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Abstract

The retroviral-like transposable element *mys* has been identified in a limited group of new-world rodents, providing a unique opportunity to assess its evolutionary origin and activity within various rodent genomes. In the course of this investigation we identified an element that appears to represent a distinct, but related, group of elements in the Mexican volcano mouse that we refer to as mys-like. We have determined the presence of this mys-like element in related species of rodents, suggesting that it likely dates back to a common ancestor of the *Reithrodontomyini* rodent tribe. The mys-like element predates the previously identified *mys* element and presumably gave rise to *mys.* Continued molecular investigations will provide insights into the evolutionary origin and history of transposable elements and their potential impacts on the mammalian genome.

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First Advisor

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Second Advisor

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Investigation of the Evolutionary Origin and History of a Newly Identified Transposable Element in Rodents

By

Ashley Larm

A Senior Thesis Submitted to the

Eastern Michigan University

Honors College

in Partial Fulfillment of the Requirements for Graduation

with Honors in Biology

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Table of Contents:

Abstract..3

Introduction..4

Materials and Methods..9

Results...13

Discussion...17

Acknowledgements..22

References...23

Abstract:

The retroviral-like transposable element *mys* has been identified in a limited group of new-world rodents, providing a unique opportunity to assess its evolutionary origin and activity within various rodent genomes. In the course of this investigation we identified an element that appears to represent a distinct, but related, group of elements in the Mexican volcano mouse that we refer to as *mys*-like. We have determined the presence of this *mys*-like element in related species of rodents, suggesting that it likely dates back to a common ancestor of the *Reithrodontomyini* rodent tribe. The *mys*-like element predates the previously identified *mys* element and presumably gave rise to *mys*. Continued molecular investigations will provide insights into the evolutionary origin and history of transposable elements and their potential impacts on the mammalian genome.

Introduction:

A varying percentage of all eukaryotic genomes and approximately 50% of most mammalian genomes are made up of transposable elements (Kazazian, 2011), mobile DNA segments that integrate into new sites within the genome. A vast majority of these mammalian elements are retrotransposons, a class of mobile genetic elements that integrate into the genome via an RNA intermediate (Morgan, 1995). These retrotransposable elements contribute to increase the genetic variation within a population because they characteristically have high copy numbers, which can increase genome size and interfere with the transcription of neighboring genes (Whitelaw and Martin, 2001), and also have the capability to induce mutations if they insert close to or into a functional gene (Kazazian, 2011). Since these integrations are stable events, they can serve as a "molecular fossil record". Molecular fossils are DNA elements, such as L1 LINE elements in rodents (Furano and Usdin, 1995), which are representative of elements that have amplified in the past but have since acquired a number of mutations. Many of these mutations cause amplification of these fossils to cease due to the location within the sequence, but some fossils are thought to have continued retrotranspositional activity (Gilbert and Labuda, 2000). Currently, about 85% of long terminal repeat (LTR) retrotransposon-derived "fossils" consist only of an isolated LTR and flanking LTRs with an internal sequence that has been lost due to homologous recombination (Lander et al., 2001).

Retrotransposons move via a "copy and paste' mechanism that utilizes an RNA intermediate derived from a "master gene" (Deininger et al., 1992) and the enzyme reverse transcriptase used to convert the RNA back to DNA upon insertion by the proposed model of target-primed reverse transcription (Moran, 1999). The notable difference between retrotransposons and other transposable elements is that other transposable elements do not copy

the segment of DNA before moving it elsewhere in the genome (Kazazian, 2011). LTR retrotransposons as well as all other transposable elements are integrated into the genome by the enzyme transposase, resulting in a staggered cut that yields short flanking direct repeats on either end of the newly integrated transposable element after ligation occurs (Kazazian, 2011). LTRs of a specific genetic element are expected to be identical at the time of retrotransposition and gather mutations after insertion over time (Sawby and Wichman, 1996). This makes LTRs very easy to detect and additionally makes the order in which they insert into the genome, as well as the mutations that they induce, easy to identify in different species which can be used to provide useful evolutionary information.

The patterns and order of the insertions of LTRs into specific genetic loci has the potential to be tracked by investigating which species contain certain retrotransposable elements at specific integration sites within the genome. If two species share an insertion at the same locus, it would support their derivation from a common ancestor. This method of presence/absence analysis can be used to provide the previously mentioned "molecular fossil record". This method has been used in previous studies to trace another transposable element called the *Alu* element which is a short interspersed element (SINE) that is LINE (long interspersed element)-dependent and that has been identified in the primate genome (Batzer et al., 1994). This study offered continued support for the hypothesis of the African origin of humans based on investigating patterns of polymorphic Alu insertions into the human genome.

