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Relationships within the *Calomys callosus* species group based on amplified fragment length polymorphisms

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Abstract

Calomys callosus was identified almost 40 years ago as the rodent reservoir of Machupo virus (MACV, Arenaviridae), which causes Bolivian hemorrhagic fever (BHF), a disease endemic to northeastern Bolivia. However, the range of *C. callosus s. l.* far exceeds the known distribution of MACV and BHF. Four sampling regions representing different mitochondrial lineages within the *C. callosus* species group and an outgroup were evaluated for their genetic relationships using amplified fragment length polymorphisms (AFLP). Four AFLP primer combinations generated 596 bands, which were used for phylogenetic and population analyses. We show, using nuclear genetic markers, that the populations of rodents responsible for the maintenance and transmission of MACV are an independent monophyletic lineage, different from other lineages in other areas of Bolivia and South America. These data support the conclusions reached using mitochondrial DNA sequence from the cytochrome *b* and control region (D-loop) genes.

Keywords: Calomys; Systematics; Amplified fragment length polymorphisms; Bolivia; Beni Department; Machupo virus; Bolivian hemorrhagic fever; Arenaviridae

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1. Introduction

Several hemorrhagic fevers of arenaviral origin in the Americas appear to demonstrate nidality (Vainrub and Salas, 1994). Nidality (Pavloskii, 1966) is a concept that describes the phenomenon by which infectious foci appear to be localized, while the distribution of the host is often quite widespread. Many pathogenic arenaviruses have an incomplete pattern of overlap with the host species range. An example of this lack of complete overlap is Bolivian hemorrhagic fever (BHF), a disease caused by Machupo virus (MACV; Arenaviridae) that was first isolated from humans almost 40 years ago (Johnson et al., 1965). The virus has been associated with the rodent host *Calomys callosus* (Johnson et al., 1966). The disease is endemic to only a handful of localities in the Beni Department of northeastern Bolivia (Kilgore et al., 1995), whereas the distribution of *C. callosus*, as currently understood (e.g. Olds, 1988), includes the lowlands and open biomes of Bolivia, Brazil, Paraguay, and Argentina. Why is BHF found in only a restricted geographic area if the apparent reservoir rodent is widespread over several South American countries? Current understanding of both host and virus phylogenetic and ecological relationships in this example is in disarray or severely limited. This limitation represents an important barrier to predictive modeling of the risks of outbreaks of vector borne diseases.

Salazar-Bravo et al. (2001) used sequence data from the mitochondrial (mtDNA) genome to address phylogenetic relationships within and among species of *Calomys* in the most comprehensive molecular analyses of the genus to date. The complete sequence of the cytochrome *b* gene was obtained from specimens of several species of *Calomys* from localities in Argentina, Brazil, Paraguay, Bolivia and Venezuela. Results of that analysis indicated that *Calomys* was a monophyletic genus and was composed of two main clades. One clade associated with specimens found in mountain habitats and referred to as the highland clade with species mostly distributed in the highlands of Bolivia, Peru, Chile and Argentina. The other clade, referred to as the lowlands clade, contained species distributed in the lowlands of Brazil, Bolivia, Argentina and Paraguay. *C. callosus*, the host of MACV, belongs to the lowlands clade.

In a complementary analysis, Salazar-Bravo et al. (2002) used sequence data from the control region and the cytochrome *b* gene of the mitochondrial genome to assess relationships among four taxa of *Calomys*, which are closely associated morphologically and have been referred to as the '*callosus* clade'. They showed that the *callosus* clade contained four closely related monophyletic lineages. One lineage was represented by specimens from the Beni Department, Bolivia where BHF occurs. A second lineage included specimens from the Andean foothills from Northern Argentina and Southern Bolivia. The third lineage was represented by specimens from Argentina that have been recognized as a distinct species, *Calomys venustus.* The fourth lineage contained *C. callosus s. s.* from the dry Chaco region of northern Paraguay and southeastern Santa Cruz Department, Bolivia.

