Polymorphic duplicate genes and persistent non-coding sequences reveal heterogeneous patterns of mitochondrial DNA loss in salamanders

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Abstract

Background: Mitochondria are the site of the citric acid cycle and oxidative phosphorylation (OXPHOS). In metazoans, the mitochondrial genome is a small, circular molecule averaging 16.5 kb in length. Despite evolutionarily conserved gene content, metazoan mitochondrial genomes show a diversity of gene orders most commonly explained by the duplication-random loss (DRL) model. In the DRL model, (1) a sequence of genes is duplicated in tandem, (2) one paralog sustains a loss-of-function mutation, resulting in selection to retain the other copy, and (3) the non-functional paralog is eventually deleted from the genome. Despite its apparent role in generating mitochondrial gene order diversity, little is known about the tempo and mode of random gene loss after duplication events. Here, we determine mitochondrial gene order across the salamander genus Aneides, which was previously shown to include at least two DRL-mediated rearrangement events. We then analyze these gene orders in a phylogenetic context to reveal patterns of DNA loss after mitochondrial gene duplication.

Results: We identified two separate duplication events that resulted in mitochondrial gene rearrangements in Aneides; one occurred at the base of the clade tens of millions of years ago, while the other occurred much more recently (i.e. within a single species), resulting in gene order polymorphism and paralogs that are readily identifiable. We demonstrate that near-complete removal of duplicate rRNA genes has occurred since the recent duplication event, whereas duplicate protein-coding genes persist as pseudogenes and duplicate tRNAs persist as functionally intact paralogs. In addition, we show that non-coding DNA duplicated at the base of the clade has persisted across species for tens of millions of years.

Conclusions: The evolutionary history of the mitochondrial genome, from its inception as a bacterial endosymbiont, includes massive genomic reduction. Consistent with this overall trend, selection for efficiency of mitochondrial replication and transcription has been hypothesized to favor elimination of extra sequence. Our results, however, suggest that there may be no strong disadvantage to extraneous sequences in salamander mitochondrial genomes, although duplicate rRNA genes may be deleterious.

Keywords: Duplication-random loss model, Gene rearrangement, Pseudogene, Mitochondrial genome evolution, Repetitive DNA, Aneides

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Background
Mitochondria are the site for the citric acid cycle and oxidative phosphorylation (OXPHOS), the final steps of ATP synthesis via cellular respiration. These intracellular organelles have their own genome, which in metazoans is a small, circular molecule averaging 16.5 kb in length [1, 2]. Metazoan mitochondrial genomes typically encode 13 electron transport proteins, two rRNAs, and 22 tRNAs [2]. This gene content is evolutionarily conserved, likely reflecting the fitness advantage of having the proteins involved in primary electron transfer (i.e. redox chemistry) co-localized with the genes that encode them, allowing direct redox control of gene expression [3]. Other hypotheses to explain why this conserved set of genes has remained in a distinct mitochondrial genome, rather than being transferred to the nuclear genome, include (1) their role in redox damage sensing [4], and (2) the hydrophobicity of their protein products, which precludes their import back into the mitochondria [5]. In contrast to such evolutionarily conserved gene content, metazoan mitochondrial genomes show a diversity of gene orders that result from gene rearrangement [1, 6–8]. Many mitochondrial gene orders are hypothesized to reflect rearrangement by neutral processes [9, 10], although tRNA positions within the vertebrate mitochondrial genome have been hypothesized to reflect selection for translational capacity [11].

The most commonly invoked model for mitochondrial gene rearrangement is the duplication-random loss (DRL) model. In the DRL model, a sequence of genes is initially duplicated in tandem. Following this initial duplication, one paralog sustains a loss-of-function mutation, resulting in strong selective pressure to retain the other gene copy functionally intact. The non-functional paralog continues to accumulate mutations and is eventually removed from the genome by deletion. Depending on which gene copy is ultimately deleted, this process may restore the original gene order or result in a novel gene order [12, 13].