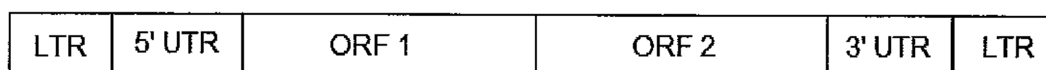


Figure 1: A representation of a generic LTR retrotransposon.

A specific retrotransposable element that has been of particular use in gaining evolutionary information regarding the rodent genome is the *mys* element which was first identified in the genus *Peromyscus* (Wichman et al, 1985). These elements are retrovirus-like elements (RLEs) , also referred to as LTR retrotransposons or LTR LINEs (Figure 1), that are often grouped with long interspersed elements (LINEs), and are similar in structure to retroviruses (Lee et al, 1996). The earliest infectious retroviruses likely arose from endogenous retroviral-like elements that acquired mechanisms for horizontal transmission and more recent retroviral elements have likely originated via retroviral infection (Lander et al., 2001).

The *mys* element has also been used as a genetic marker to track the evolutionary history of various species of mice through time (Killian, 2011). A number of different *Peromyscus* species (deer mice and their relatives) have been analyzed for the presence of the *mys* element in their genomes. The species *Neotomodon alstoni* was found to have an apparently related, but distinct, previously unidentified element in addition to the *mys* element (Killian, 2011). This was based on the cloning and sequencing of the *Peromyscus maniculatus* and *Neotomodon alstoni* presumptive *mys* PCR-amplified products. All 5 *P. maniculatus* clones were clearly *mys* but 2 out of 5 of the *N. alstoni* clones were found to be distinct in sequence. We determined that these differences were not based on random mutations because these clones shared many distinct nucleotides. They also shared about a 94% sequence identity with *mys*, and therefore, we refer to this family of elements as *mys*-like (Killian and Kass, unpublished data).

The identification of this new *mys*-like element in *Neotomodon alstoni* was significant because it is closely related to the *Peromyscus* genus as part of the *Neotominae* subfamily of *cricetid* rodents. Therefore, this finding triggered the question of whether more distantly related genera may contain this other element as well. These two genetic elements together could be

used to help to increase understanding of the phylogeny of the rodent species within the *Neotominae* subfamily, which has been fairly controversial (Lee et al, 1996), as well as providing a way to further understand origins and evolution of retrotransposon in general.

The discovery of the *mys*-like element opened up a new potential for the study of the origins of retrotransposon families, amplification capabilities (mechanisms), contributions to the dynamics of mammalian genomes and genetic impacts, as well as the potential use of the element as a tool in phylogenetic analysis and population studies. Using a previously proposed phylogeny of rodents (Miller and Engstrom, 2008), including those in *Peromyscus* as well as other related genera and using DNA samples isolated from the tissue of a number of these rodent species, we utilized various sets of primers for "intra-*mys*" PCR to investigate the origin and evolutionary history of this newly discovered LINE family. *Mys* was presumably found in all *peromyscine* species but was not identified in the house mouse, *Mus* (Killian, 2011). Since *mys* and *mys*-like elements are found in a narrower taxonomic group than other mammalian retrotransposons, they provide a unique opportunity to trace their history and gain insights into the origins and evolution of this group of transposable elements.

Until recently, retrotransposons were included in the category of "junk DNA" in the context of the genome because they did not code for a specific function that was beneficial to the host (Kazazian, 2011). Investigations of these DNA segments determined these to be retrotransposable elements that do not necessarily provide a favorable role within the organism that they are present in, with some encoding for the genetic machinery needed to move themselves to another location within the genome (Kazazian, 2011). Therefore, although they do not appear to have an immediate function, they can alter the specific functions of other genes and induce genetic variation within organisms on an evolutionary timescale (Kazazian, 2011). One

example of this was shown in a study that demonstrated that another retroviral-like retrotransposon in mice, VL30, complexes with a specific PSF protein to regulate steroidogenesis and possibly other physiological functions in mice (Song et al., 2003). The same study also suggests that these retrotransposons may have evolved not as "junk DNA" but instead as transcriptionally active non-coding DNA that acquired physiological and pathological function over time (Song et al., 2003).