When other types of data (i.e. karyology and ecology) were considered, it was clear that the four reciprocally monophyletic clades (Beni, Andes, *C. callosus s. s.*, and *C. venustus*) could be recognized as distinct taxonomic units. For example, the habitat occupied by the Beni specimens is composed of savanna and savanna-associated habitats in northeastern Bolivia, immediately south of the Amazon basin proper. Animals from this region of Bolivia have a chromosome diploid number ($2n$) of 50. Populations from the mountain forests (Salazar-Bravo, unpublished observations) at middle elevations (above 600 m and below 2000 m) along the eastern side of the Andes Mountains have a $2n = 54$. The dry Chacoan forest and associated habitats, located in the southeastern region of the Bolivian department of Santa Cruz and the northeastern region of Paraguay is the habitat of *C. callosus s. s.*, which also have a $2n = 50$, but occur in a completely different ecological setting than the animals from Beni. The fourth group, *C. venustus* with a $2n = 56$ (Espinosa et al., 1997; Vitullo et al., 1990), is a species that has been long recognized as an independent taxonomic lineage found in the northern pampas region of Argentina. Three of the four lineages within *C. callosus s. l.* already have binomial names available: *C. venustus* (Thomas) from Argentina, *Calomys fecundus* (Thomas) from the Andean foothills from southern Bolivia and northern Argentina, and *C. callosus s. s.* (Rengger) from Paraguay and Bolivia (see Salazar-Bravo et al., 2002).

Because lineage sorting and ‘gene tree vs. species tree’ could compromise the results of Salazar-Bravo et al. (2002), we use the amplified fragment length polymorphisms (AFLP) technique to test the phylogenetic hypotheses presented by them. AFLP is a relatively new method for generating DNA fingerprints (Vos et al., 1995) and allows the analyses of the nuclear genome of an organism when little information is available for that genome. We investigate AFLP as a first attempt to examine the nuclear genome of these rodents and as an independent data set to test the maternally inherited mtDNA hypothesis presented by Salazar-Bravo et al. (2002). The AFLP banding patterns allowed us to better understand phylogenetic relationships and population dynamics within and among populations of *Calomys*.

2. Methods

Total genomic DNA used in this study was isolated from 25 frozen tissue samples (Table 1). AFLP fragments were generated using an AFLP Selective Amplification Start-up Module kit provided by Perkin–Elmer Applied Biosystems. Following the protocols of Vos et al. (1995), DNA was double-digested using *Eco*RI and *Mse*I restriction enzymes. Fragments were ligated to adapters designed specifically for the restriction sites, and then were used in a pre-selective PCR amplification containing primers complementary to the adapter sequence. Five specimens were selected from the pre-selective PCR products and were then subjected to a combination of 64 different selective primer pairs in PCR amplifications. From those results, we selected four of the primer combinations that would provide a large number of fragments per primer combination. The pre-selective products for all 25 DNA specimens were then amplified with the following selective primer combinations: MseI-CAT/EcoRI-ACG; MseI-CAC/EcoRI-ACG; MseI-CTA/EcoRI-ACG; and MseI-CAG/EcoRI-ACG. The EcoRI-ACG primer was labeled with a fluorescent dye. The dye labeled selective amplification products were loaded on an ABI 377 automated DNA sequencer with

Table 1
Specimens examined

mtDNA lineage[a]	Specific locality	Identification[b]
Beni Department	Bolivia: Beni; Bolpebra	NK27659 NK27668
Chaco (*C. callosus s.s.*)	Bolivia: Beni; La Republica	NK37735
	Bolivia: Beni; San Antonio	NK37739
	Bolivia: Beni; Chumano	NK37787
	Bolivia: Beni; Villa Olga, 6 km by road from San Miguel de Chavez	NK37800
	Bolivia: Santa Cruz; 4 km N 1 km W Santiago de Chiquitos	NK12310
	Bolivia: Santa Cruz; 10 km N San Ramon	NK13009
	Paraguay: Amambay; Parque Nacional Cerro Corá	NK22523 NK22532
	Paraguay: Boqueron; Monte Palma	NK72344 NK72378
Andes foothills (*C. fecundus*)	Bolivia: Tarija; 1 km E Tucumilla	NK23650
	Bolivia: Tarija; 1 km S Camatindy	NK23354
	Bolivia: Chuquisaca; Porvenir	NK12564
	Bolivia: Chuquisaca; 2 km SE Monteagudo	NK21355
	Argentina: Santiago del Estero; Guasayan Virgen del Valle picnic area, HWY 64 between Santa Catalina and La Puerta Chiquita	AK15376 AK15382
C. venustus	Argentina: Córdoba; 2 km S Espinillo	TK49115 TK49116
C. laucha	Argentina: Santiago del Estero; Quebrachos Buena Vista 15 km NE Va. Ojo de Agua off HWY 13	AK15337
	Argentina: Santa Fe; Maximo Paz	NK15988
	Argentina: Santa Fe; Casilda	NK15989
	Bolivia: Tarija; Estancia Bolivar	NK25156 NK25158

