement fixation test** generally detects nucleoprotein antibodies, a marker for the **S RNA** segment.
In this study, some of the homologous **CF** titers of four dose hyperimmune ascitic fluid were high (**512-1024**), probably explaining the more extensive, low titer heterologous relationship obtained.
^ MDD stands for Madre de Dios, an unofficial name proposed here to refer to isolate FMD 1303.

* CQ stands for Cat Que, an unofficial name proposed here to refer to isolate VN 04-2108.
Other abbreviations: ORO-Oropouche, IQT-Iquitos, JAT-Jatobal, UTI-Utinga, UV-Utive, BUT-Buttonwillow, ING-Ingwavuma, MER-Mermet, MAN-Manzanilla, FP-Facey's Paddock.

Table 3. Hemagglutination inhibition results.

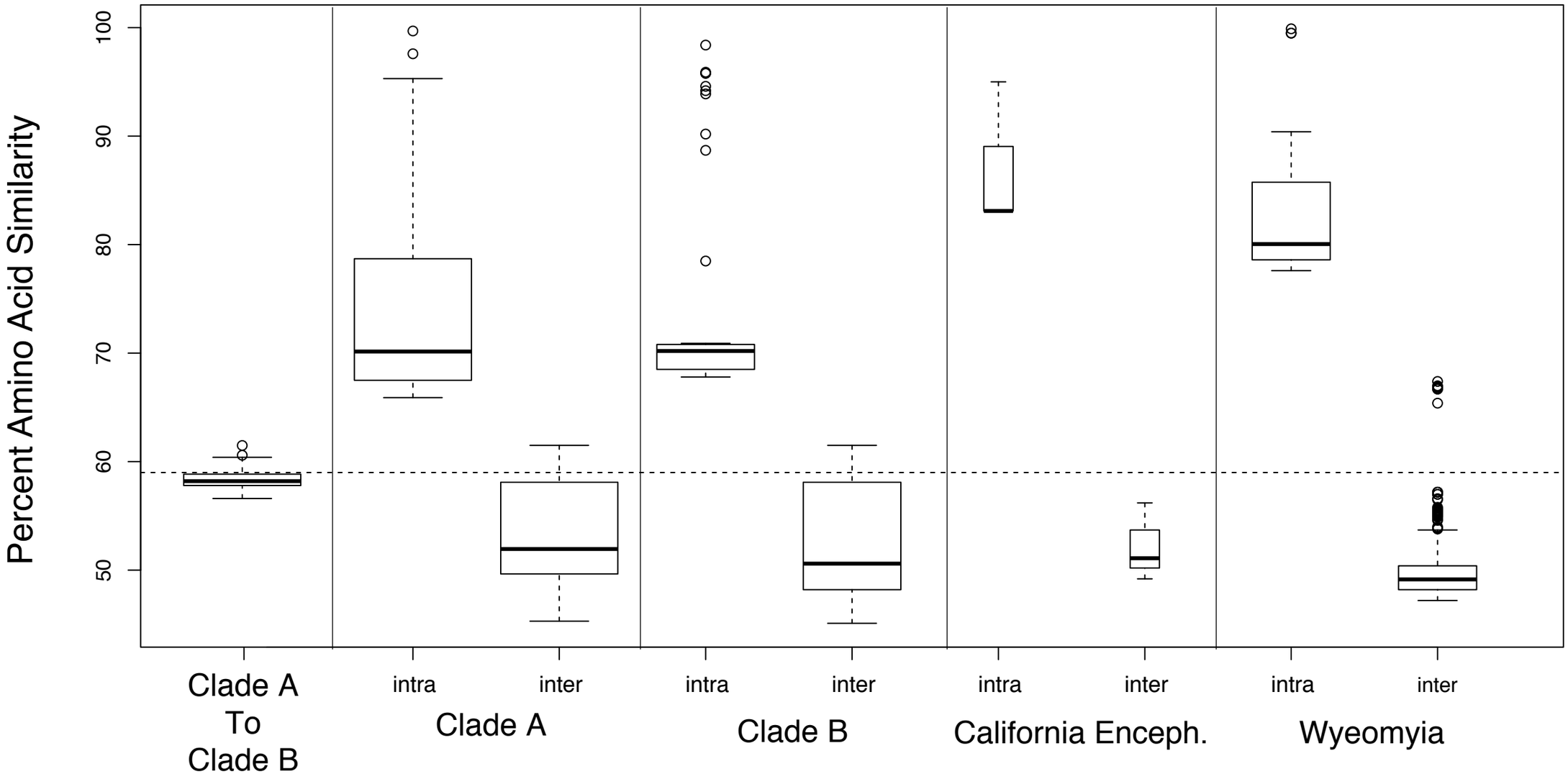
Antibody	Hemagglutination Inhibition test Antigen 4u.				
	ORO	**UTI**	**CQ***	**ING**	**MER**
ORO	**1:10240**	1:160	1:80	1:20	1:40
IQT	1:320	1:80	1:80	1:40	1:80
MDD^	1:320	1:80	1:80	1:40	1:40
JAT	0	0	1:40	0	0
UTI	1:20	**1:20**	0	0	0
UV	1:80	1:80	1:40	0	0
BUT	0	0	1:20	0	0
CQ*	1:160	1:80	**1:2560**	1:640	1:320
ING	1:80	1:40	1:80	**1:160**	1:160
MAN	0	0	1:40	1:40	1:40
MER	1:40	1:40	1:160	1:160	**1:640**
FP	1:20	0	1:20	0	0