The goal of this project is to study this newly discovered element in more detail, to investigate its pattern of occurrence in different rodent species, when it originated and if there is any evidence that it has competed with the *mys* element at any point during the evolution of the species in which it is present. If this element can be identified in species that are more distantly related to *Peromyscus,* it may be possible that this element actually arose prior to the *mys* element, perhaps giving rise to it. An apparent lack of *mys* in species that are not part of the *Peromyscus* genus in which this element was initially identified (Wichman et al., 1985) supports this hypothesis.

Materials and Methods:

The experimental procedures that were utilized in this study included polymerase chain reaction (PCR) using internal *Neotomodon mys*-like primers (Figure 2) of which the numbering was based on nucleotide positions in ORF 1, and analyzed using gel electrophoresis. DNA used in this study was isolated from tissue samples obtained from The Museum of Southwestern Biology and the *Peromyscus* Stock Center in a previous study (Killian, 2011). Since this was the first study specifically involving the isolation of this element, conditions for carrying out these procedures were altered multiple times in order to generate maximum visualization of the desired products on an agarose gel. This included altering the amounts of DNA used, the amounts of reagents used and the cycling conditions for PCR.

Figure 2: A representation of one of the sets of intra-*mys* primers within ORF 1 that were utilized in this study.

The species tested included *Reithrodontomys fulvescens* (Fulvous harvest mouse), *Peromyscus leucopus* (White footed mouse), *Peromyscus aztecus* (Aztec mouse), *Peromyscus melanophrys* (Plateau mouse), *Peromyscus eremicus* (Cactus mouse), *Neotomodon alstoni* (Mexican volcano mouse), *Onychomys leucogaster* (Northern grasshopper mouse), *Oryzomys palustris* (Marsh rice rat), *Sigmodon hispidus* (Hispid cotton rat), *Tylomas tumalensis* (Tumbala climbing rat), *Neotoma albigula* (White-throated wood rat) and *Mesocricetus auratus* (Golden hamster), all of which are part of the family *Cricetidae* as well as some species in the family *Muridae,* including *Rattus rattus* (Rat), *Meriones sp* (Gerbil) and *Mus musculus* (House mouse). The species selected for analysis in this study were chosen based on the findings of a previous

mys element study (Killian, 2011) as well as a previously proposed phylogeny by Miller and Engstrom (2008) (Figure 3) based on parsimony analysis of DNA sequences of the interphotoreceptor retinoid-binding protein (IRBP), growth hormone receptor (GHR), and Cytochrome b (Cytb). Since the *mys* element had been identified in all *Peromyscine* rodents, the phylogeny was used to select *Peromyscine* species as well as species with varying degree of relatedness to these species for analysis in order to see the relationships between the *mys* and *mys*-like elements.

PCR was set up in a 20 ul reaction with either 20 ng DNA (*P. leucopus, R. fulvescens, N. alstoni* and *O. leucogaster)* or 100 ng DNA (remaining species) with 1x Dreamzyme Buffer (Fermentas), 200 uM dNTP, 250 nM forward primer or LINE forward primer (F60, F40, LINE 633 or LINE 136 – table 1), 250 nM of neo*mys* reverse primer or LINE reverse primer (R430, R375, LINE 1053 or LINE 633 – table 1), 1 uM DreamTaq DNA polymerase (Fermentas) and 1.5 mM $MgCl_2$. The most effective PCR minicyler conditions were as follows: Step 1: 94° C for 2 minutes, Step 2: 94° C for 30 seconds, Step 3: 57° C for 30 seconds (annealing temperature), Step 4: 72° C for 30 seconds, Step 5: back to step 2 and repeat 29 times, Step 6: 72° C for 5 minutes and Step 7: keep samples at 4° C until program is stopped.

Table 1: Table showing primer sequences used in this study.

Primer name	Primer Sequence
F60	5' - TAA AGT TGG GGC ACC TGT TTT CA - 3'
F40	5' - AGT CAT TCA AAG TTC TCT TG - 3'
R375	5' - GGA TTT ACT TCA GAG GAG GA - 3'
R430	5' - TTT AAA AAT TTA GAC GTT CTG - 3'
LINE 633	5' - ATT GGA AAG GAA GAA GTC A - 3'
LINE 1053	5' - ATT TGT AGA TTG CTT TTG G - 3'