[a] Mitochondrial lineage defined in Salazar-Bravo et al. (2002).

[b] AK—Frozen tissue housed at the Texas Co-operative Wildlife Collections, Texas A&M University. Voucher specimens housed at Division of Mammals, Sam Noble Oklahoma Museum of Natural History, University of Oklahoma, Norman, OK. NK—Division of Genomic Resources, Museum of Southwestern Biology, University of New Mexico, Albuquerque, NM. TK—Museum of Texas Tech University, Lubbock, TX.

an internal size standard in each lane. Fragments in the range of 100–500 base pairs were analyzed using the GeneScan Analysis Software 3.1.

Relationships among members of the *C. callosus* clade and the outgroup (*Calomys laucha*) were assessed by the use of maximum parsimony (MP) and neighbor-joining (NJ) using PAUP∗4.0b6 (Swofford, 2000). For the MP analysis all characters were treated as unordered and equally weighted. The data set was subjected to 1000 heuristic searches of 100 random addition replicates with TBR branch swapping. Homoplasy was evaluated using the consistency index (CI; Kluge and Farris, 1969) and the retention index (RI; Farris, 1989). Tree length was used to determine the most parsimonious solution, and support for individual clades was evaluated using both the decay index (Bremer, 1988) and bootstrap resampling using 10,000 replicates with heuristic searches set with 10 random addition replicates, and TBR branch swapping. Decay indices were generated using AutoDecay 4.0.2 (Eriksson, 1998). The NJ distance analysis was performed on a matrix generated from the Nei–Li distance measure (Nei and Li, 1979). Bootstrap resampling using 10,000 replicates was conducted to test the support of individual clades.

Analysis of Molecular Variance (AMOVA) was performed to study genetic structure of *Calomys* lineages using the program ARLEQUIN (Schneider et al., 2001). Population genetic structure was investigated using pairwise population Fst significance tests. Fst was estimated using the methods of Wright (1931), Weir and Cockerham (1984), and Lynch and Milligan (1994). The Fortran code of the program RAPDFST (Black, 1998) had to be converted to SAS/BASE software, version 8.1 of the SAS System for Windows in order to accommodate the large number of loci examined. Weir and Cockerham's (1984) Theta (Fst) corrects for small and unequal sample sizes as well as for small numbers of individuals. Lynch and Milligan's (1994) Fst was developed to evaluate population structure using dominant (lack of complete genotypic information) genetic markers such as AFLPs.

3. Results

A total of 596 bands were analyzed. The ACG/CAT primer combination produced 153 fragments, the ACG/CAC combination produced 135 fragments, the ACG/CTA pair produced 157 fragments, and the ACG/CAG primer combination produced 151 fragments. Forty of these bands were constant among all lineages and 97 were phylogenetically uninformative; leaving 459 phylogenetically informative characters.

