Sullivan et al. BMC Zoology (2017) 2:11
DOI 10.1186/s40850-017-0020-3

BMC Zoology

RESEARCH ARTICLE Open Access

Whole mitochondrial genomes provide increased resolution and indicate paraphyly in deer mice

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Abstract

Background: Recent phylogenies of deer mice, genus *Peromyscus*, have relied heavily on mitochondrial markers. These markers provided resolution at and below the level of species groups, but relationships among species groups and *Peromyscus* affiliated genera have received little support. Here, we present the mitochondrial genomes of 14 rodents and infer the phylogeny of *Peromyscus* and related taxa.

Results: Our analyses support results from previous molecular phylogenies, but also yield support for several previously unsupported nodes throughout the *Peromyscus* tree. Our results also confirm several instances of paraphyly within the clade and suggest additional taxonomic work will be required to clarify some relationships.

Conclusions: Our findings greatly enhance our understanding of the evolution of *Peromyscus* providing support for previously unsupported relationships. However, the results also highlight the need to address paraphyly that may exist in this clade.

Keywords: Mitochondrial genome, Paraphyletic, *Peromyscus*, Phylogenetics

Background

Mitochondrial loci have been the most popular phylogenetic markers in animals for over three decades. Their ease of amplification [1, 2], uniparental inheritance, lack of recombination in mammals [2–4], differential selection among genes [2], and general synteny [5] combine to make mitochondrial phylogenetic markers an excellent choice for many study systems. Recent increases in DNA sequencing capabilities have provided an opportunity to sequence full mitochondrial genomes (mitogenomes) rather than partial, one, or a few mitochondrial genes. As a result, previous phylogenies that relied on a portion of the mitochondrion are now undergoing re-analysis in an effort to expand support for or re-affirm previous conclusions [6].

The genus *Peromyscus*, commonly referred to as North American deer mice, encompasses more than 70 species that diverged within the last 6–10 million years [7]. Species including *P. maniculatus* [8] and *P. leucopus* [9] are among the most common mammals in North America and have been studied extensively for over 100 years [10]. Despite extensive study, new species [11] and subspecies [12] are being described on a regular basis. The large number of species, both described and undescribed, as well as substantial cryptic variation, has yielded numerous distinct phylogenetic hypotheses.

Previous classifications of *Peromyscus* recognized seven morphologically distinct subgenera (*Habromys, Haplomylomys*, *Isthmomys, Megadontomys*, *Osgoodomys*, *Peromyscus*, and *Podomys*) [13, 14]. Many of those subgenera (*Habromys, Isthmomys*, *Megadontomys*, *Osgoodomys*, and *Podomys*) have at times been elevated to generic status [15]. Some recent molecular phylogenies, however, show paraphyly [7]. *Isthmomys* is sister to the genus *Peromyscus*, representing a distinct genus, as is currently accepted, but *Habromys*, *Megadontomys*, *Osgoodomys*, *Neotomodon*, and *Podomys*, distinct genera, were found within *Peromyscus*, rendering the genus paraphyletic [7, 16, 17], though *Neotomodon alstoni* [18] previously had been *Peromyscus alstoni* [14, 19].

Recent attempts to resolve the history of this clade by Bradley et al. [16], Miller and Engstrom [17] and Platt et al. [7] were based on various combinations of nuclear

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and mitochondrial genes. All three studies arrived at similar topologies, but varied in their levels of support at some nodes. Species-level relationships were well-supported but the relationships among some genera and species groups were not. Given that approaches that use cytochrome b (*cytb*) alone were somewhat successful in resolving *Peromycus*, we reasoned that the use of whole mitogenomes, a more data-rich approach, may be an avenue to more robust results at the generic and species group level, thus providing additional clarity. Here, we analyze whole mitogenomes from *Habromys, Isthmomys*, *Neotomodon*, *Peromyscus, Podomys*, and three outgroups, *Sigmodon*, *Neotoma*, and *Reithrodontomys*, as identified from previous molecular phylogenies [7, 16, 17].

Methods

Sampling, DNA preparation and sequencing

Taxa selected for this study were identified based on the findings of Platt et al. [7]. Our objective was to reduce taxon sampling for some groups to isolate specific clades. To do so, we chose representatives from selected subclades to serve as proxies. Specifically, one individual here is a proxy for a larger clade (generally represented as a species group). Members of *Neotoma*, *Reithrodontomys*, and *Isthmomys,* which have determined to be closely related clades by several morphological and molecular analyses [7, 15, 16] were included. Species identifications, museum catalog numbers and are included in Table 1.