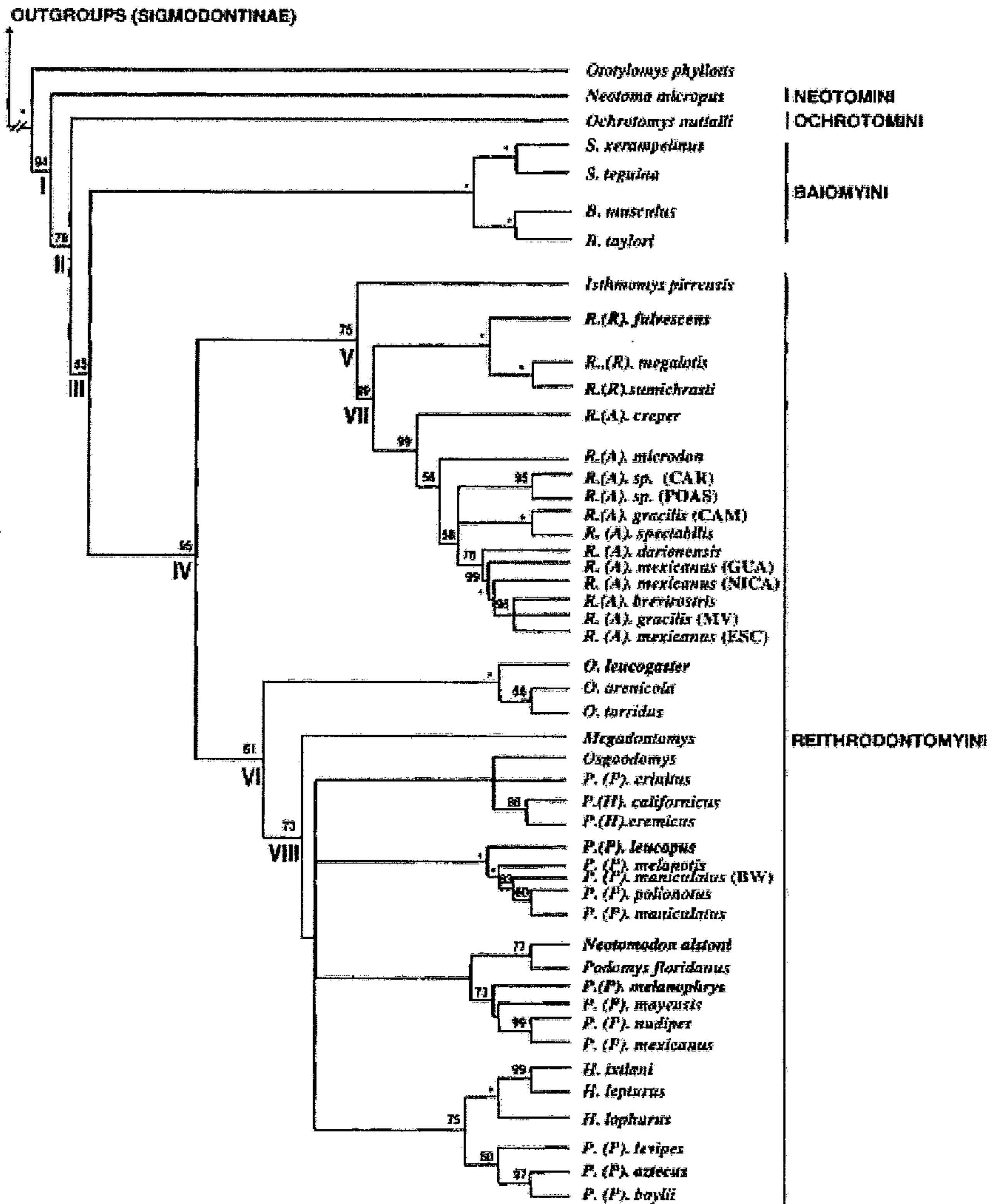


Figure 3: Previously proposed rodent phylogeny generated by Miller and Engstrom (2008) to which the species in this study (highlighted) were compared.

Identification of PCR products was done using agarose gel electrophoresis. 2.5 ul of 6x loading dye were added to the samples before running them on 2% agarose gels. 2.5 ul of GelRed was added to the gel mixture prior to pouring for visualization purposes and after pouring, the gel was put into a 1x TAE (Tris base, acetic acid and EDTA) buffer. The gel was then run at 150 volts for approximately 1-1.5 hours and visualized under UV radiation with a gel documentation system. Amplification product sizes were compared to a Labnet 100 bp DNA ladder.

Results:

The determination of the presence of the *mys*-like element was carried out in a number of different North American rodent species. In the current study, this element was able to be amplified and visualized in a number of different species in the family *Cricetidae*, including *R. fulvescens, P. leucopus, P. aztecus, P. melanophrys, P. eremicus, N. alstoni, O. leucogaster, O. palustris* and *S. hispidus*. The *mys*-like element was not able to be amplified and visualized in a number of species within the family *Cricetidae,* including hamster, T. *tumbalensis* and *N. albigula* as well as some species in the family *Muridae*, including rat, gerbil and house mouse.

