Gairo virus, a novel arenavirus of the widespread *Mastomys natalensis*: Genetically divergent, but ecologically similar to Lassa and Morogoro viruses

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ABSTRACT

Despite its near pan-African range, the Natal multimammate mouse, *Mastomys natalensis*, carries the human pathogen Lassa virus only in West Africa, while the seemingly non-pathogenic arenaviruses Mopeia, Morogoro, and Luna have been detected in this semi-commensal rodent in Mozambique/Zimbabwe, Tanzania and Zambia, respectively. Here, we describe a novel arenavirus in *M. natalensis* from Gairo district of central Tanzania, for which we propose the name "Gairo virus". Surprisingly, the virus is not closely related with Morogoro virus that infects *M. natalensis* only 90 km south of Gairo, but clusters phylogenetically with Mobala-like viruses that infect non-*M. natalensis* host species in Central African Republic and Ethiopia. Despite the evolutionary distance, Gairo virus shares basic ecological features with the other *M. natalensis*-borne viruses Lassa and Morogoro. Our data show that *M. natalensis*, carrying distantly related viruses even in the same geographical area, is a potent reservoir host for a variety of arenaviruses.

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Introduction

Arenaviruses are single-stranded, negative-sensed RNA viruses with mostly rodents as natural hosts. Their bi-segmented genome consists of four open-reading frames (ORF) in ambisense orientation. The small (S) segment encodes for precursor glycoprotein (GPC) and nucleoprotein (NP), the large (L) segment contains genes for matrix protein Z and the L protein (L), respectively (Fig. 1). Most arenaviruses appear restricted to a single rodent species as natural host, but several host-switching events have likely occurred during their evolutionary history (Jackson and Charleston, 2004; Coulibaly-N'Golo et al., 2011; Bowen et al., 1997). The majority are not pathogenic to humans, but seven arenavirus species are known to cause hemorrhagic fevers in humans, of which two in Africa (Lassa and Lujo viruses) and five in South America (Junin, Machupo, Guanarito, Sabia and Chapare viruses). Lymphocytic choriomeningitis virus, carried by house mice worldwide, causes lymphocytic choriomeningitis in humans.

With thousands of human cases annually (WHO, 2005), Lassa virus has a tremendous public health impact. Peculiarly, while its reservoir host, the Natal multimammate mouse *Mastomys natalensis*, is distributed throughout most of Sub-Saharan Africa, human Lassa fever cases have only been reported in West Africa (Fig. 2). In other African regions the sporadic sampling of *M. natalensis* has led to the detection of related, but distinct arenaviruses: Mopeia virus (MOPV) in Mozambique (Wulff et al., 1977) and Zimbabwe (Johnson et al., 1981), Morogoro virus (MORV—a strain of MOPV) in Tanzania (Günther et al., 2009) and Luna virus (LUNV) in west Zambia (Ishii et al., 2011, 2012) (Fig. 2). Living commensally or as an agricultural pest prone to population irruptions (Leirs et al., 1996), *M. natalensis* is in frequent contact with humans throughout sub-Saharan Africa, but no human infections with *M. natalensis*-borne arenaviruses have been reported from southern or eastern Africa. Also none of the other arenaviruses detected in different African rodents have been linked to human diseases.

To extend our knowledge on the geographical distribution, diversity, and ecology of African arenaviruses, we screened the
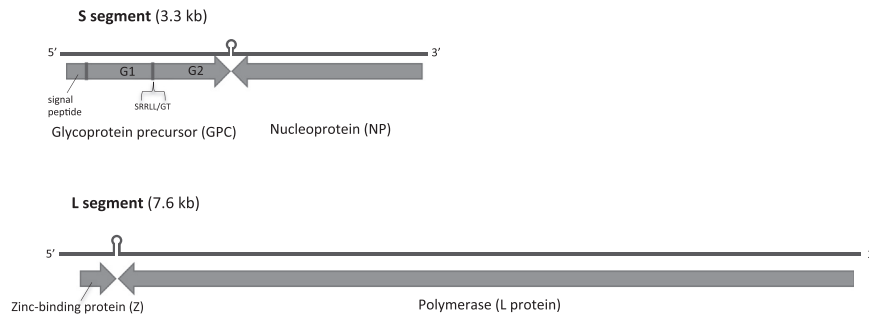

Fig. 1. Schematic genome organization of Gairo virus, according to the general arenavirus template.

small mammal community in Central Tanzania for arenaviruses. We detected and characterize a novel arenavirus, Gairo virus (GAIV), in *M. natalensis*. The virus was found in close proximity to the distribution area of Morogoro virus and its ecology appears similar to that of other *M. natalensis*-borne arenaviruses, yet it is genetically divergent.

Results

We captured a total of 371 small mammals, 332 of which were *M. natalensis*. One animal died inside the trap and was not further processed; yielding a total dataset of 370 small mammals of which 331 were *M. natalensis* (Table 1). Morphological identification of animals as *M. natalensis* was confirmed by cytochrome *b* sequences. Of the 247 *M. natalensis* individuals for which kidney samples were available, 18 (7.3%) were arenavirus RT-PCR positive in both dried blood spots (DBS) and kidney samples while a further 6 individuals (2.4%) had only positive kidney samples (Table 1). Of the remaining 84 *M. natalensis*, 5 (6.0%) had RT-PCR positive DBS samples. Thirty out of 331 individuals (9.1%) carried anti-arenavirus antibodies, of which two were also RT-PCR positive in both blood and kidneys, and of which one was RT-PCR positive in kidneys alone (Table 1). Sequencing of the RT-PCR products of partial L and partial NP gene and the resulting phylogenetic analyses (Fig. 3A, Supplementary Fig. 1A) revealed that all 29 PCR-positive samples contained a novel arenavirus, for which we propose the name Gairo virus (GAIV). GAIV was not detected in any of the other sampled rodent species (Table 1). One *Arvicanthis neumanni* individual from Chakwale was arenavirus RT-PCR positive, but a GenBank BLAST search of the 340 nt partial L gene sequence indicated the presence of an arenavirus related to Ippy virus, which was previously isolated from *Arvicanthis sp.* in Central African Republic (Digoutte, 1970; Swanepoel et al., 1985).