A single most parsimonious tree was obtained (Fig. 1). The tree length was 1444 steps, and the CI and RI were 0.3850 and 0.6280, respectively. The level of resolution offered by the analysis of these data indicated the existence of three reciprocally monophyletic lineages within currently recognized *C. callosus s. l.*, while supporting the differentiation of *C. venustus* as a distinct species. Specimens assigned to *C. callosus s. l.* from the lowlands of central, south and southeast Santa Cruz Department in Bolivia, and west and northeast Paraguay form the first group (Chaco). The second group is formed by specimens from the Beni Department, Bolivia. The third group (Andes foothills, *C. fecundus*) is composed of specimens from intermediate

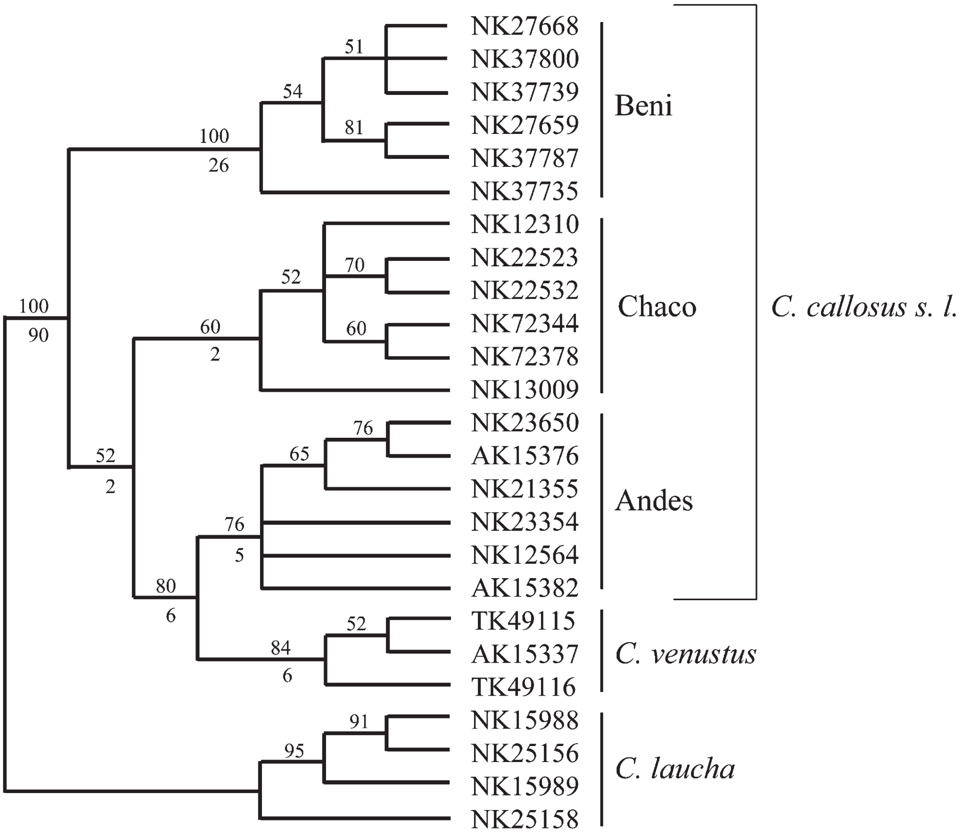

Fig. 1. Phylogenetic relationships among five lineages of *Calomys* based on parsimony analysis of AFLP banding patterns (tree length = 1444; CI = 0.3850; RI = 0.6280). Numbers above branches represent bootstrap values and numbers below branches represent decay indices.

elevations in the Bolivian Departments of Tarija and Chuquisaca (southern Bolivia) and northern Argentina. Bootstrap values and decay indices indicated less support for the Chaco, Andes, and *C. venustus* clades than for the clade containing the Beni Department specimens.

The NJ analyses produced a tree with a slightly different topology (Fig. 2). However, the four clades found in the MP analyses still are present in the NJ tree. The only difference is the topology of the clades. *C. venustus* and *C. fecundus* still are sister taxa, but *C. callosus s. s.* formed a sister relationship with the Beni Department specimens. Bootstrap values indicate strong support for the individual clades.