The DRL model has been invoked to explain the variation in mitochondrial gene order observed in diverse animal clades including lizards, insects, and caecilians [8, 14–18]. Evidence for the DRL model includes the presence of mitochondrial pseudogenes that can be identified as paralogs of rearranged genes [19]. Despite its apparent role in generating mitochondrial gene order diversity, little is known about the tempo and mode of random gene loss after duplication events. For example, does loss of gene duplicates occur slowly, reflecting rare deletion events and/or their fixation solely by genetic drift? Alternatively, does loss of gene duplicates occur quickly, reflecting frequent deletion events and/or positive selection for deletions?

Selection would play a role in the deletion of duplicate genes if their presence in the genome negatively impacted mitochondrial function. This could occur by increasing the time required to complete transcription of mitochondrial genes and/or replication of the mitochondrial genome [20, 21]. It could also occur by increasing susceptibility to harmful gain-of-function mutations [22] or by shifting genes to a more vulnerable position along the mutation gradient [23]. In such cases, selection would favor the elimination of all extraneous sequence, irrespective of its identity. Alternatively, expression of mitochondrial pseudogenes could result in the production of non-functional proteins [24]. Finally, expression of additional functional gene copies could result in changes to the stoichiometry of mitochondrial proteins [25, 26]. In these latter two cases, selection would favor mutations that delete these specific sequences or otherwise diminish their contribution to the mitochondrial proteome.

Much of our current understanding of mitochondrial gene order variation comes from comparisons across highly divergent clades such as bird families and amphibian orders, where rearrangements have been fixed deep in evolutionary time [1, 19, 27, 28]. In these systems, gene loss occurred far enough in the past that little or no trace of duplicated sequences remain in the genomes of extant taxa; this precludes the study of the tempo and mode of paralog loss. In contrast, the study of more recent gene rearrangements — where duplicates and intermediate stages of loss are polymorphic within species or fixed between closely related lineages — allow the study of random loss “in action.” Such studies remain relatively rare [29, 30].

Salamanders, an amphibian clade of 708 species [31], include both typical vertebrate mitochondrial genomes and a number of independently derived gene rearrangements consistent with origination through the DRL model [19]. Because this mitochondrial genomic diversity is unusual among vertebrates, salamanders are a promising taxon for identifying recent gene rearrangements appropriate for studies of tempo and mode of duplicate gene loss. Within salamanders, the six-species genus Aneides includes two species — A. hardii and A. flavipunctatus — that have distinct, derived mitochondrial gene orders resulting from gene duplications [19, 32]. The duplication in A. flavipunctatus encompassed protein coding genes ND6 and cyt b, tRNA genes E, T, and P, and an intergenic spacer found in diverse salamander clades [19, 28, 33, 34]. The mitochondrial genome of A. hardii shows evidence of having undergone two duplication events. The first duplication involves a nearly identical region of the genome duplicated in A. flavipunctatus, suggesting that it may have occurred in the common ancestor of these two species. The second duplication in A. hardii involves part of the first duplication as well as the control region, 12S, 16S, ND1, and tRNAs F, V, and L [19].

For this study, we examined mitochondrial gene order in multiple population-level lineages of Aneides in a
phylogenetic context to pinpoint the evolutionary origins of gene duplications. Our goals were to (1) determine whether the similar duplications in *A. hardii* and *A. flavipunctatus* are, in fact, a synapomorphy for some/all species in the genus, (2) determine whether variation in gene order exists between any closely related lineages or within species, and (3) examine the tempo and mode of duplicate gene loss. We identified two separate duplication events that resulted in mitochondrial gene rearrangements. One such rearrangement occurred at the base of the clade tens of millions of years ago, while the other occurred much more recently (i.e. within a single species), resulting in gene order polymorphism and paralogs that are readily identifiable. We demonstrate that near-complete removal of duplicate rRNA genes has occurred since the recent duplication event, whereas duplicate protein-coding genes persist as pseudogenes and duplicate tRNAs persist as functionally intact paralogs. In addition, we show that non-coding DNA duplicated at the base of the clade has persisted across species for tens of millions of years. Taken together, our results suggest that, although there may be no strong disadvantage to extraneous sequences in salamander mitochondrial genomes, duplicate rRNA genes may be deleterious.