In order to verify the DNA samples were adequate for assessing the presence of *mys* we analyzed the species for the L1 LINE known to be found in all mammals (Singer, 1982). This analysis was done with two different primer sets in order to verify results. With the use of the LINE 633 and LINE 1053 primers for intra-line PCR, a 420 base pair band is expected in visualization of the L1 LINE element. This element was visualized in *N.alstoni, R. fulvescens, P. leucopus*, gerbil and hamster (Figure 4). The lack of the LINE element *in O. leucogaster* (Figure 4) is notable because there was a problem initially with the DNA that was being used. Due to this, it was initially thought that this species did not contain the *mys*-like element, but after LINE analysis, the problem with the DNA was recognized and rectified.

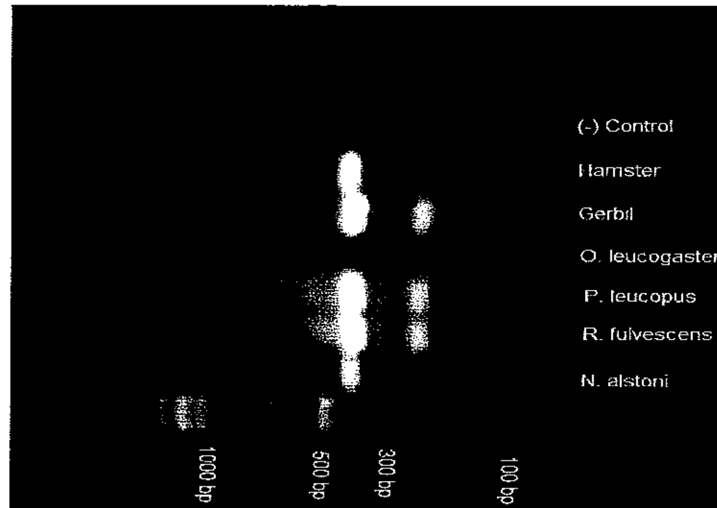


Figure 4: Identification of the LINE-1 element among rodent species. PCR was performed using primers LINE633 and LINE 1053 (LINE-1 expected band of 420 bp) and analyzed on a 2% agarose gel.

To assess the presence of the *mys*-like element, DNA was amplified using the *mys*-like specific primers, F40 and R375 (Figure 5): the expected band size is 370 base pairs. *O. palustris* and *R. fulvescens* were found to contain the *mys*-like element while *T. tumbalensis* and *N. albigula* do not (Figure 5). Distinct size variants were observed at approximately 350 base pairs in *S. hispidus* in addition to the expected band of 370 base pairs and at approximately 300 base pairs in *N. albigula* without the presence of the expected band.

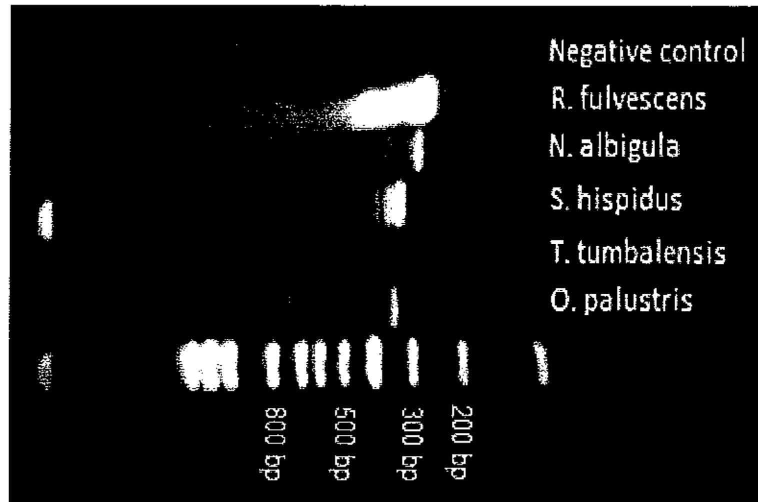


Figure 5: Identification of the *mys*-like element among rodent species. PCR was performed using *Neotomodon* specific *mys*-like primers F40 and R375 (*mys*-like expected band of 335 bp) and analyzed on a 2% agarose gel.