Note: Hemagglutinating antigen preparation was unsuccessful for BUT, MAN, UV, JAT, IQT, FP, and MDD.

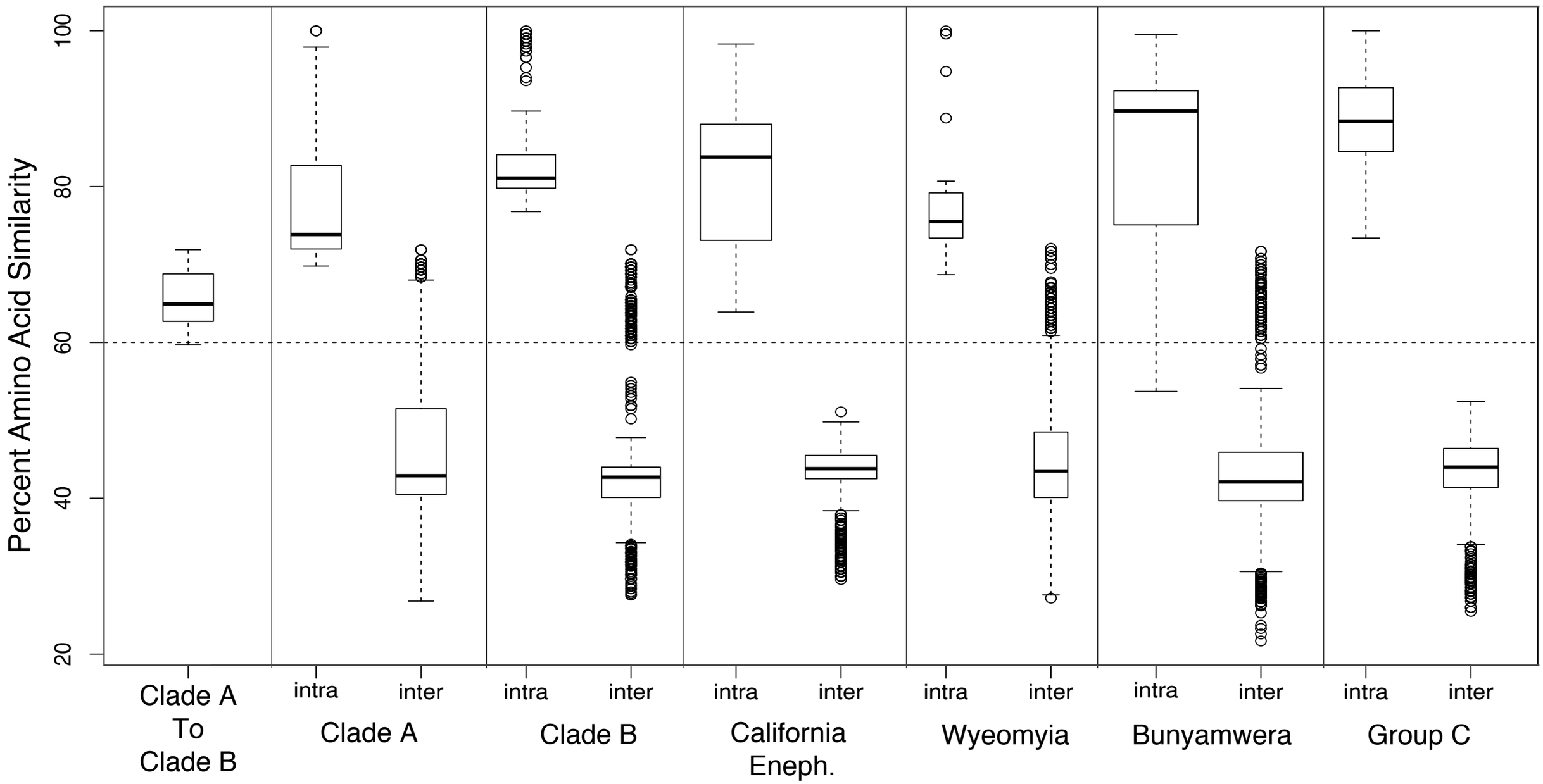
^ MDD stands for Madre de Dios, an unofficial name proposed here to refer to isolate FMD 1303.
* CQ stands for Cat Que, an unofficial name proposed here to refer to isolate VN 04-2108.
Other abbreviations: ORO-Oropouche, IQT-Iquitos, JAT-Jatobal, UTI-Utinga, UV-Utive, BUT-Buttonwillow, ING-Ingwavuma, MER-Mermet, MAN-Manzanilla, FP-Facey's Paddock.