**Methods**

**Taxon selection and sampling strategy**

Our first objective was to estimate a comprehensive phylogeny for *Aneides* to provide the evolutionary context for analysis of mitochondrial gene rearrangements. To this end, we collected nuclear gene sequence data from four individuals for all six species in the clade (Table 1). We included additional samples of *A. flavipunctatus* because previous studies have documented high levels of divergence across populations in this species [35–37]. We also included an additional sample of *A. hardii* because a previous study identified a novel gene order in this species [19]. We included four related plethodontid salamander species as outgroups (*Ensatinia eschscholtzii*, *Hydromantes brunus*, *Plethodon elongatus*, and *Desmognathus fuscus*) [32, 38, 39]. All work was completed in compliance with Colorado State University’s Institutional Animal Care and Use Committee (Protocol 08-184A-02).

Our second objective was to compare gene order both within and among species of *Aneides* to pinpoint the evolutionary origins of the gene duplications underlying gene rearrangement. To this end, we collected mitochondrial genome sequence data from individuals that represent nine divergent population-level lineages of *Aneides* (Table 2). Wherever possible, we collected mitochondrial genome sequence data from individuals sampled in the phylogenetic analysis to maximize overlap between the two datasets. As above, we sampled multiple individuals from both *A. flavipunctatus* and *A. hardii*.

**DNA amplification and sequencing for phylogenetic analyses**

For each sample, we extracted total genomic DNA from either flash-frozen or RNAlater-preserved (Qiagen) liver or tail tissue using the Puregene kit (Qiagen) according to the manufacturer’s protocols. We amplified a portion of the nuclear-encoded genes BDNF, POMC, and RAG1 using the Polymerase Chain Reaction (PCR), which produced amplicons of 720 bps, 489 bps, and 837 bps for each gene, respectively. Primers and PCR reaction conditions were obtained from previously published studies [38]. Briefly, approximately 10 ng of genomic DNA was amplified using 0.25 mM dNTPs, 0.5 μM of each primer, 0.5 U Taq, 25 μM MgCl₂, and 10× reaction buffer (no MgCl₂) in a 12.5 μL reaction volume. Genes were PCR-amplified for 35 cycles (95 °C 45 s, 56–58 °C 1 min, and 72 °C 1 min). All PCR products were electrophoresed on a 1% agarose SB gel stained with Gel Red (Biotium Inc.) and visualized under ultraviolet light. PCR products were purified using ExoSAP-IT (Affymetrix USB products). PCR products were sequenced in both directions by the University of Chicago Comprehensive Cancer Center’s DNA sequencing and genotyping facility using direct double-strand cycle sequencing with Big Dye v3.1 chemistry (Perkin-Elmer) and an ABI 3730 automated sequencer. Contigous DNA sequences were aligned and edited using Geneious version R7 (Biomatters), and multiple sequence alignments were initially generated using Muscle v.3.6 [40] and subsequently verified by eye. The open reading frames were also verified for all genes using Geneious R7. All sequences are deposited in GenBank (accession numbers MF946473-MF946547).

**Phylogenetic analyses**

We estimated phylogenetic relationships within the genus *Aneides* using both Maximum Likelihood (ML) and Bayesian Inference (BI) on the aligned, concatenated nuclear-gene dataset (BDNF, POMC, and RAG1). We evaluated multiple data partitioning strategies in order to incorporate evolutionary information specific to each gene and codon position. We determined three appropriate data partitions using PartitionFinder v.1.1.1 [41]: (1) codon positions 1 and 2 for BDNF, (2) codon positions 1 and 2 for POMC and RAG1 combined, and (3) codon position 3 for all three genes combined. Using the Akaike Information Criterion to select the best model of nucleotide substitution, we determined JC, F81 + I, and GTR + Γ for each partition, respectively.