Virological characterization

GAIV was isolated from four *M. natalensis* samples in Vero E6 cell culture. The culture supernatants reached infectious particle titers of 3.5×10^4 FFU/mL (TZ-27317), 2.1×10^4 FFU/mL (TZ-27370), 5.1×10^4 FFU/mL (TZ-27421) and 2.5×10^3 FFU/mL (TZ-27457) 13 days post inoculation. Virus particles in the supernatant of the second passage of TZ-27421 (2.1×10^5 FFU/mL) were used for 454 sequencing. Of the 110,822 reads generated by 454 GS-FLX, 1.36% mapped to the preliminary GAIV consensus genome generated in Geneious 6.1.7. The average coverage was 88.5 (SD=33.0) for the GPC, 54.6 (SD=15.5) for the NP, 44.3 (SD=8.6) for the L and 23.7 (SD=3.4) for the Z gene.

Assembly allowed reconstruction of the entire Gairo virus S and L segments, apart from 17 non-coding nucleotides at the 3′ end of the S segment, 8 non-coding nucleotides at the 5′ end of the L

segment and 17 non-coding nucleotides at the 3′ end of the L-segment.

In the S segment region encoding G1, 5 nucleotide sites were polymorphic among the reads (neither base occurring at frequency > 75%). While two polymorphisms (C67T and A258C) were synonymous, three others were non-synonymous of which two occurred at residues that are otherwise strongly conserved in Old-World arenaviruses. It is therefore possible that these represent mutations that occurred by growth in green monkey-derived Vero E6 cells: the A265C mutation caused an aspartate (conserved in all OWA except Lujo) to histidine change and the A914G mutation caused a glutamic acid (conserved in all OWA except Merino Walk) to glycine change. The C1043T polymorphism was associated with a threonine-isoleucine polymorphism (not a conserved residue in OWA). The one polymorphism observed on the L segment (at nucleotide position 1855 in the L gene) was synonymous.

Each of the two segments of the GAIV genome shows the typical two open reading frames, separated by the typical stem-loop structures. In the L ORF of GAIV the canonical polymerase domains (pre-A, A, B, C, D and E) (Poch et al., 1989; Vieth et al., 2004) and the endonuclease motif (P_{88}, D_{89}, E_{102}, K_{115}) (Vieth et al., 2004) are well conserved. The Z ORF contained two late domains, PSAP and PPPY, like most arenaviruses (Supplementary Table 2). GPC of LCMV is post-translationally cleaved into a signal peptide and the mature G1 and G2 at the consensus sequence R(R/K/H)L(A/L/S/T/F)(Beyer et al., 2003). This motif is found in all Old-World and some New-World arenaviruses (Rojek et al., 2008), with several sequence variations, and thus now also in GAIV, with RRLL as sequence (Supplementary Table 2). GAIV G1 contains 5 potential N-glycosylation sites in positions analogous to other OWA.

Phylogenetic characterization

Phylogenetic trees from the complete sequences of all 4 arenavirus genes placed GAIV in a monophyletic group with Mobala virus (MOBV, Fig. 3B–D, Supplementary Fig. 1B and C). Nucleotide sequence identities between GAIV and MOBV were 71.6% (GPC), 74.2% (NP), 67.7% (L) and 63.1% (Z). Identity at amino acid (AA) sequence level was 81.3% (GPC), 84.6% (NP), 71.0% (L) and 59.4% (Z) (Supplementary Table 2). The tree based on 340 nucleotides of the L gene, which includes other African arenaviruses for which only this sequence is available, further grouped GAIV and MOBV with Mobala-like viruses from *Mastomys awashensis* (average 79.1/92.1% nucleotide/AA sequence identity) and *Stenocephalemys albipes* (average 78.2/92.0% nucleotide/AA sequence identity) from Ethiopia in a monophyletic clade of Mobala-like viruses (Fig. 3A). The sequence identity between GAIV and Morogoro virus, which infects *M. natalensis* only 90 km further south of our sampling localities, was considerably lower, with 70.4/77.7% (GPC), 70.9/78.3% (NP), 61.2/60.7% (L) and 58.3/55.3% (Z) (Supplementary Table 2).
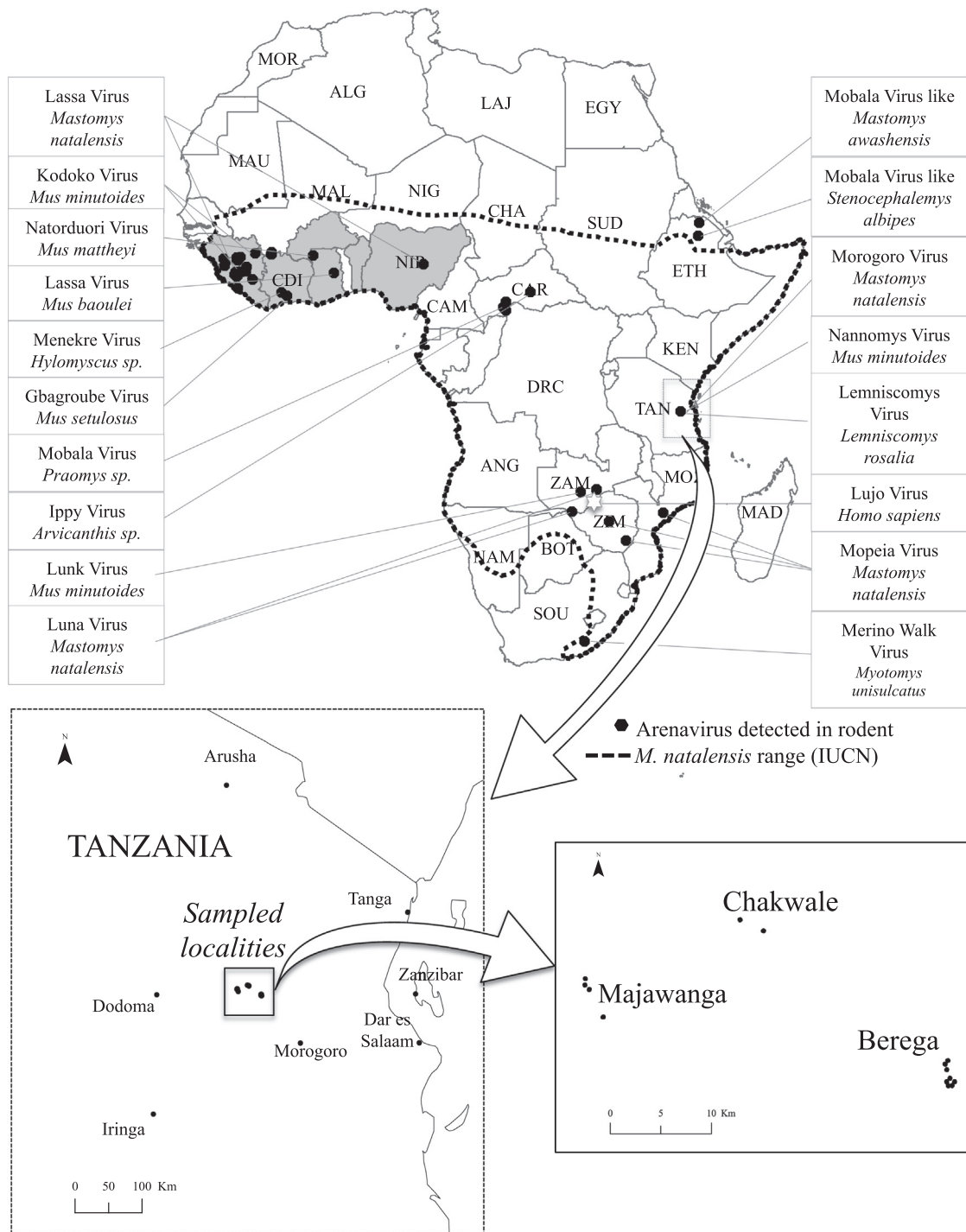
Fig. 2. Up: Occurrences of African arenaviruses detected in rodents (dots); countries with Lass-fever cases are shaded grey (Coulibaly-N'Golo et al., 2011; Johnson et al., 1981; Günther et al., 2009; Ishii et al., 2011, 2012; Gonzalez et al., 1983; Goüy de Bellocq et al., 2010; Swanepoel et al., 1985; Meheretu et al., 2012; Fichet-Calvet and Rogers, 2009; Lecompte et al., 2007; Safronetz et al., 2013). Below, left: north-east quadrant of Tanzania. Below, right: sampling sites (small dots) of this study.