Whole genomic DNA was isolated using a Qiagen DNeasy Blood and Tissue kit (Qiagen, Valencia, CA) via the manufacturer's protocol and fragmented to ~ 400 bp. Sizes were verified on an Agilient 2100 Bioanalyzer. Illumina compatible sequencing libraries were prepared from the fragmented DNA using the NEB/KAPA library preparation. Each sample was tagged with a unique index and pooled in equal proportions, after which, the pooled libraries were sequenced on single run of an Illumina MiSeq (2 × 250 bp). Raw data are available in GenBank under BioProject ID PRJNA308567.

Mitochondrial genome assembly and annotation

Raw sequence reads were filtered and processed using Trimmomatic version 0.35 [20]. Specifically, we clipped Illumina adapters, disregarded read ends whose Phred scores fell below 20, and utilized a four-base sliding window to trim reads once the average quality fell below 25. We assembled mitogenomes through a custom Bash script (https://github.com/KevinAMSullivan/Mitochondrial_Genomes/tree/MIRA) The script utilized two major programs, MITObim [21] and MIRA [22] to map the filtered reads to a reference genome before assembling the mitogenomes. We selected *Akodon montensis* [23], a Sigmodontine rodent and the most closely related organism with a fully sequenced mitogenome, as the reference (GenBank accession number KF769456).

MITOS [24] was used to annotate the mitochondrial genes for each genome. Putative genes were submitted to BLASTn to confirm sequence length. When gaps were noted, we manually checked for frameshifts but none were observed. Acceptable results were those whose top hits were to a *Peromyscus* or closely related species and that covered nearly 100% of the putative gene. The putative gene was shortened or lengthened to match the length of the BLAST hit if a different size. All

Table 1 Basic read, coverage and size data for each taxon. Museum ID refers to a special identification or catalogue number for speciments at the Natural Science Research Laboratory, Museum of Texas Tech University

Taxon	Museum ID	Reads	Avg. coverage	Genome size
Habromys ixtlani (Goodwin, 1964) [35]	TK 93158	1,912,125	22	16,515
Isthmomys pirrensis (Goldman, 1912) [31]	TK 22583	1,689,780	106	16,628
Neotomadon alstoni (Merriam, 1898) [16]	TK 148849	1,401,025	106	16,776
Neotoma mexicana (Baird, 1855) [30]	TK 93257	2,967,246	102	16,697
Peromyscus attwateri (Allen, 1895) [37]	TK 116711	1,082,677	122	16,679
Peromyscus aztecus (Saussure, 1860) [32]	TK 93385	2,568,999	50	16,692
Peromyscus crinitus (Merriam, 1891) [27]	TK 119629	1,389,297	65	16,703
Peromyscus megalops (Merriam, 1898) [48]	TK 93381	1,444,002	33	16,925
Peromyscus mexicanus (Sassure, 1860) [32]	TK 93144	1,524,641	243	16,860
Peromyscus pectoralis (Osgood, 1904) [36]	TK 148816	1,771,386	238	16,833
Peromyscus polionotus (Wagner, 1984) [33]	TK 24228	1,059,501	30	16,413
Podomys floridanus (Chapman, 1889) [49]	TK 92501	1,738,547	62	16,517
Reithrodontomys mexicanus (Sassure, 1860) [32]	TK 104488	1,576,095	190	16,606
Sigmodon hispidus (Say and Ord, 1825) [26]	160,320	1,055,119	27	16,394
Mean		1,655,746	114	16,664

sequences were deposited in GenBank under the accession numbers KY707299–707312. Genome coverage was estimated using bedtools genomecov [25] in combination with mapping of the processed reads to each MITObim assembly with BWA via a custom script (https://github.com/KevinAMSullivan/Mitochondrial_Genomes/tree/MIRA).

Assembly validation

To test the reliability of mitogenome assemblies, we used two *cytb* genes per taxon from GenBank as controls (Additional file 1: Table S1), and worked under the assumption that our reference assembled *cytb* gene and those from GenBank should form monophyletic clades. We chose full length, non-identical entries from the selected species, aligned them with *cytb* from our assemblies, and used RAxML [26] to estimate the best tree from 1000 maximum likelihood (ML) searches. Support for each node was generated with bipartition frequencies from 10,000 bootstrap replicates. The GTR+GAMMA+I model of nucleotide substitution was used for both ML analyses.