We conducted partitioned Bayesian analyses in MrBayes v.3.2 [42] for the concatenated nuclear-gene dataset. We conducted two independent searches consisting of three
we heated and one cold Markov chain for 10 million generations with every 1000th sample retained. A rate multiplier was used to allow substitution rates to vary among partitions, and default priors were applied to all model parameters. We assessed convergence of the MCMC using several diagnostics. We viewed trace plots of tree-lnL values and other parameters in Tracer v.1.5 [43]. Trees sampled prior to stationarity (i.e. the first 1000 tree samples) were considered as burn in and were discarded. To determine whether the two independent runs converged on similar results, we examined the split standard deviation for -lnL tree values among chains; values <0.01 were taken to indicate convergence. We also used the program Are We There Yet? (AWTY) [44] to compare changes in the posterior probabilities of split frequencies across the independent runs.

Partitioned Maximum Likelihood analyses were conducted using RAxML v.7.2.5 [45] under the GTR + Γ model of nucleotide substitution for all data partitions identified in the Bayesian analysis. Support values for the inferred relationships were obtained from 1000 nonparametric bootstrap pseudoreplicates.

Mitochondrial genome sequencing and assembly
We obtained cellular DNA shotgun sequence data, which includes both nuclear and mitochondrial DNA, using the

| Species                | Voucher | State: County | BDNF   | POMC   | RAG1    | Source       |
|------------------------|---------|---------------|--------|--------|---------|--------------|
| Aneides aeneus         | DH74978 | GA: Chatooga  | MF946473 MF946498 MF946523 | This study |
| Aneides aeneus         | DH74985 | GA: Chatooga  | MF946474 MF946499 MF946524 | This study |
| Aneides aeneus         | DH77583 | KY: Letcher   | MF946475 MF946500 MF946525 | This study |
| Aneides aeneus         | DH77584 | KY: Letcher   | MF946476 MF946501 MF946526 | This study |
| Aneides ferreus        | MVZ219942 | CA: Siskiyou  | MF946477 MF946502 MF946527 | This study |
| Aneides ferreus        | MVZ219953 | OR: Douglas  | MF946478 MF946503 MF946528 | This study |
| Aneides ferreus        | MVZ219958 | OR: Linn     | MF946479 MF946504 MF946529 | This study |
| Aneides ferreus        | RCT545  | CA: Del Norte | MF946480 MF946505 MF946530 | This study |
| Aneides flavipunctatus | AGC299  | CA: Santa Cruz| MF946481 MF946506 MF946531 | This study |
| Aneides flavipunctatus | MVZ219973 | CA: Siskiyou  | EU275895 EU275849 EU275809 Vieites et al. [38] |
| Aneides flavipunctatus | MVZ219977 | CA: Sonoma   | MF946482 MF946507 MF946532 | This study |
| Aneides flavipunctatus | RAC080  | CA: Mendocino | MF946483 MF946508 MF946533 | This study |
| Aneides flavipunctatus | RCT481  | CA: Shasta    | MF946484 MF946509 MF946534 | This study |
| Aneides flavipunctatus | RLM172  | CA: Del Norte | MF946485 MF946510 MF946535 | This study |
| Aneides hardii         | MVZ226110 | NM: Otero    | EU275857 EU275811 EU275780 Vieites et al. [38] |
| Aneides hardii         | RAC020  | NM: Lin      | MF946486 MF946511 MF946536 | This study |
| Aneides hardii         | RAC025  | NM: Otero    | MF946487 MF946512 MF946537 | This study |
| Aneides hardii         | RAC042  | NM: Lin      | MF946488 MF946513 MF946538 | This study |
| Aneides hardii         | RAC054  | NM: Lin      | MF946489 MF946514 MF946539 | This study |
| Aneides lugubris       | MVZ230722 | CA: San Diego | MF946490 MF946515 MF946540 | This study |
| Aneides lugubris       | MVZ249828 | CA: Mariposa | MF946491 MF946516 MF946541 | This study |
| Aneides lugubris       | RAC060  | CA: Santa Clara| MF946492 MF946517 MF946542 | This study |
| Aneides lugubris       | RAC081  | CA: Mendocino | MF946493 MF946518 MF946543 | This study |
| Aneides vagrans        | HBS26688 | CA: Mendocino | MF946494 MF946519 MF946544 | This study |
| Aneides vagrans        | MVZ219886 | CA: Del Norte | MF946495 MF946520 MF946545 | This study |
| Aneides vagrans        | MVZ220091 | CA: Humboldt | MF946496 MF946521 MF946546 | This study |
| Aneides vagrans        | RAC073  | CA: Humboldt  | MF946497 MF946522 MF946547 | This study |
| Ensatina eschscholtzii | MVZ236171 | CA: San Luis Obispo | EU275862 EU275816 EU275785 Vieites et al. [38] |
| Desmognathus fuscus    | MVZ224931 | MA: Franklin | EU275858 EU275812 EU275781 Vieites et al. [38] |
| Hydromantes brunus     | MVZ238576 | CA: Mariposa | EU275871 EU275825 EU275790 Vieites et al. [38] |
| Plethodon elengatus    | MVZ220003 | CA: Del Norte | EU275882 EU275836 AY650120 Wiers et al. [58]; Vieites et al. [38] |
Illumina MiSeq sequencing platform for a total of eight individuals representing all six species of *Aneides*, with additional sampling within *A. hardii* and *A. flavipunctatus* (Table 2). Illumina sequencing libraries were produced for each sample using the IntegenX PrepX-DNA 24 library prep kit (IntegenX). Libraries were pooled equimolar for sequencing, allocating 1/3 of a 2 × 250 cycle MiSeq run (Illumina) for all samples. Library preparation and sequencing were performed by the University of Idaho Institute for Bioinformatics and Evolutionary Studies (IBEST) Genomics Resources Core facility. All sequences are deposited in the GenBank SRA (PRJNA407969). We screened all shotgun reads to eliminate sequencing adapters, identify and remove contaminants, and trim reads based on quality scores using the bioinformatics pipeline “SeqyClean.py” provided by the IBEST Computational Resources Core. We assembled the remaining shotgun reads into contigs using Newbler v.2.6. We also included published shotgun sequence data for an additional sample of *A. flavipunctatus* [46], resulting in shotgun datasets for nine total individuals.