While the identity percentages of the entire gene/protein sequences between GAIV and MOBV were substantially higher than between GAIV to other OWAs, for the G1 part of GPC, which is involved in host receptor recognition (Cao et al., 1998), the 64.3/68.2% nucleotide/AA identity were similar to comparisons between GAIV and other *M. natalensis*-borne arenaviruses and Gbagroube (Supplementary Table 2). Also in the phylogenetic tree of only the G1 part of the GPC gene (Fig. 3C), GAIV failed to form a monophyletic group with MOBV, and instead the position of neither GAIV nor

Mobala virus can be resolved. The phylogenetic tree of the G2 part of the GPC was on the other hand well resolved (Fig. 3D).

Ecological characterization

The probability of GAIV infection significantly decreases with the age (as measured by eye lens weight) of the animals ($\chi^2 = 4.95$, $p = 0.026$), but none of the other host characteristics (sex, weight, sexual maturity, BMI) was significantly associated with GAIV RT-

Table 1
Number of small mammal captures at each locality and grid therein. Between brackets: % Gairo virus positive (detected by RT-PCR assay)—% anti-arenavirus antibody positive (detected by IFA). In each grid 100 traps were set for one night.

	Number of captures and blood samples screened (% RT-PCR positive—% IFA positive)								Number of kidney samples screened (% RT-PCR positive)
	Acomys sp.	Aethomys sp.	Arvicanthis neumannii	Crocidura sp.	Lemniscomys zebra	Nannomys sp.	Gerbilliscus sp.	Mastomys natalensis	Mastomys natalensis
Berega total	**6 (0–0)**	**8 (0–0)**	**7 (0–0)**	**2 (0–0)**	**0 (0–0)**	**0 (0–0)**	**3 (0–0)**	**141 (2.84–3.55)**	**141 (3.55)**
2012_01	5 (0––0)	5 (0–0)	0 (0–0)	0 (0–0)	0 (0–0)	0 (0–0)	1 (0–0)	3 (0–0)	3 (0)
2012_02	0 (0–0)	0 (0–0)	0 (0–0)	1 (0–0)	0 (0–0)	0 (0–0)	0 (0–0)	10 (0–0)	10 (0)
2012_03	0 (0–0)	0 (0–0)	0 (0–0)	0 (0–0)	0 (0–0)	0 (0–0)	0 (0–0)	28 (3.57–3.57)	28 (3.57)
2012_04	0 (0–0)	0 (0–0)	0 (0–0)	0 (0–0)	0 (0–0)	0 (0–0)	0 (0–0)	24 (0–0)	24 (0)
2012_05	1 (0–0)	3 (0–0)	1 (0–0)	0 (0–0)	0 (0–0)	0 (0–0)	0 (0–0)	4 (0–25)	4 (0)
2012_06	0 (0––0)	0 (0–0)	0 (0–0)	0 (0–0)	0 (0-0)	0 (0–0)	2 (0–0)	18 (11.11–0)	18 (16.67)
2012_13	0 (0–0)	0 (0–0)	5 (0–0)	1 (0–0)	0 (0–0)	0 (0–0)	0 (0–0)	39 (0–5.13)	39 (0)
2012_14	0 (0–0)	0 (0–0)	1 (0–0)	0 (0–0)	0 (0–0)	0 (0–0)	0 (0–0)	15 (6.67–6.67)	15 (6.67)
Chakwale total	**0 (0–0)**	**4 (0–0)**	**2 (0–0)**	**1 (0–0)**	**1 (0–0)**	**0 (0–0)**	**0 (0–0)**	**84 (1.19-1.19)**	**28 (3.57)**
2012_07	0 (0–0)	4 (0–0)	1 (0–0)	1 (0–0)	0 (0–0)	0 (0–0)	0 (0–0)	42 (2.38–0)	15 (6.67)
2012_08	0 (0–0)	0 (0–0)	1 (0–0)	0 (0–0)	1 (0–0)	0 (0–0)	0 (0–0)	42 (0–2.38)	13 (0)
Majawanga total	**0 (0–0)**	**0 (0–0)**	**0 (0–0)**	**1 (0–0)**	**1 (0–0)**	**3 (0–0)**	**0 (0–0)**	**106 (16.98–22.64)**	**78 (23.08)**
2012_09	0 (0–0)	0 (0–0)	0 (0–0)	0 (0–0)	1 (0–0)	1 (0–0)	0 (0–0)	15 (0–20)	15 (6.67)
2012_10	0 (0–0)	0 (0–0)	0 (0–0)	0 (0–0)	1 (0–0)	0 (0–0)	0 (0–0)	23 (17.39–43.48)	23 (30.43)
2012_11	0 (0–0)	0 (0–0)	0 (0–0)	0 (0–0)	0 (0–0)	0 (0–0)	0 (0–0)	46 (26.09–19.57)	18 (38.89)
2012_12	0 (0–0)	0 (0–0)	0 (0–0)	1 (0–0)	1 (0–0)	0 (0–0)	0 (0–0)	22 (9.09–9.09)	22 (13.64)
Total	**6 (0-0)**	**12 (0–0)**	**9 (0–0)**	**4 (0–0)**	**2 (0–0)**	**3 (0–0)**	**3 (0–0)**	**331 (6.95–9.06)**	**247 (9.72)**