Phylogenetic analysis of whole mitochondrial genomes

We concatenated the mitochondrial protein coding sequences and aligned them with Muscle [27]. That alignment is available as Additional file 2. Bayesian and ML phylogenetic analyses were accomplished using MrBayes [28] and RAxML [26], respectively. For the Bayesian analysis, four independent runs on five chains were implemented for 3,000,000 generations. We evaluated stability of the final tree by continuing to an average standard deviation of split frequency less than 0.01. The data were also partitioned by codon. No substitution model was specified as a prior to let the program search across all possible models to determine the most appropriate model of evolution. We then used the selected model, GTR+GAMMA, for our ML analysis, for which we implemented 1000 bootstrap replicates. In both analyses, the *A. montensis* and *S. hispidus* [29] mitogenomes were specified as outgroups when drawing the trees as Neotominae and Sigmodontinae are sister subfamilies whose relationships have been supported by previous analyses [30, 31]. Our unrooted tree supports this relationship. The implementation scripts for both analyses are available on github (https://github.com/KevinAMSullivan/MIRA-MitoBim/tree/Phylogenies).

Results

We sequenced and assembled whole mitogenomes of 14 rodents in the subfamily Neotominae and one member of Sigmodontinae, *Sigmodon hispidus*. Taxon sampling included seven genera and six of the 13 species groups within *Peromyscus* [16]. Genome assembly data, including accession numbers, read coverage, and mean read totals across all taxa, are listed in Table 1.

Each assembled mitogenome comprises 22 tRNA, 2 rRNA, and 13 protein coding regions, totaling 37 genes. Although most genes are transcribed from the positive (heavy) strand, several genes, mainly tRNAs but also including *nad6*, are transcribed from the negative (light strand). Additionally, the 3′ end of *atp6* overlaps with the 5′ end of *atp8*, a common characteristic in mitogenomes. Noncoding regions typical of mitogenomes are present, including a control region downstream of *cytb*. As these mitogenomes were not closed assemblies, such variation in mitogenome size is thought to be due to the highly variable and often heteroplasmic control region.

Validation of MITObim assembled and MITOS-annotated *cytb* took three forms. First, reconstructed genes clustered with loci amplified and sequenced using more traditional methods (Additional file 3: Figure S1), suggesting that the remaining protein coding sequences were accurately assembled and annotated for each taxon. Second, differences that were present are within acceptable ranges for such rapidly evolving loci. Despite forming monophyletic clades for each species, some of the *cytb* genes differed from those in GenBank. For example, *cytb* from our *P. crinitus* [32] mitogenome exhibited 43 and 44 differences, respectively, when compared to their GenBank counterparts, i.e. ~96% identity. Divergences in the range may be expected for such a rapidly evolving locus within a species [33]. Third, we have high confidence in our base calls. For example, average coverage of our *cytb* loci is 142× (data not shown), and the average percentage of those reads indicating any given nucleotide is 99.3%. Given such high support, as well as the fact that *P. crinitus* is the top BLAST hit to the gene (96% identify, 0.0 E value), we have confidence in the veracity of our *cytb* sequences over alternate hypotheses for such sequence dissimilarity. Similar results were observed for *cytb* sequences in all taxa (Additional file 4: Table S2). The single exception was for *Neotoma mexicana*. The best match (100%) for our reconstructed *cytb* sequence was to *N. isthmica*. However, *N. isthmica* is closely related to *N. mexicana* [34], and the second best hit was to *N. mexicana* (99%), confirming its validity as an outgroup [35].

ML and Bayesian phylogenies inferred from mitochondrial protein coding genes recovered identical topologies (Fig. 1). This topology is similar to recent molecular phylogenies. First, *S. hispidus, N. mexicana, I. pirrensis* [36], and *R. mexicanus* [37] are positioned outside of *Peromyscus*. Unlike previous phylogenies however, high posterior probabilities are found at these nodes. In fact, every node, save that linking *P. crinitus* and *P. polionotus* [8], has high support. Second, our analyses indicated a paraphyletic *Peromyscus*. Finally, *Isthmomys* and *Reithrodontomys* comprise a clade sister to what is currently considered to *Peromyscus* and its