For each of the nine shotgun sequence datasets, we identified contigs of mitochondrial sequences using tBLASTx; the mitochondrial genome reference sequences of *A. hardii* (AY728226) and *A. flavipunctatus* (AY728214) were used as queries to BLAST against the assembled contigs with an e-value cutoff of 1e-5. Depending on the focal individual, complete (or nearly complete) mitochondrial genomes were represented by one to eight contigs. Several genome assemblies included gaps across regions of low/non-existent sequencing coverage. To close these gaps, as well as verify the assembly of multiple contigs, we developed genome-specific primers for PCR amplification and Sanger sequencing. PCR amplification and Sanger sequencing were performed as described above for the nuclear genes used in the phylogenetic analysis.

### Mitochondrial gene order identification

We annotated mitochondrial genome sequences for each individual using Geneious R7. rRNAs and tRNAs were identified based on sequence similarity with published genomes. All 13 protein-coding sequences were verified by eye for appropriate vertebrate mitochondrial open reading frames and stop codons. For each sample, we determined mitochondrial gene order based on our genome annotations and alignment to reference mitochondrial genomes. We also identified pseudogenes based on sequence similarity to inferred functional gene copies as well as position relative to annotated gene sequences.

### Gene loss analysis

Within *A. hardii*, individuals have different mitochondrial genome haplotypes; one haplotype has additional duplicate genes not present in the other. We thus focused on *A. hardii* to study random gene loss “in action.” To this end, we measured (1) substitutions within duplicate gene sequences and their effects on open reading frame, if applicable, and (2) deletions within duplicate sequences. To perform these measures, we created pairwise alignments of pseudogene sequences and their intact paralogs from within the same genome using ClustalW; the intact paralog serves as a proxy for the sequence immediately following duplication. We scaled deletions of duplicate sequences by substitutions, as has been done in other studies, because the limited salamander fossil record precludes precise estimates of absolute rates; salamander divergence date estimates have large 95% confidence intervals [28, 38, 47–49]. More specifically, we estimated the average number of mitochondrial nucleotide