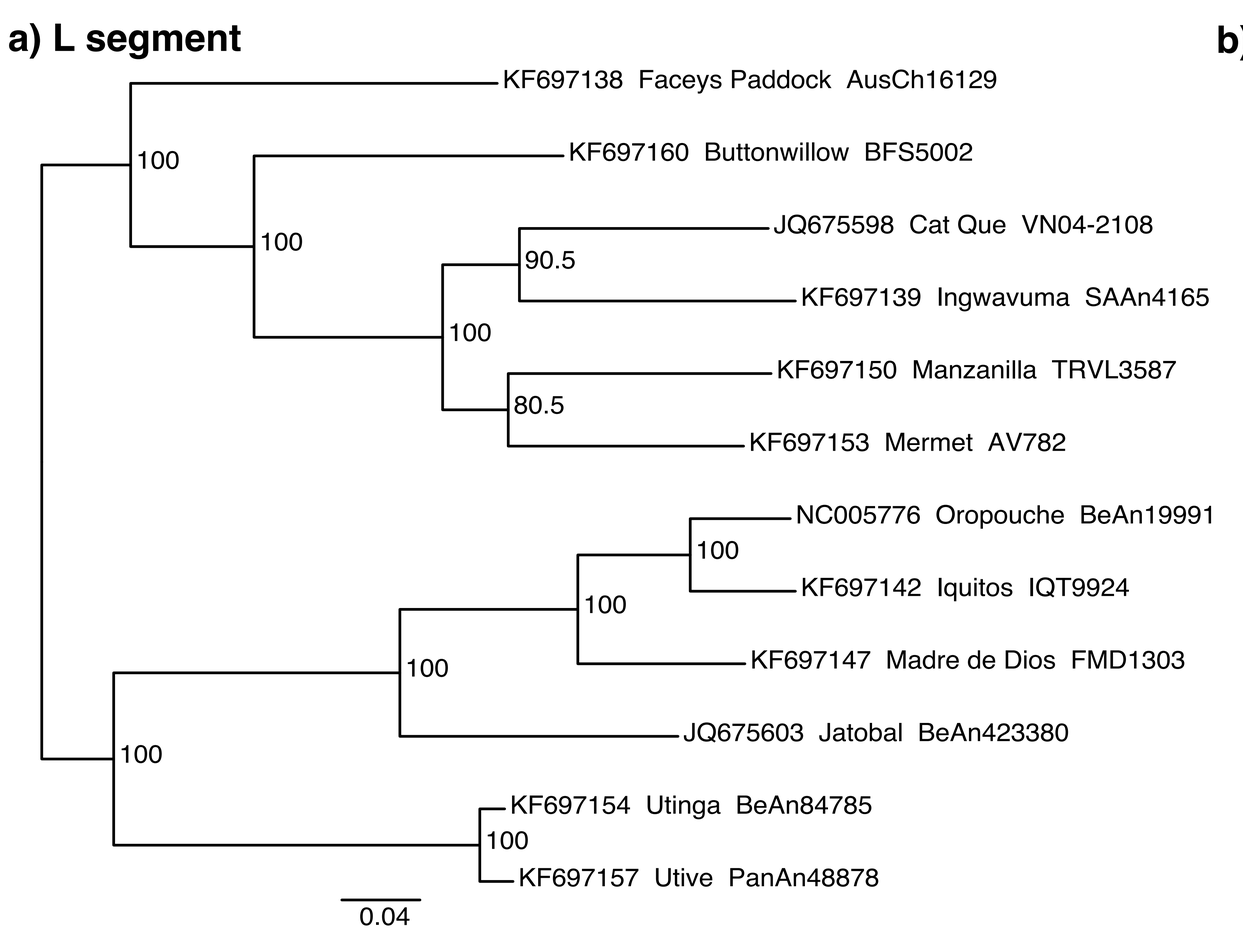
KF697142 Iquitos IQT9924
NC005776 Oropouche BeAn19991
KF697147 Madre de Dios FMD1303
JQ675603 Jatobal BeAn423380
KF697154 Utinga BeAn84785
KF697157 Utive PanAn48878
KF697138 Faceys Paddock AusCh16129
KF697160 Buttonwillow BFS5002
KF697150 Manzanilla TRVL3586
JQ675598 Cat Que VN04-2108
KF697139 Ingwavuma SAAn4165
KF697153 Mermet AV782
HM627178 Leanyer NT 16701
HE649912 Schmallenberg BH80/11-4
HE795105 Shamonda IbAn5550
HE795090 Douglas 93-6
HE795102 Sathuperi
HE795096 Sabo IbAn9398
AB297885 Akabane MP496L
NC009894 Akabane OBE-1
AB297887 Tinaroo CSIRO153
HE795108 Simbu SAAr53
HE795087 Aino 38K
HE795093 Peaton CSIRO110
HE795099 Sango An5077
NC 022039 Brazoran original
JN157805 Zungarococha IQE7620
JN572080 Wyeomyia Original
JN572074 Taiassui BeAr671
JN572077 Tucunduba BeAr278
JN801038 Wyeomyia Darien
JN801035 Wyeomyia TRVL8349
JN572068 Macaua BeAr306329
JN572062 Anhembi SPAr2984
JN572065 Iaco BeAn314206
JN572071 Sororoca BeAr32149
JN968590 Cachoeira Porteira BeAr328208
AB257769 Ilesha KO-2
AY593726 Mboke DakArY357/6e
AY593728 Ngari DakArD28542/4e
NC001925 Bunyamwera
AB257766 Batai MM2222
EU004194 Potosi 89-3380
EU004192 Main Drain BFS5015L
EU026160 Maguari BeAr7272L
EU004193 Northway 0234L
JN801039 Guaroa BeH22063
EF687077 Jamestown Canyon 61V2235L
EU789573 Inkoo KN3641L
NC004108 La Crosse Human/78
EU203678 Snowshoe Hare Original
EF687079 Melao TRVL9375L
AF393326 California Encephalitis LEIV-TAH
AF393328 California Encephalitis LEIV-11483L
EU621833 Chantanga 12718L
0.04
Oropouche
Utinga
Facey's Paddock
Buttonwillow
Manzanilla
Clade A
Clade B
Simbu
Wyeomyia
Bunyamwera
California Encephalitis