PCR positivity. The probability of antibody presence significantly increases as animals get older ($\chi^2 = 19.22$, $p = 0.002$) and heavier (Weight: $\chi^2 = 21.53$, $p < 0.001$; BMI: $\chi^2 = 12.93$, $p < 0.001$). Sexually mature animals were 1.98 times as likely to carry antibodies than sub-adults/juveniles ($\chi^2 = 15.92$, $p < 0.001$). Antibody (13/109 male vs. 12/121 female) or RNA (10/109 male vs. 14/121 female) prevalence did not differ significantly between sexes ($\chi^2 = 0.21$, $p = 0.65$). An overview of prevalences in categories of the different host characteristics is given in Table 2. Summaries of the statistics can be found in the Supplementary Table 3.
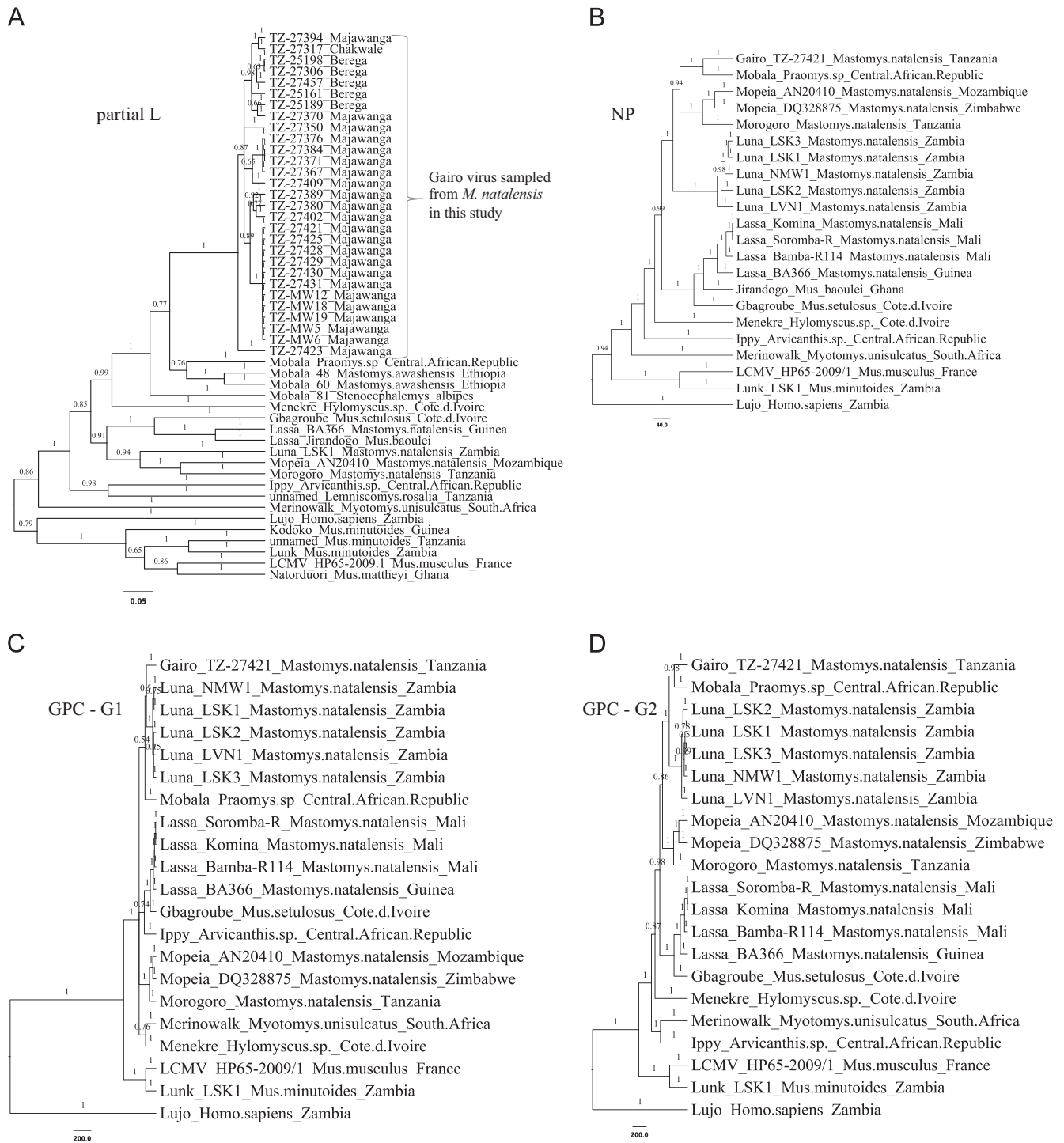
Discussion

Besides the well-known pathogen Lassa virus, the African rodent *M. natalensis* carries several other arenaviruses of which no infections in humans have ever been reported. Phylogenies of each of the 4 entire gene sequences revealed that the novel *M. natalensis*-borne Gairo virus (GAIV) forms a monophyletic group with Mobala virus (MOBV), which was previously isolated from *Praomys sp.* in Central African Republic (CAR) (Fig. 3) (Gonzalez et al., 1983). Other relatives of MOBV have previously been genetically detected in *M. awashensis* and *S. albipes* in the Ethiopian highlands (Meheretu et al., 2012) (Figs. 2 and 3).