| Species          | Voucher       | Number of reads | Total base pairs | Mitochondrial contigs | Mitochondrial genome annotation (kb) |
|------------------|---------------|----------------|------------------|-----------------------|--------------------------------------|
| *A. aeneus*      | DH77584       | 1,648,165      | 510,564,223      | 1                     | 16.8                                 |
| *A. ferreus*     | RCTS45        | 1,181,785      | 365,727,056      | 5                     | 16.4                                 |
| *A. flavipunctatus* | MVZ219977    | 1,165,899      | 349,989,009      | 5                     | 17.1                                 |
| *A. flavipunctatus* | RLM172       | 1,044,399      | 308,615,225      | 4                     | 17.5                                 |
| *A. hardii*      | AYT728214     | –              | –                | 1                     | 20.2                                 |
| *A. hardii*      | RAC25         | 931,319        | 284,267,433      | 6                     | 18.7                                 |
| *A. hardii*      | RAC42         | 1,708,754      | 507,960,897      | 5                     | 17.9                                 |
| *A. hardii*      | RACS4         | 1,280,531      | 378,635,736      | 1                     | 17.0                                 |
| *A. hardii*      | AYT728226     | –              | –                | 1                     | 22.2                                 |
| *A. lugubris*    | MVZ249828     | 728,185        | 199,882,489      | 7                     | 17.0                                 |
| *A. vagrans*     | MVZ220991     | 832,332        | 252,211,876      | 5                     | 16.5                                 |

Table 2: Individuals used in this study and their voucher numbers, total number of Illumina MiSeq reads, total number of base pairs, total number of contigs used in mitochondrial genome assembly, and sequence length (kb) of mitochondrial genome annotation.
substitutions per site ($D_{XY}$) between the two different A. hardii gene orders. We generated multiple sequence alignments including two individuals representing each gene order for each of the protein-coding genes using translational ClustalW alignment [50] and verified open reading frames and stop codons by eye (see Additional file 1). We then estimated $D_{XY}$ for a concatenated dataset of all 13 mitochondrial protein-coding genes using DnaSP v 5.10.1 [51], which uses the Jukes and Cantor’s model of nucleotide substitution to estimate genetic divergence between populations and is likely sufficient for such shallow divergences.

**Results and discussion**

**Aneides phylogeny estimated from nuclear genes**

Despite recent work on salamander phylogenies [38, 39, 52, 53], the phylogenetic relationships within Aneides were previously unknown. From our analysis of 2046 bp from three nuclear genes (BDNF, POMC, and RAG1), we estimated a resolved phylogeny for the genus that includes all six species represented by several divergent lineages (Fig. 1). Our results show A. aeneus to be sister to the rest of the clade; A. aeneus is found in eastern North America, while the remaining five species are only found in western North America. The two Pacific Northwest species — A. ferreus and A. vagrans — are sister taxa, which together form the clade sister to A. flavipunctatus. Aneides flavipunctatus + A. ferreus + A. vagrans is sister to A. lugubris; these four species are found in the coastal regions of Western North America. Aneides hardii, which is restricted to a few isolated mountaintops in southern New Mexico, is sister to the coastal clade. Our results are consistent with the relationships suggested by other studies that included some species of Aneides [32, 38, 39, 53].

**Mitochondrial gene order**

We identified mitochondrial gene orders for nine Aneides samples using shotgun sequence data. The total amount of sequence and average read length varied among samples, but overall, the datasets represent ~0.5–1.5% of the nuclear genomes at 1× coverage (Table 2). We produced nearly complete mitochondrial genome sequences from a single contig for two samples. For each of the remaining samples, we produced four to eight contigs that represent large fractions of the mitochondrial genome. We eliminated several assembly gaps using PCR; however, several regions of the genome remain absent from our assemblies. Specifically, the control region and the region between

| Duplication & Rearrangement |
|-----------------------------|
| 0.01 substitutions/site      |

Fig. 1 Nuclear phylogeny of Aneides with nodes labeled with Maximum Likelihood bootstrap support (MLBP) above and Bayesian posterior probabilities (BPP) below. Both analyses resulted in congruent tree topologies, depicted above, with highly supported nodes (MLBP >99 and BPP > 0.99) denoted with an asterisk, while weakly supported nodes (MLBP <70 or BPP < 0.70) are not labeled. Aneides hardii branch lengths are all <0.00001. Orange bars indicate inferred mitochondrial gene duplication and rearrangement events.
CYTB and ND6 were difficult to assemble, likely reflecting the highly repetitive nature of these two regions [19]. Despite these gaps, our contigs include all 37 mitochondrial genes for each individual; thus, we were able to infer gene order from all nine individuals with confidence. We combined these data with previously published genomes from two additional Aneides individuals (one A. hardii and one A. flavipunctatus) for a total of 11 focal Aneides mitochondrial genome sequences (Table 2).