AMOVA suggests that there is significant variation among the lineages examined (Table 2). Slightly less than 60% of the genetic variation observed in this data set occurs among lineages. The remaining genetic variation is a result of variation within lineages. The overall pattern of Fst values among populations were similar regardless of the method used to derive them (Table 3). Although the pattern was similar,

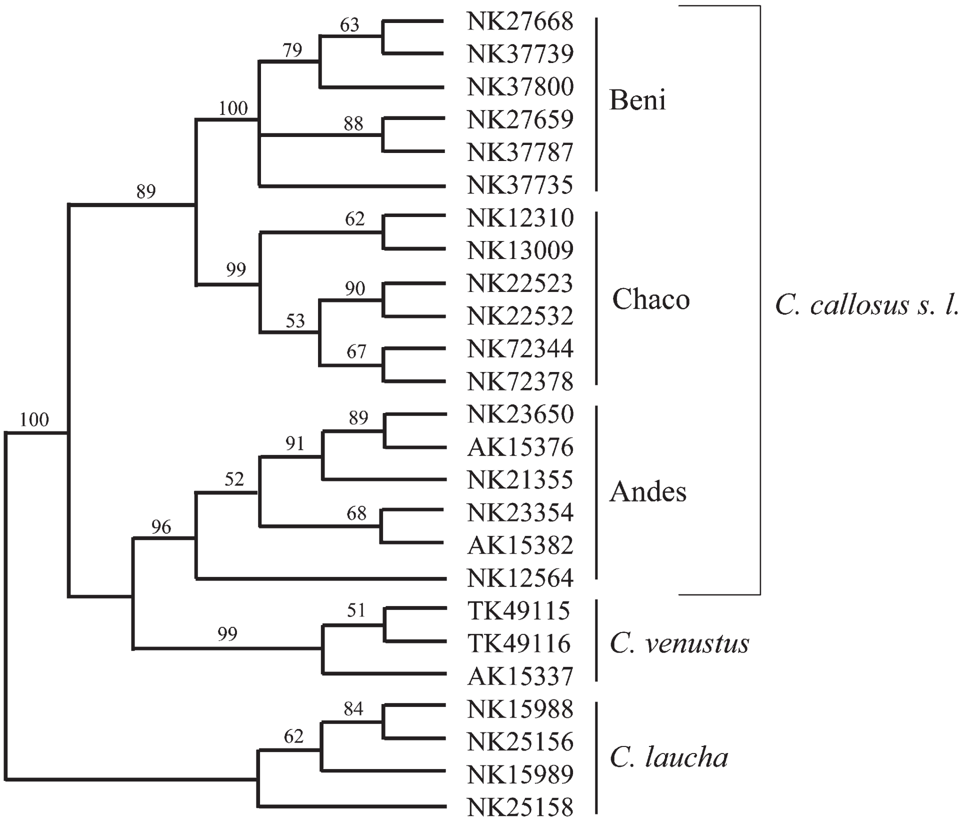

Fig. 2. Phylogenetic relationships among five lineages of *Calomys* based on NJ analysis of AFLP banding patterns. Distance measure used was Nei–Li (Nei and Li, 1979). Numbers above branches represent bootstrap values.

Table 2
AMOVA of the five taxa of *Calomys* examined

Source of variation	df	Sum of squares	Variance components	Percentage of variation
Among populations	4	1229.333	54.16667	57.02
Within populations	20	816.667	40.83333	42.98
Total	24	2046.000	95.00000	100.00

Wright’s Fst values were slightly lower than the values for both Weir and Cockerham’s Theta (for small sample sizes) and Lynch and Milligan’s Fst (for dominant markers).

Table 3
Population pairwise Fst comparisons using the methods of Wright (1931), Weir and Cockerham (1984), and Lynch and Milligan (1994)

	Beni	*C. callosus*	*C. fecundus*	*C. venustus*
C. callosus				
Fst	0.278			
Theta	0.491			
LM Fst	0.481			
C. fecundus				
Fst	0.341	0.258		
Theta	0.573	0.443		
LM Fst	0.565	0.430		
C. venustus				
Fst	0.404	0.334	0.280	
Theta	0.637	0.547	0.455	
LM Fst	0.631	0.697	0.455	
C. laucha				
Fst	0.454	0.428	0.440	0.491
Theta	0.723	0.697	0.693	0.720
LM Fst	0.724	0.697	0.694	0.712

4. Discussion

Salazar-Bravo et al. (2002) have suggested, based on mtDNA, that the population of *C. callosus* that is found in the Beni of Bolivia represents a unique monophyletic lineage closely related to other lineages within the *callosus* clade. The nuclear markers presented here support their conclusions. However, the topologies among the lineages found in the mtDNA analysis and the different methods for analyzing the AFLP nuclear markers are not identical (Fig. 3). In the mtDNA tree the Beni Department specimens were sister to the Andean foothills specimens, and *C. venustus* was sister to that clade. *C. callosus s.s.* was at the base of that tree.