Delineating species in the genus *Arenavirus* follows multiple criteria that are based on antigenic cross-reactivity, geographic distribution, host species, pathogenicity to humans, and divergence of amino-acid sequences (Emonet et al., 2006). While there is no published data on the occurrence of GAIV and MOBV and its relatives outside of the study areas in CAR, Ethiopia and Tanzania, they were each detected thousands kilometers apart and each in different host species. The nucleoprotein AA sequence of GAIV differs 15.4% from that of MOBV, while for Old-World arenaviruses interspecies cut-off divergences of 10.2% or 12% are generally recognized (Emonet et al., 2006; Bowen et al., 2000). We therefore propose that GAIV be recognized as a distinct arenavirus species within the MOBV clade.

The evolutionary histories of the arenaviruses within this MOBV-clade thus appear to include several host-switches. Adaptation to novel hosts is likely at least partially mediated through substitutions in the G1 part of GPC, which is known to be involved in host cell receptor recognition. Indeed, the G1 AA sequence of GAIV and MOBV were only 68% identical, and in the G1 phylogenetic tree the relationship between GAIV and all other non-LCMV OWAs remained unresolved (Fig. 3C). The discrepancy between trees of G1 and other genes was also apparent for some other OWAs, but the tight monophyletic cluster of Morogoro and Mopeia virus (that have a phylogenetic distance similar to that between GAIV and MOBV virus but both infect *M. natalensis*) is just as well resolved in the G1 tree as in the other gene trees. While G1 phylogenies thus do not accurately reflect the evolutionary histories of the arenaviruses, they may contain information on current host species. Rapid adaptation of arenaviruses to novel hosts through substitutions in G1 region might also explain the 5 polymorphic positions observed in this region in the GAIV strain cultured in Vero E6 cells (originally derived from African green monkeys), of which 3 were non-synonymous.

Despite the high genetic divergence between *M. natalensis* arenaviruses, infection by these viruses seems to result in similar ecological patterns in their respective *M. natalensis* populations. The general prevalences of GAIV RNA and anti-arenavirus antibodies in this study were similar than reported previously for other *M. natalensis*-borne arenaviruses (Lassa, Morogoro and Luna) in different regions (Ishii et al., 2011, 2012; Fichet-Calvet et al., 2007; Borremans et al., 2011). The small proportion of animals that contain both GAIV RNA (either in blood, kidneys, or both) and antibodies suggests that most animals clear the active infection soon after onset of antibody production. This pattern is also apparent for the other studied *M. natalensis* arenaviruses (Lassa in Guinea (Fichet-Calvet et al., 2007; Demby et al., 2001) and Morogoro virus in Tanzania (Borremans et al., 2011)), as well as MOBV in *Praomys sp.* Gonzalez et al., (1983). In contrast, ecological studies on the New-World arenaviruses Junin (Mills et al., 1994), White-Water Arroyo (Fulhorst et al., 2002), Bear-Canyon (Fulhorst et al., 2002) and Catarina virus (Milazzo et al., 2013) show that most rodents with an active arenavirus infection simultaneously carry the antibodies against it (and vice versa), suggesting that these arenaviruses get cleared later from rodent host bodies after the antibody response than the studied *M. natalensis* and *Praomys*-borne viruses.

A

partial L

TZ-27394_Majawanga
TZ-27317_Chakwale
TZ-25198_Berega
TZ-27306_Berega
TZ-27457_Berega
TZ-25161_Berega
TZ-25189_Berega
TZ-27370_Majawanga
TZ-27350_Majawanga
TZ-27376_Majawanga
TZ-27384_Majawanga
TZ-27371_Majawanga
TZ-27367_Majawanga
TZ-27409_Majawanga
TZ-27389_Majawanga
TZ-27380_Majawanga
TZ-27402_Majawanga
TZ-27421_Majawanga
TZ-27425_Majawanga
TZ-27428_Majawanga
TZ-27429_Majawanga
TZ-27430_Majawanga
TZ-27431_Majawanga
TZ-MW12_Majawanga
TZ-MW18_Majawanga
TZ-MW19_Majawanga
TZ-MW5_Majawanga
TZ-MW6_Majawanga
TZ-27423_Majawanga

Gairo virus sampled
from *M. natalensis*
in this study

Mobala_Praomys.sp_Central.African.Republic
Mobala_48_Mastomys.awashensis_Ethiopia
Mobala_60_Mastomys.awashensis_Ethiopia
Mobala_81_Stenocephalemys_albipes
Menekre_Hylomyscus.sp._Cote.d.Ivoire
Gbagroube_Mus.setulosus_Cote.d.Ivoire
Lassa_BA366_Mastomys.natalensis_Guinea
Lassa_Jirandogo_Mus.baoulei
Luna_LSK1_Mastomys.natalensis_Zambia
Mopeia_AN20410_Mastomys.natalensis_Mozambique
Morogoro_Mastomys.natalensis_Tanzania
Ippy_Arvicanthis.sp._Central.African.Republic
unnamed_Lemniscomys.rosalia_Tanzania
Merinowalk_Myotomys.unisulcatus_South.Africa
Lujo_Homo.sapiens_Zambia
Kodoko_Mus.minutoides_Guinea
unnamed_Mus.minutoides_Tanzania
Lunk_Mus.minutoides_Zambia
LCMV_HP65-2009.1_Mus.musculus_France
Natorduori_Mus.mattheyi_Ghana

0.05

B

NP

Gairo_TZ-27421_Mastomys.natalensis_Tanzania
Mobala_Praomys.sp_Central.African.Republic
Mopeia_AN20410_Mastomys.natalensis_Mozambique
Mopeia_DQ328875_Mastomys.natalensis_Zimbabwe
Morogoro_Mastomys.natalensis_Tanzania
Luna_LSK3_Mastomys.natalensis_Zambia
Luna_LSK1_Mastomys.natalensis_Zambia
Luna_NMW1_Mastomys.natalensis_Zambia
Luna_LSK2_Mastomys.natalensis_Zambia
Luna_LVN1_Mastomys.natalensis_Zambia
Lassa_Komina_Mastomys.natalensis_Mali
Lassa_Soromba-R_Mastomys.natalensis_Mali
Lassa_Bamba-R114_Mastomys.natalensis_Mali
Lassa_BA366_Mastomys.natalensis_Guinea
Jirandogo_Mus.baoulei_Ghana
Gbagroube_Mus.setulosus_Cote.d.Ivoire
Menekre_Hylomyscus.sp._Cote.d.Ivoire
Ippy_Arvicanthis.sp._Central.African.Republic
Merinowalk_Myotomys.unisulcatus_South.Africa
LCMV_HP65-2009/1_Mus.musculus_France
Lunk_LSK1_Mus.minutoides_Zambia
Lujo_Homo.sapiens_Zambia