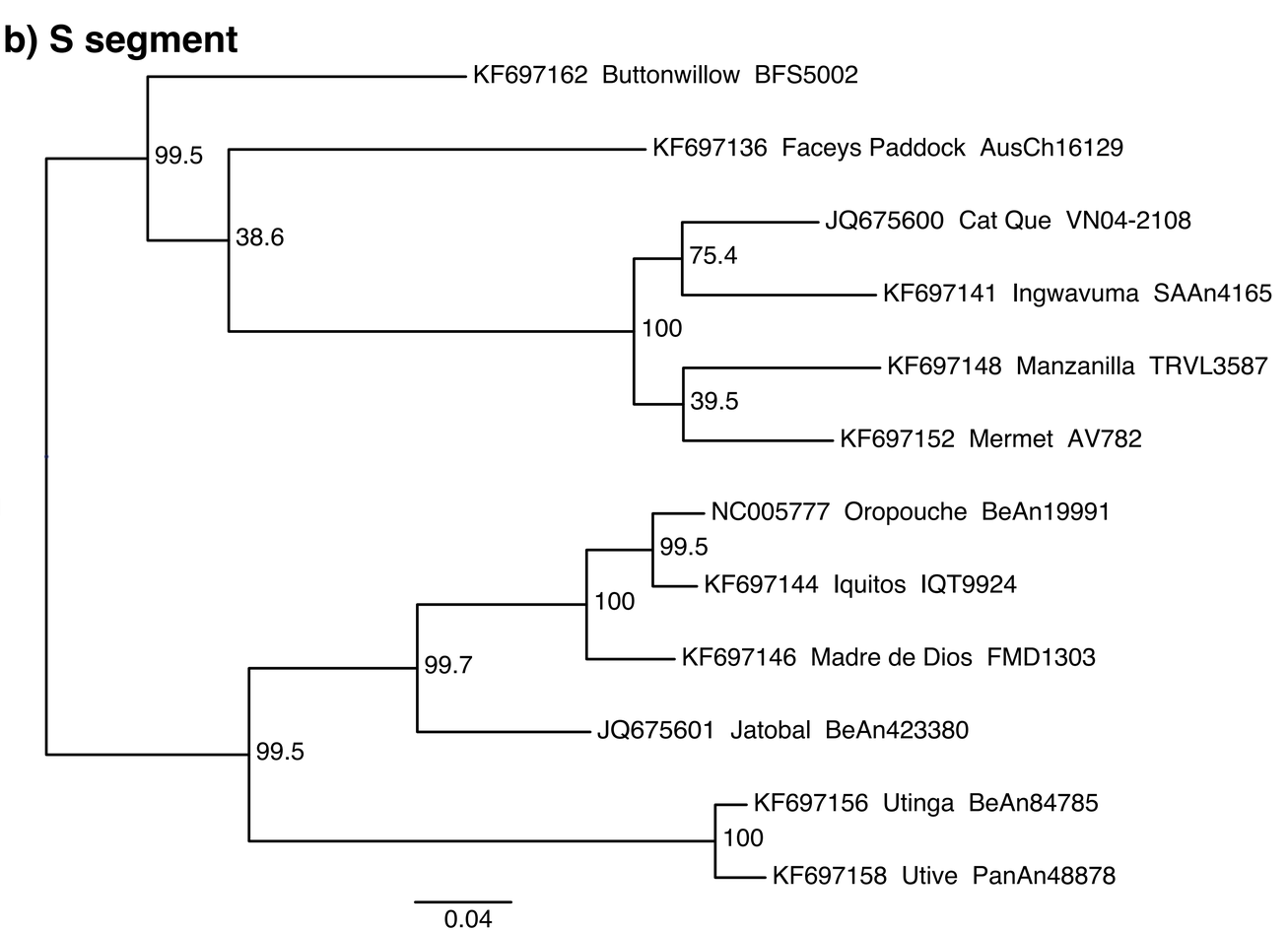
a) L segment

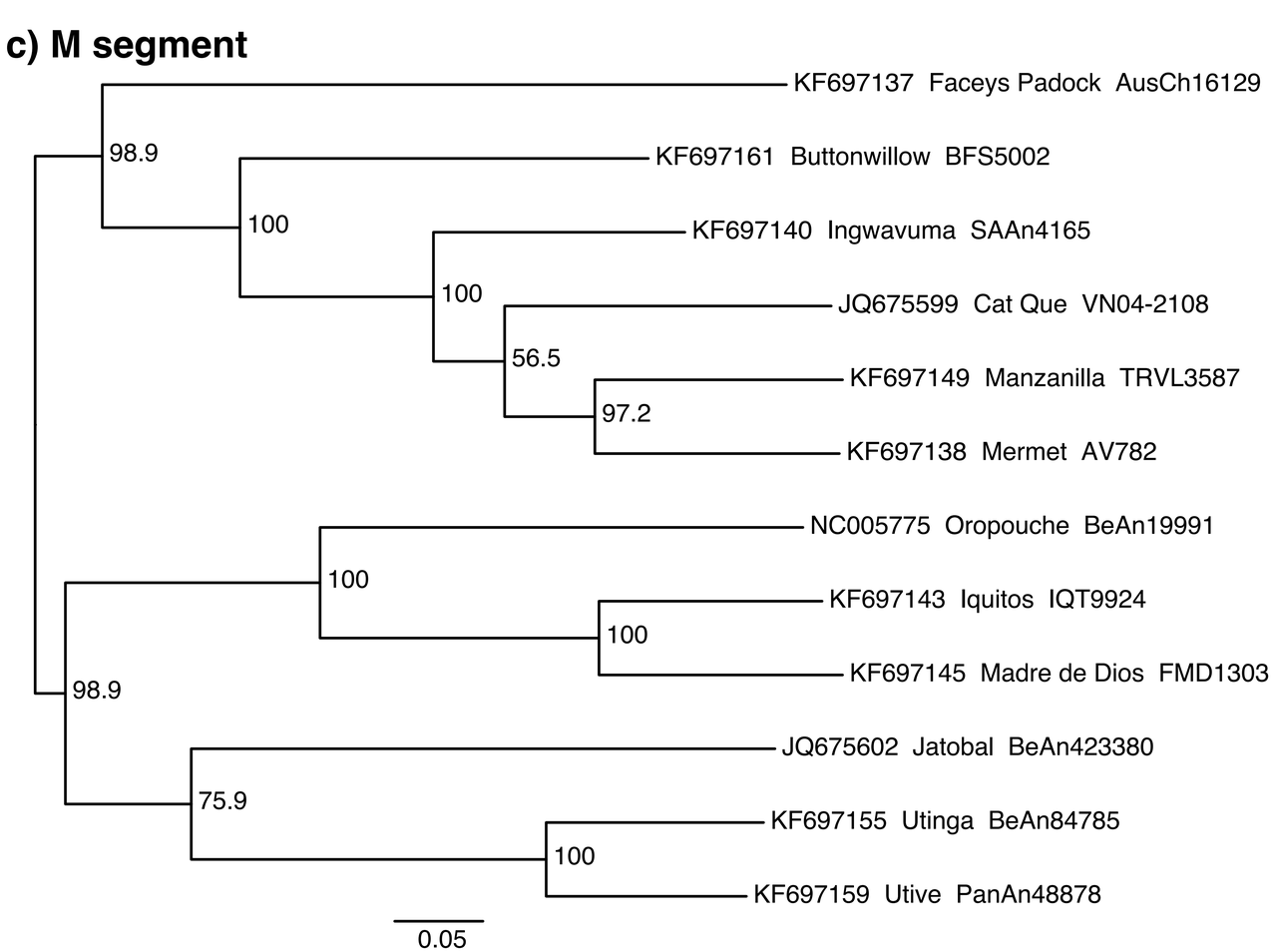


b) S segment




a) L segment
KF697138 Faceys Paddock AusCh16129
KF697160 Buttonwillow BFS5002
JQ675598 Cat Que VN04-2108
KF697139 Ingwavuma SAAn4165
KF697150 Manzanilla TRVL3587
KF697153 Mermet AV782
NC005776 Oropouche BeAn19991
KF697142 Iquitos IQT9924
KF697147 Madre de Dios FMD1303
JQ675603 Jatobal BeAn423380
KF697154 Utinga BeAn84785
KF697157 Utive PanAn48878
100
100
90.5
100
80.5
100
100
100
100
100
0.04


b) S segment
KF697162 Buttonwillow BFS5002
KF697136 Faceys Paddock AusCh16129
JQ675600 Cat Que VN04-2108
KF697141 Ingwavuma SAAn4165
KF697148 Manzanilla TRVL3587
KF697152 Mermet AV782
NC005777 Oropouche BeAn19991
KF697144 Iquitos IQT9924
KF697146 Madre de Dios FMD1303
JQ675601 Jatobal BeAn423380
KF697156 Utinga BeAn84785
KF697158 Utive PanAn48878
99.5
38.6
75.4
100
39.5
99.5
100
99.7
99.5
100
0.04


c) M segment
KF697137 Faceys Padock AusCh16129
KF697161 Buttonwillow BFS5002
KF697140 Ingwavuma SAAn4165
JQ675599 Cat Que VN04-2108
KF697149 Manzanilla TRVL3587
KF697138 Mermet AV782
NC005775 Oropouche BeAn19991
KF697143 Iquitos IQT9924
KF697145 Madre de Dios FMD1303
JQ675602 Jatobal BeAn423380
KF697155 Utinga BeAn84785
KF697159 Utive PanAn48878
98.9
100
100
56.5
97.2
100
100
98.9
75.9
100
0.05