The AFLP markers show strong support for the monophyly of the Beni Depart-

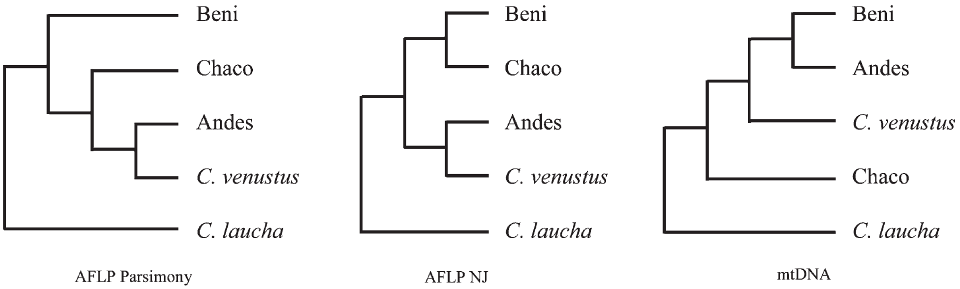

Fig. 3. Comparison of the relationships of the five Calomys lineages based on AFLP parsimony, AFLP NJ, and mtDNA (Salazar-Bravo et al., 2002) sequence parsimony and maximum likelihood.

ment clade, which is consistent with the conclusions of Salazar-Bravo et al. (2002) based on mtDNA analyses. There is good support for the sister relationship between *C. venustus* and the clade representing the Andes specimens, but weaker support for the *C. callosus s. s*. clade joining at the base of the *C. venustus*/Andes clade.

Swofford et al. (1996) do not recommend the use of restriction endonuclease data for phylogenetic analyses because of the violation of the character independence assumption. For example, if a new restriction site appears between two existing sites, the longer band will be cut into two separate bands. As a result, no bands will be shared even though two of three restriction sites are shared. It can be argued that enough fragment data can be examined in order to swamp the kind of example above (see for example, Bremer, 1991) and can be used to estimate phylogenetic relationships of closely related species (Parsons and Shaw, 2001). However, the violation of this assumption even with closely related taxa may explain why the mtDNA tree topology and the AFLP parsimony tree topology do not agree. Albeit, the phylogenetic analyses, as well as the NJ analysis, do support the monophyly of the lineages within *C. callosus s. l.*

Our question regarding the nidality of *C. callosus* and BHF are at the interface between phylogenetics and population genetics. Closely related taxa still may retain the ability to exchange genes. However, the Fst values indicate that there is a high degree of genetic differentiation among the three lineages that represent *C. callosus s. l.* The high Fst values may represent a historical view of gene flow among these lineages rather than current, on going gene flow as these lineages likely shared a common ancestor within the last million years (based on mtDNA; Salazar-Bravo et al., 2002). A larger sample size for each of these lineages will allow for a more thorough test of this hypothesis. This study does, however, demonstrate the utility of AFLP analyses for population genetic studies of *Calomys* species.

Interpreting the patterns of evolution is critical in terms of understanding the processes of evolution. For example, the monophyly of the genus *Calomys* has been contested based on morphological (Steppan, 1993), as well as molecular data (Engel et al., 1998). Salazar-Bravo et al. (2001) using well-identified and properly vouchered specimens as suggested by Ruedas et al. (2000), show unequivocally that *Calomys* is a monophyletic genus. Determining which of these hypotheses is correct is an important issue in understanding virus/host relationships, as well as relationships among viruses. Not only do these finding have important ramifications for public health but a highly corroborated tree of life will provide critical infrastructure for all of comparative biology.

Regardless of the taxonomic status (species, subspecies, etc.) of these lineages it is clear that the populations that host MACV have had an independent evolutionary history, which helps explain why this disease only occurs in northeastern Bolivia and not throughout the range of *C. callosus s. l.*. This result provides additional support to help resolve the apparent lack of overlap between the distribution of MACV and *C. callosus*.

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