An alternative set of *mys*-like primers, F60 and R430, was used to verify the presence of the *mys*-like element in the different species tested. With this, the *mys*-like element was also visualized in a member of *Peromsycus*, *P. aztecus* (Figure 6). The more distantly related species of rat, mouse, gerbil and hamster that were found not to contain the *mys* element in previous studies (Killian, 2011), also do not contain the *mys*-like element (Figure 6).

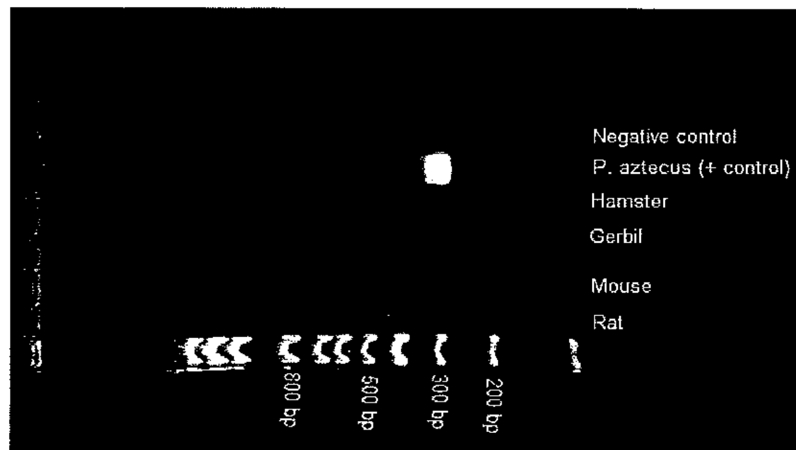


Figure 6: Identification of the *mys*-like element among rodent species. PCR was performed using *Neotomodon* specific *mys*-like primers F60 and R430 (*mys*-like expected band of 370 bp) and analyzed on a 2% agarose gel.

We were also able to estimate copy numbers of the *mys*-like element in the species tested based on their band intensities seen on the agarose gels. Of the species tested, four had much more intense bands when visualized on the agarose gels and, therefore, significantly higher copy numbers: *R. fulvescens, P. leucopus, N. alstoni* and *O. leucogaster* (Figure 5) which initially prompted the further dilution of the DNA stock solution in future procedures. This was based on the intensity of the amplified products (Figure 7) even with the use of 1/5 of the concentration of the DNA used with the other samples.

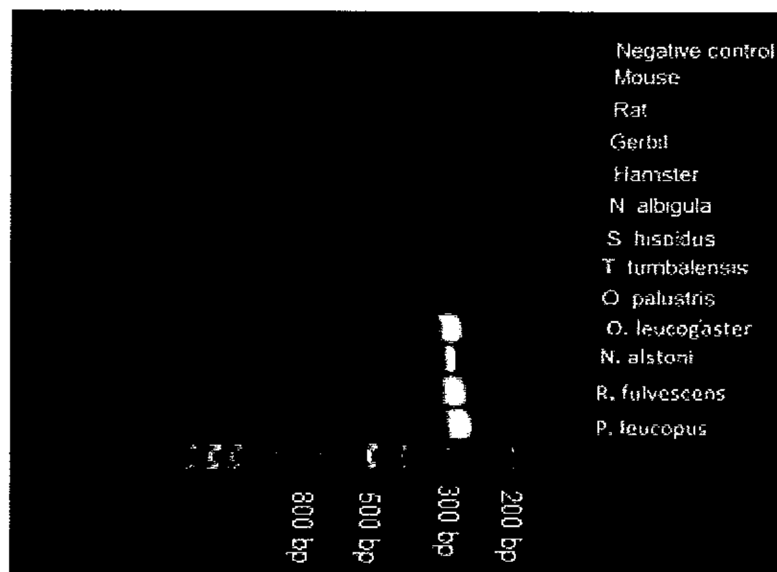


Figure 7: Identification of the *mys*-like element among rodent species. PCR was performed using *Neotomodon* specific *mys*-like primers F60 and R375 (*mys*-like expected band of 315 bp) and analyzed on a 2% agarose gel.

Contamination was a difficult obstacle in this analysis as well. Multiple gels revealed varying amounts of contamination prompting multiple changes in reagents and materials in order to attempt to solve this problem. Generating amplified products without evidence of DNA contamination could potentially verify the findings of this study, including the proposed presence of the size variations in *S. hispidus* and *N. albigula* that were previously mentioned.