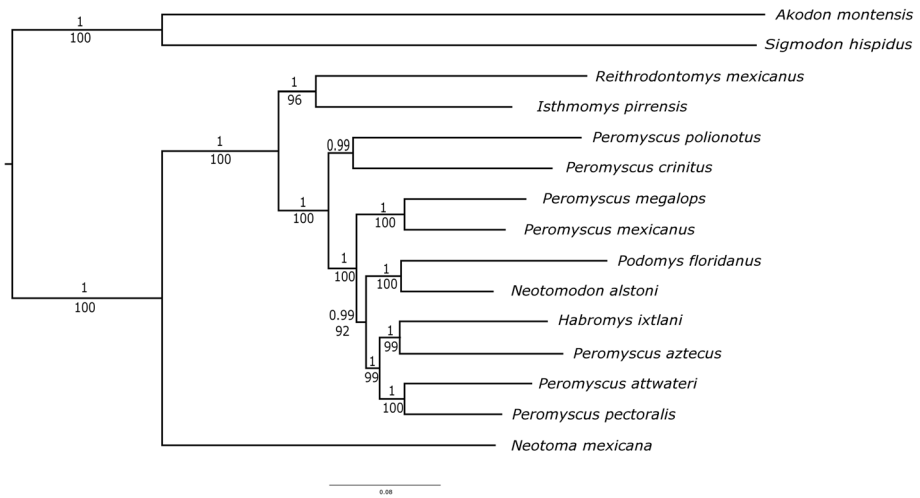

Fig. 1 Phylogeny inferred by the partitioned maximum likelihood analysis. The topology of the Bayesian tree was identical. *A. montensis* was included as a previously sequenced outgroup. Bootstrap values (*above*) and posterior probabilities (*below*) are provided at all internodes

affiliated genera. This pairing is of note, as both genera have shown conflicting relationships depending on the marker used [7, 16, 17, 38].

Our phylogeny using whole mitogenomes largely mirrors that of Platt et al. [7], save the quartet of *H. ixtlani* [39], *P. aztecus* [37], *P. pectoralis* [40], and *P. attwateri* [41]. Platt et al. found *P. attwateri* and *P. aztecus* to form a clade, with *P. pectoralis* as sister to this grouping and *H. ixtlani* being the most closely related species to a clade containing all three. The whole mitogenome tree is in partial agreement with Bradley et al. [16] with regard to these same four species. Our close pairing of *P. attwateri* and *P. pectoralis* matches, but their phylogeny places *P. aztecus* as a sister group to a clade that encompasses *H. ixtlani*, *P. attwateri*, and *P. pectoralis*.

Discussion

Regardless of the differences or similarities, our tree (Fig. 1) provides support for previously unsupported nodes within the *Peromyscus* phylogeny. Previous trees were largely resolved, but lacked significant support at key nodes, mainly those in middle regions of the tree that identify relationships among species groups [7, 16, 17]. Platt et al. [7] provided substantial support (posterior probabilities ≥ 95% and bootstrap values ≥ 85) at nodes grouping the *crinitus*, *eremicus*, and *californicus* species groups; the *mexicanus, megalops,* and *melanophrys* species groups; and the *aztecus* and *boylii* species groups. However, support in the Bayesian mitogenome phylogeny has posterior probabilities (> 0.95) at every node and bootstrap support (> 90) in the ML phylogeny at all nodes save one.

Two branches of our inferred phylogeny had not yet been reinforced by any molecular analysis. The first concerns *H. ixtlani*, *P. aztecus*, *P. attwateri*, and *P. pectoralis*. Although some analyses had suggested a close relationship among these taxa, no node in this clade had received substantial support in any previous molecular phylogeny. Our analysis is the first to provide such support for these relationships.

The second branch of interest suggests a close relationship between *Isthmomys* and *Reithrodontomys*. Although no phylogenetic analysis ever suggested the genus *Reithrodontomys* should be nested within *Peromyscus*, *Isthmomys* had previously been subsumed as a subgenus [13]. The pairing is counterintuitive given their morphological differences. Although both occupy the same geographic region, there are obvious incongruities in size. *Isthmomys pirrensis* can reach well over 100 g (averaging 140 g in one study) and 300 mm [42, 43], whereas rodents in *Reithrodontomys* are much smaller. *R. mexicanus* ranges from 167 to 190 mm in length [19], and a collection of different surveys gave a range from 7.9–9.5 g [42]. Although their pairing has been previously suggested [7, 16, 17], our analysis is the first to provide high support values.