40.0

C

GPC - G1

Gairo_TZ-27421_Mastomys.natalensis_Tanzania
Luna_NMW1_Mastomys.natalensis_Zambia
Luna_LSK1_Mastomys.natalensis_Zambia
Luna_LSK2_Mastomys.natalensis_Zambia
Luna_LVN1_Mastomys.natalensis_Zambia
Luna_LSK3_Mastomys.natalensis_Zambia
Mobala_Praomys.sp_Central.African.Republic
Lassa_Soromba-R_Mastomys.natalensis_Mali
Lassa_Komina_Mastomys.natalensis_Mali
Lassa_Bamba-R114_Mastomys.natalensis_Mali
Lassa_BA366_Mastomys.natalensis_Guinea
Gbagroube_Mus.setulosus_Cote.d.Ivoire
Ippy_Arvicanthis.sp._Central.African.Republic
Mopeia_AN20410_Mastomys.natalensis_Mozambique
Mopeia_DQ328875_Mastomys.natalensis_Zimbabwe
Morogoro_Mastomys.natalensis_Tanzania
Merinowalk_Myotomys.unisulcatus_South.Africa
Menekre_Hylomyscus.sp._Cote.d.Ivoire
LCMV_HP65-2009/1_Mus.musculus_France
Lunk_LSK1_Mus.minutoides_Zambia
Lujo_Homo.sapiens_Zambia

200.0

D

GPC - G2

Gairo_TZ-27421_Mastomys.natalensis_Tanzania
Mobala_Praomys.sp_Central.African.Republic
Luna_LSK2_Mastomys.natalensis_Zambia
Luna_LSK1_Mastomys.natalensis_Zambia
Luna_LSK3_Mastomys.natalensis_Zambia
Luna_NMW1_Mastomys.natalensis_Zambia
Luna_LVN1_Mastomys.natalensis_Zambia
Mopeia_AN20410_Mastomys.natalensis_Mozambique
Mopeia_DQ328875_Mastomys.natalensis_Zimbabwe
Morogoro_Mastomys.natalensis_Tanzania
Lassa_Soromba-R_Mastomys.natalensis_Mali
Lassa_Komina_Mastomys.natalensis_Mali
Lassa_Bamba-R114_Mastomys.natalensis_Mali
Lassa_BA366_Mastomys.natalensis_Guinea
Gbagroube_Mus.setulosus_Cote.d.Ivoire
Menekre_Hylomyscus.sp._Cote.d.Ivoire
Merinowalk_Myotomys.unisulcatus_South.Africa
Ippy_Arvicanthis.sp._Central.African.Republic
LCMV_HP65-2009/1_Mus.musculus_France
Lunk_LSK1_Mus.minutoides_Zambia
Lujo_Homo.sapiens_Zambia

200.0

Fig. 3. Phylogenetic trees from nucleotide sequences of 340 nt of the L gene (A), entire NP gene (B), G1 part of GPC gene (C) and G2 part of GPC gene (D). Numbers on the branches represent Bayesian posterior probabilities that the taxa form monophyletic groups and the scale bar represents the number of substitutions per site. In the tree of the partial L gene (A), all GAIV positive samples from this study are included, as well as one representative of each published arenavirus taxon (if the L sequence was available). For the other trees (B–D), all available entire gene sequences of published OWA taxa described from their natural hosts (apart from Lujo for which the natural host is unknown) are included. See Supplementary Table 1 for a list of the arenavirus sequences used and their GenBank Accession numbers.

Nevertheless, for nearly all ecologically studied arenaviruses (both New-World: Junin Mills et al., 1994, Oliveros Mills et al., 2007 and Old-World: Lassa Demby et al., 2001, Morogoro Borremans et al., 2011) the probability to carry anti-arenavirus antibodies increases with the age of the host individuals, and we found this also for GAIV (Table 2). In other words, the older an animal becomes, the higher chance it has to have acquired an arenavirus infection at some point in its life. While not excluding sexual or vertical transmission, this observation suggests that horizontal transmission is important for most arenaviruses. It also implies that relatively higher proportions of animals in younger age categories are still susceptible; and indeed we found a significant negative relationship between GAIV RT-PCR positivity and age. This was previously also observed in *M. natalensis* populations with Lassa (Fichet-Calvet et al., 2008) and Morogoro viruses (Borremans et al., 2011).

Table 2
Prevalence of anti-arenavirus antibodies, of GAIV RNA in blood samples (viremic animals) and of GAIV RNA in kidney samples, given as percentages. Between brackets: 95% confidence intervals of the binomial probability (using the profile method), calculated in the R binom package. *N*: sample size per category (note that not all host characteristics could be determined for each of the total of 331 captured *M. natalensis*).

Host characteristic	Category	Antibody prevalence in blood samples	N	GAIV RNA prevalence in blood samples (viremia)	N	GAIV RNA prevalence in kidney samples	N
Sex	Female	9.4 (5.6–14.4)	170	7.6 (4.3–12.3)	170	10.8 (6.2–16.9)	130
	Male	8.8 (5.1–13.9)	158	6.3 (3.2–10.8)	158	8.6 (4.4–14.6)	116
Reproductive maturity	Immature	4.7 (2.4–8.1)	211	7.6 (4.5–11.6)	211	10.8 (6.5–16.5)	148
	Mature	17.1 (11–24.6)	117	6.0 (2.6–11.2)	117	8.2 (3.8–14.7)	98
Body weight (g)	0–15	6.9 (1.2–19.8)	29	10.3 (2.7–24.7)	29	27.8 (11.0–50.5)	18
	15–30	4.7 (2.3–8.3)	191	6.3 (3.4–10.3)	191	8.5 (4.7–13.9)	141
	30–45	10.8 (5.4–18.7)	83	9.6 (4.5–17.2)	83	9.0 (3.7–17.3)	67
	45–60	33.3 (15.9–54.6)	21	0 (0–14.4)	21	5.6 (0.6–22.3)	18
	60–80	75 (27.7–98)	4	0 (0–51)	4	0 (0–71.1)	2
Eye lens weight (mg)	0–5	12.5 (1.2–44.5)	8	12.5 (1.2–44.5)	8	37.5 (11–71)	8
	5–7.5	6.7 (2.4–13.8)	75	8.0 (3.3–15.6)	75	10.7 (5–18.9)	75
	7.5–10	7.5 (3.5–13.6)	106	13.2 (7.7–20.5)	106	10.8 (5.8–17.8)	102
	10–12.5	18.2 (8.8–31.2)	44	4.5 (0.8–13.4)	44	4.7 (0.8–13.7)	43
	12.5–18	75 (27.7–98)	4	0 (0–51)	4	0 (0–51)	4