Discussion:

The *mys*-like family of retrotransposable elements was identified upon the investigation of the related *mys* element in *P. leucopus* (Killian and Kass, unpublished data). This newly discovered element was found to be ancestral to *Peromyscus* (Killian, 2011) by identification in all analyzed *Peromyscus* species, as well as related genera (Killian, 2011; Killian and Kass, unpublished data). The genera containing this *mys*-like element include those more distantly related than those that harbor the *mys* element. We were able to amplify and visualize the *mys*-like in a number of *Peromyscus* and related species. Although *R. fulvescens, P. leucopus, P. aztecus, P. melanophrys, P. eremicus, N. alstoni, O. leucogaster, O. palustris* and *S. hispidus* appear to have the *mys*-like element, the species *P. leucopus, R. fulvescens, N. alstoni* and *O. leucogaster* might have significantly higher copy numbers based on the level of intensity of their amplified products. Therefore, *mys*-like may have become very active in a common ancestor of these species. This supports the *mys*-like element likely predating the *mys* element that was identified strictly in *Peromyscus* (Killian, 2011) and perhaps the *mys* element was derived from *mys*-like. Based on the findings of this study, some preliminary patterns can be identified and, with further investigation, there is potential to contribute to a better understanding of the evolution of this retrotransposon family.

The identification of the *mys*-like element in members of the rodent tribe *Reithrodontomyini* (see branch IV in Figure 3) but not in other analyzed *cricetid* rodents such as *N. albigula* and *T. tumbalensis* suggests that it originated in a common ancestor of this rodent tribe. Therefore, we have preliminary evidence indicating the origin of this retrotransposon family. These findings can also be considered in contrast to the identification of the *mys* element

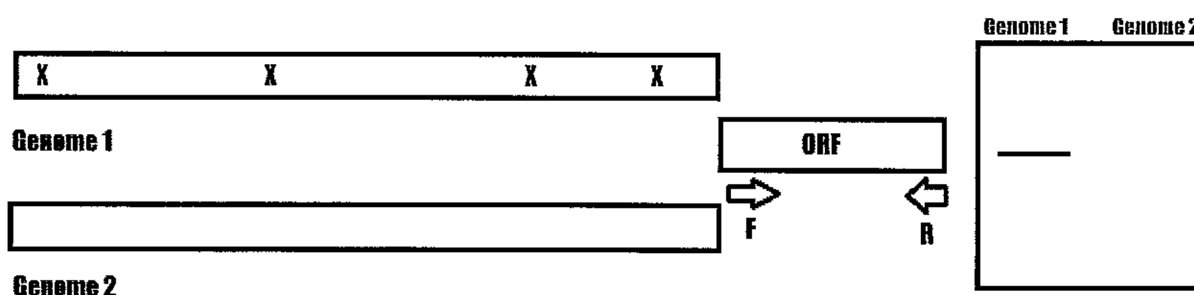
in the limited group of *peromyscine* rodents which suggests that the *mys*-like element likely pre-dated *mys*.

Cloning and sequencing of these *mys*-like loci must be done to ensure that the *mys*-like element is being amplified by using the *mys*-like primers and visualized in this study. Also, the determination of variations that have occurred over time among species that appear to contain this family of LINEs is also needed. This is important because it allows for dating genetic changes in order to see which aspects of the genome have been conserved over evolutionary time, and which have evolved more recently (Furano and Usdin, 1995). It was this type of phylogenetic analysis between L1 elements from different species that revealed that the 5' UTR region of the L1 element is independent of the rest of the element and therefore has been repeatedly acquired by various L1 families in mammals (Furano and Usdin, 1995).

Further investigations of the presence/absence of the *mys*-like element within the genomes of different species as well as the presence/absence of variants at individual loci will help elucidate the relationships between the *mys* element and the *mys*-like element by creating a basis of comparison between the two in terms of evolution over time. The identification of "younger" elements allows for assessing presence/absence at the level of the entire genome with primers specific for the ORF of the element in question as well as the identification of presence/absence at a particular locus by doing PCR with primers flanking the "young element" at an orthologous locus (Figure 8). A previous mobile element study of *Alu* insertion events in humans utilized this method of identifying presence/absence variants within the genome in order to provide further insights on population genetics (Batzer, et al., 1994). The rate of insertion and fixation of *Alu* elements in the genome is very slow which allows for presence/absence analysis to be able to reveal patterns of these events within the genomes as individuals who have the same

Alu insertions obtained them from a common ancestor (Batzer, et al., 1994). A study carried out at particular loci in L1 LINEs revealed that about 17% of variable L1 integration sites are altered due to continued accumulation of mobile elements within a small region of the genome (Vincent et al., 2003). The same study showed an absence of insertion site homoplasy and therefore, the use of L1 and Alu elements as useful tools in population genetics.