One major weakness of our study is its lack of complete taxon sampling. This makes comprehensive phylogenetic reconstruction uncertain due to missing critical (i.e. representing additional ingroups) taxa. The addition of excluded taxa, especially *Osgoodomys* and *Megadontomys*, should provide a more comprehensive view of phylogenetic relationships within the genus. That being said, the high support values we recover suggest that the relationships at the deeper nodes are valid.

The *Peromyscus* phylogeny has been studied repeatedly and revised often, using data from several sources [42]. This latest attempt, using next-generation sequencing, continues to suggest paraphyly. Indeed, every molecular phylogeny has been unequivocal in suggesting paraphyly.

Given this observation, addressing the status of genera subsumed within *Peromyscus* should be seen as a priority within rodent taxonomy.

Aside from acknowledging paraphyly, future work on *Peromyscus* phylogenetics should focus on further clarifying the true phylogeny via more taxa and markers. The inclusion of additional mitogenomes will elucidate relationships within the genus to provide increased detail. However, despite their advantages, mitochondrial loci are imperfect markers. Mitochondria genomes are susceptible to incomplete lineage sorting (ILS) and introgression [44]. Hybrids, especially asymmetric hybrids, exemplify a similar problem in that they may all have the same mitochondrial DNA and species boundaries in those cases would not be reflected [45–47]. Supplementing the mitochondrial phylogeny with large numbers of nuclear markers such as ultraconserved elements (UCEs) [48, 49] or retrotransposons [50, 51] is an important next step in understanding phylogenetic relationships within *Peromyscus*.

Conclusions

Here, we present analyses of whole mitochondrial genomes, for 14 species, ten of which are *Peromyscus* or close relatives. The data yield phylogenies with significant support at previously unsupported nodes, particularly the pairing of *Isthmomys* and *Reithrodontomys*, but also suggest paraphyly within the genus that could be resolved by elevating monophyletic groups to genera or subsuming currently recognized genera as subgenera. Our analyses provide evidence that additional data will help clarify the evolutionary history of this genus.

Additional files

Additional file 1: Table S1. Title of data: Cytochrome b GenBank information. Taxa names and accession numbers for cytochrome b genes downloaded from GenBank in Additional file 3: Figure S1. (XLS 28 kb)

Additional file 2: Whole mitochondrial exomes. Description: Alignment of the protein coding genes of each assembled mitogenome along with *Akodon montensis*, downloaded from GenBank. (TXT 257 kb)

Additional file 3: Figure S1. Cytochrome b phylogenetic validation. ML tree of the assembled cytochrome b sequences compared with cytochrome b sequences downloaded from GenBank. These results showed if the sequences from our data correlated with known *cytb* sequences. (PNG 60 kb)

Additional file 4: Table S2. v2. Cytochrome b blast validation. Best hits to GenBank entries for cytochrome b genes from assembled mitochondrial genomes. For *N. mexicana*, the best hit was to a closely related species, with the second best hit to *N. mexicana*. (XLS 38 kb)

Abbreviations

Cytb: Cytochrome-b; Mitogenome: Mitochondrial genome; ML: Maximum likelihood

Acknowledgements

The Natural Sciences Research Laboratory at the Museum of Texas Tech University kindly provided tissues via the Genetic Resources Collection. Additional thanks goes to RTL Genomics for sequencing efforts.

Funding

This work was supported by the National Science Foundation, DEB-1355176. Additional support was provided by College of Arts and Sciences at Texas Tech University.

Availability of data and materials

The raw reads have been uploaded to NCBI's SRA via accession numbers SAMN04393360–04393374. A nexus file used for phylogenetic analysis is available through Dryad, doi:10.5061/dryad.c2j05.

Authors' contributions

DAR conceived the project, aided in mitochondrial genome assembly and assisted in writing. RDB provided essential information regarding *Peromyscus* and related taxa, devised the taxon sampling scheme and assisted writing the manuscript. RNP aided in the phylogenetic analyses. KAMS analyzed the data and led writing of the manuscript. All authors read, edited, and approved the final manuscript.

Ethics approval and consent to participate

No ethical approval was required from an institutional or national ethics review board.

Consent for publication

Not applicable.

Competing interests

The authors declare they have no competing interests.

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Received: 16 December 2016 Accepted: 2 August 2017
Published online: 18 August 2017

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