In conclusion, the discovery of GAIV in *M. natalensis* highlights the great arenavirus diversity a single rodent species can harbor. This is clearly not solely due to the wide, near pan-African distribution of the rodent, as we detected GAIV in *M. natalensis* populations just 90 km from the locality where previously Morogoro virus, an outlier strain of Mopeia virus from Mozambique, was described. *M. natalensis'* arenavirus diversity is also markedly distinct from that of the house mouse, *Mus musculus*, from which only one arenavirus species (Lymphocytic choriomeningitis virus—LCMV) has been described across its cosmopolitan range. A more detailed sampling across the transition zone between two divergent arenaviruses of *M. natalensis* should allow a better understanding of the ecological processes that keep the occurrence of its arenaviruses limited to distinct geographical regions.

Material and methods

Rodent trapping and sample collection

We captured rodents at three localities in central Tanzania: Berega (6.18S, 37.14E), Majawanga (6.11S, 36.82E) and Chakwale (6.05S, 36.6E) (Fig. 2) between 18/08/2012 and 28/08/2012. In each locality, respectively 8, 4, and 2 grids of live traps (Sherman Live Trap Co., Tallahassee, FL, USA) were set for one night in harvested maize fields. Each grid consisted of 100 traps, baited with peanut butter mixed with maize flour, and placed at 10 m intervals. At least 500 m separated each grid from others. All 370 live captured animals were weighed, their blood was collected from the retro-orbital sinus (for released animals) and from the heart (for sacrificed animals) and dried on filter paper. Their reproductive status (sexual maturity) was examined following Leirs et al. (1993), with males recorded as immature when their testes were abdominal and not externally visible, and as mature when their testes were externally visible (in either abdominal or scrotal position); sexual maturity of females was based on vaginal perforation. A random subset of animals was sacrificed (a total of 283, of which 247 *M. natalensis*), their body length measured, and kidney, spleen, heart and liver samples were taken after dissection and immediately frozen in liquid nitrogen, as well as preserved in RNAlater (kidney and spleen only) and ethanol (liver only). *M. natalensis* eyes were preserved in 10% formalin. Dissected lenses were cleaned, dried at 80 °C for > 36 h and weighed; their weight serving as a relative estimate of the age of the animals (Leirs et al., 1990). The remaining animals (a total of 87) were released after weighing, reproductive status examination, blood collection and

clipping of two toes (preserved in ethanol) under anesthesia. All animal work was approved by the University of Antwerp Ethical Committee for Animal Experimentation (2011-52), and followed regulations of the Research Policy of Sokoine University of Agriculture as stipulated in the "Code of Conduct for Research Ethics" (Revised version of 2012).

Genetic characterization of rodent species

For those animals morphologically identified as *M. natalensis* in the field, DNA was extracted from liver or toe samples with DNeasy DNA extraction kit (Qiagen), according to manufacturer's instructions. Species identification was then confirmed by sequencing a portion of the cytochrome *b* gene, after a PCR using primers L14723 and H15915 (Lecompte et al., 2002) in a 0.02 μM concentration, among 1 × Buffer (GoTaq®Flexi Buffer, Promega), 0.1 μM MgCl₂, 0.02 μM dNTP (Fermentas) and 1.35 units GoTaq® DNA Polymerase (Promega). Sequencing was done using the L14723 primers at the Genetic Sequencing Facility of the Vlaams Instituut voor Biotechnologie (Belgium). Cytochrome *b* sequences were subsequently entered in the BLAST search engine (http://blast.ncbi.nlm.nih.gov/) and compared with known sequences. The sequences for which at least 700 base pairs could be reliable called were deposited in GenBank (Accession numbers KP140966–KP141215).

Assay for anti-arenavirus antibody

All 370 dried blood spots (DBS, pre-punched spaces on the filter paper that each absorb approximately 10 μL of whole blood) were screened for anti-arenavirus IgG antibodies using an indirect immunofluorescence assay (IFA) as described in (Günther et al., 2009). Each DBS was eluted in 300 μL PBS overnight, of which 10 μL was pipetted on slides covered with Vero cells infected with Morogoro virus. All IgG antibodies against known arenaviruses from the Lassa virus complex cross-react (Wulff et al., 1977; Günther et al., 2009; Gonzalez et al., 1983). On each slide, PBS was used as negative control and a DBS sample known to be anti-MORV antibody positive was used as a positive control. After incubating at 37 °C for 1 h and washing thrice in PBS, FITC-conjugated rabbit anti-mouse IgG secondary antibodies were added. After another incubation and washing round, slides were manually verified for fluorescent labeling at 10 × 40 magnification.

Arenavirus RNA screening

All 370 DBS were pooled by two before RNA extraction, using the QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) and following the protocol described in (Goüy de Bellocq et al., 2010). RNA was also extracted from kidney samples of all 247 *M. natalensis* individuals for which these were available, also pooled by two, using the Nucleospin Total RNA Isolation Kit (Macherey Nagel) according to manufacturer's instructions. Subsequently RT-PCRs were performed on both the blood and kidney extracts using the SuperScript One-Step RT-PCR System and universal African arenavirus primers targeting a 340-nt fragment of the L protein gene (Vieth et al., 2007). Positive pools were resolved by performing RNA extraction on separate kidney samples of the individuals represented in the positive pools, or, when no kidney samples were available, on the separate DBS samples. On positive resolved samples, an additional one-step RT-PCR was performed targeting a 558-nt fragment of the nucleoprotein (NP) gene (primers OWS2805-fwd, OWS2810-fwd, OWS3400-rev and OWS3400A-rev Ehichioya et al., 2011). All amplicons were Sanger-sequenced at the Genetic Service Facility of the Vlaams Instituut voor Biotechnologie (Antwerp, Belgium), and corresponding sequences deposited in GenBank (accession numbers KJ856580–KJ856623 and KP141216–KP141229).