A) Presence/Absence of a particular element within the genome:



B) Presence/Absence at a particular locus within the genome:

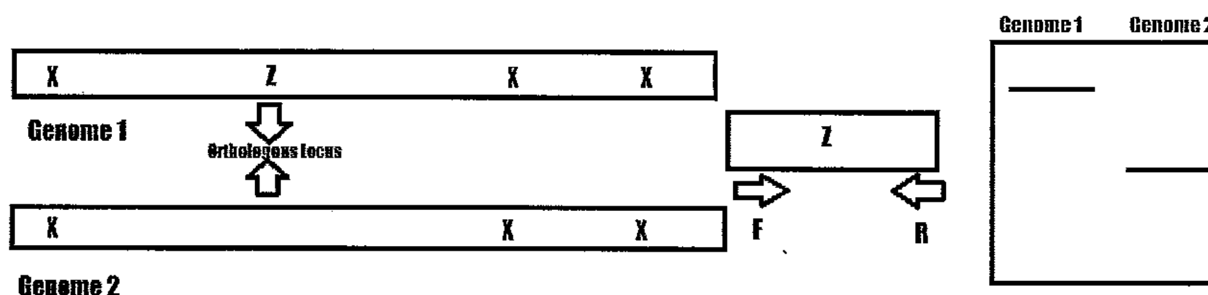


Figure 8: A comparison of two methods of phylogenetic analysis. In A, genome 1 contains transposable element "X" and genome 2 does not. When primers that flank the ORF of this element are used and an agarose gel is run with the resulting PCR products, genome 1 shows a band showing that it does have the element while genome 2 shows no band since its genome does not contain this particular element. In B, genome 1 contains young retrotransposable element "Y" where genome 2 does not have this element at the same locus. When primers that flank the young element are used and the PCR products are run on a gel,

genome 1 shows a larger band than genome 2 showing that genome 2 does not contain this element at that particular locus.

We will also contrast *mys*-like evolution to that of the related *mys* element to learn more about the relationships between retrotransposon families, such as if they competed with each other during amplification (based on copy number) or if certain rodents are more prone to allow activity of retrotransposons. A previous study done with species from the *Sigmodontinae* subfamily of rodents supported the idea that the rapid expansion of another retrotransposable element in the *mys* family, *mys*TR, correlates directly with the loss of L1 activity followed by the loss of SINE activity in these species which brings up the possibility of competition of these elements during evolution (Erickson et al., 2011).

Also, the size variants observed in *N. albigula* and *S. hispidus*, possibly a *mys*-like variant shared in this clade, will be explored to determine if additional *mys*-related families have been generated in certain species. By analyzing numerous elements, a consensus sequence could be derived which would represent a "master gene" allowing for the depiction of more recently integrated elements. A "master gene" has been defined as a genetic element that generates numerous copies of itself since it is present for a long period of time (Deininger et al., 1992). It is possible that the master gene that gave rise to the *mys*-like element has been altered or modified in ways that gave rise to additional related genetic elements.

A number of recent studies have strived to show the importance of retrotransposable elements, such as the *mys*-like element, in an effort to further our understanding of evolution. It has been found that mammalian genomes have evolved mechanisms against retroviral-like element activity through histone modifications and genomic cytosine methylation in order to prevent the negative effects of their transposition (Maksakova, et al, 2008 and Yoder, et al.,

1997). A related element to the *mys* element, *mys*TR, has also been found in very high copy numbers recently (Cantrell et al., 2005), which the authors proposed could be related to lack of LINE-1 activity and its survival through mammalian evolutionary radiation and it has also been proposed that some LINEs, such as L1 in megabats, have "died" and no longer have activity (Cantrell et al, 2008). This shows that these elements have the potential to reveal new information about the genome and evolutionary history that may not have been revealed about certain phylogenetic relationships.

This preliminary identification provides a strong framework for continued investigation leading to a better understanding of the relatively new field of studying retrotransposon evolution on a broader scale. This field of biological study is relatively new and has a great amount of potential to advance our understanding of population genetics. This information can be applied further to the study of different mammalian diseases and disorders that have been proposed to be linked to retrotransposon activity such as hemophilia, congenital muscular dystrophy and the blood disorder hereditary ellipotocytosis (Ostertag et al., 2003). In mice, Maksakova et al. (2008) estimated 10% of spontaneous mutations are due to retrotransposons. Therefore, retrotransposons continue to exhibit an active role in the dynamics of the mammalian genome.

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