Virus isolation

Virus isolation was attempted for 15 RT-PCR positive samples, under BSL-3 conditions at the Bernard-Nocht Institute for Tropical Medicine. An approximately 0.5 cm^3 mixture of organ samples were lysed in 1 mL Dulbecco's Modified Eagle's medium (DMEM) containing 2% fetal bovine serum (FBS) using a bead mill. The lysed tissue was centrifuged for 2 min at $> 10,000 \times g$, and 250 µL of the supernatant was used to inoculate a monolayer of 1×10^5 Vero E6 cells in DMEM−2% FBS. After 1 h incubation at 37 °C, the medium was replaced with DMEM−10% FBS containing penicillin–streptomycin. Aliquots of supernatant were taken every 1 to 3 days, and RNA was extracted using the QIAamp Viral RNA Mini Kit. Viral RNA concentration was monitored by quantitative RT-PCR using the QuantiTect SYBR Green RT-PCR Kit (Qiagen, Hilden, Germany) and primers GAIV_S287_F (TCGACAATGCCYCTCTCTTG) and GAIV_S456_R (ATYGC-CATCATGGARTGRTC). Titers of infectious arenavirus particles was determined by immunofocus assay using Old World arenavirus N protein specific monoclonal antibodies for detection of infected cells, as previously described (Rieger et al., 2013). In order to produce sufficient material for next generation sequencing, Vero E6 cells in 75 cm^2-flask were infected with supernatant of the first passage of strain TZ-27421, at an MOI of 0.01.

Whole genome sequencing and assembly of Gairo virus isolate TZ-27421

Twenty-eight 140-µL supernatant samples of the second passage of Gairo virus TZ-27421 were each mixed with 560 µL AVL buffer (without carrier RNA) and in combination passed through a total of 12 QIAmp Viral RNA Mini Kit columns. RNA extraction was performed according to manufacturer's instructions, and RNA was eluted in 27 µL of water per column. Eluted RNA was combined into 3×100 µL that were each cleaned up and concentrated to a volume of 14 µL using the RNeasy Mini Elute Clean up kit (Qiagen, Hilden, Germany). The cDNA library construction and 454 sequencing performed at Macrogen (Seoul, Korea). The cDNA library was constructed using cDNA Rapid Library Preparation Kit (GS FLX Titanium Series, Roche) according to manufacturer's instructions. Preparation of template beads for 454 sequencing followed the emPCR method manual for rapid libraries (Lib-L) and for large volume emulsions (LV) (Roche

Applied Science). Enriched DNA capture beads were then multiplexed with another cDNA library and sequenced on 1/8 region of a 454 GS FLX instrument (Roche Applied Science) according to manufacturer's instructions.

Using PRINSEQ webserver (Schmieder and Edwards, 2011), reads with a PHRED quality score < 20 were removed. First, reads were de novo assembled in Geneious 6.1.7. (Biomatters). The preliminary consensus sequences of the S and L segments were then subsequently used as a reference in bowtie2 (Langmead and Salzberg, 2012) to map all reads of the sample to. The resulting assembly was then processed in Geneious 6.1.7. The final consensus nucleotide at each position of the sequence was called as the nucleotide represented by at least 75% of the reads (otherwise it was called as an ambiguity) and when coverage was at least 4 times. To obtain the sequence of the 3′end terminal part of L segment, we performed a SuperScript One-Step RT-PCR with primers L3end_F (5′-GTCAGGGA-CAACCAAGGGTA-3′) and L3end_R (5′-GCCTAGGATCCCCGGTGCG-3′) of which the amplicon was Sanger sequenced as indicated above. Genome segment sequences were deposited in GenBank under accession numbers KJ855308 (S) and KJ855307 (L).

Potential N-glycosylation sites were searched for with NetNGlyc 1.0 Server (http://www.cbs.dtu.dk/services/NetNGlyc/). Secondary structure of the intergenic regions was predicted on the RNAfold webserver (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi).

Phylogenetic analysis

Phylogenetic trees for the partial L (340 nt) and NP (518 nt coding region trimmed from 558 nt fragment) gene alignments were inferred in MrBayes (Huelsenbeck, 2001) with the parameters: general time reversible (GTR) model of nucleotide substitution and 6 categories of gamma distributed rate variation among sites (partitioned into the 3 codon positions); 2 Markov chain Monte-Marlo (MCMC) chains with 2000,000 generations. The nucleotide sequences of the complete genes (GPC, NP, Z and L) of GAIV were aligned with available entire gene sequences of other Old World arenaviruses (OWA) using the TranslatorX server (Abascal et al., 2010). Phylogenetic trees for each gene besides Z, as well as the parts of GPC gene encoding for G1 and G2, were inferred by MrBayes (Huelsenbeck, 2001). For all genes, we used a GTR substitution model with 6 categories of gamma distributed rate variation among sites and a birth-death model as prior on relaxed clock determined branch lengths. Nucleotide sequence data were partitioned into three codon positions. The 2 MCMC chains were run between 10,000,000 and 30,000,000 generations and convergence was verified in Tracer (Rambaut and AJ, 2007). All sequences used from GenBank are listed in Supplementary Table 1.

Ecological statistics

Presence of antibody and Gairo virus RNA in wild-caught *M. natalensis* individuals was related to several host characteristics (sex, weight, body mass index − BMI[weight/body-length^2], reproductive status and lens weight: an estimate for the age of the animals Leirs et al., 1990) using separate generalized linear mixed models with logit link function in the lme4 package in R (R Core Team, 2014), always including trapping locality as a random effect. Likelihood ratio testing was used to assess statistical significance of a parameter by comparing full and reduced models. For these analyses, a subset of the dataset ($n=230$) was used for which the necessary data was available (RT-PCR of kidney samples, eye lens weight, body length).

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.virol.2014.12.011.

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