We found evidence of gene rearrangement, relative to the typical vertebrate mitochondrial gene order, in all 11 mitochondrial genome sequences. The mitochondrial gene order in A. aeneus, A. lugubris, A. vagrans, A. ferreus, all sampled lineages of A. flavipunctatus, and a subset of the sampled lineages of A. hardii includes a gene order exchange between ND6 and CYTB as well as the accumulation of repetitive, non-coding DNA in this region (Fig. 2a). This pattern is consistent with (1) an initial duplication of ND6, trn-E, CYTB, trn-T, an intergenic spacer, and trn-P, followed by (2) complete excision of one copy of ND6 + trn-E and decay of the remaining sequences to either recognizable pseudogenes (i.e. ψtrn-P) or unrecognizable tandem repeats [19]. Based on these results, we infer that this gene rearrangement is a synapomorphy for Aneides that occurred at the base of the clade (Fig. 1). Divergence date estimates for the split between Aneides and its sister taxon (i.e. Desmognathus + Phaeognathus) range from 36 to 69 mya, providing maximum and minimum dates for this duplication [38]. Thus, non-coding DNA resulting from the gene duplication at the base of Aneides has persisted in the genome of the descendant lineages for tens of millions of years.

**Gene order is polymorphic within A. hardii**

Of the four new A. hardii mitochondrial genomes we sequenced for this study, two individuals have the gene order we show here to be ancestral for Aneides (Fig. 2a). The other two individuals have the gene order previously reported for this species that reflects two duplication events [19] (Fig. 2b). Thus, within A. hardii, we identified a recent gene rearrangement where duplicates and intermediate stages of paralog loss are polymorphic within the species. Although our sampling found the gene orders in geographically isolated populations, our sample size is too small to infer that the populations are fixed for the difference in gene order.

**Patterns of gene loss following duplication in A. hardii**

Since the duplication event occurred within the A. hardii lineage, paralogous sequences within A. hardii genomes have begun to diverge from one another, accumulating point substitutions, deletions, and, to a lesser extent, insertions. Here, we summarize patterns of divergence between duplicates for different types of sequences.

**Protein-coding genes**

All of ND6 and at least some portion of ND1 were included in the A. hardii-specific duplication (Fig. 2a). The functional copy of ND6 in A. hardii is 519 bp long. In contrast, the putative pseudogene copy of ND6 we identified in RAC25 is 521 bp long, reflecting one insertion of a total length of two bp. We were unable to assemble this region of the genome in RAC20. Sequence identity between ND6 and ψND6 is 97%. ψND6 differs from the functional copy by ten point substitutions and two insertions, which result in several amino acid replacements to
stop codons. The longest predicted ORF within ψND6 is 139 amino acids long.

The functional copy of ND1 in A. hardii is 960 bp. ψND1 is a 676 and 685 bp fragment that shares 91.7% and 94.6% sequence identity with the first 682 bp of the functional ND1 in RAC25 and RAC20, respectively. Specifically, ψND1 differs from the functional copy in RAC20 by 30 point substitutions, a 2 bp deletion, and two insertions (5 bp total), which result in several amino acid replacements to stop codons. Similarly, ψND1 differs from the functional copy in RAC25 by 42 point substitutions, four deletions (11 bp total), and two insertions (5 bp total), resulting in several amino acid replacements to stop codons. The longest predicted ORF within ψND1 is 105 amino acids long in RAC25 and 205 amino acids in RAC20. The ψND1 sequences are 94.7% identical between the two individuals. Because we are unable to confirm the total amount of ND1 that was included in the initial duplication, we are unable to determine the total amount of deleted sequence in ψND1. If ND1 were only partially duplicated, it would have become a pseudogene immediately because of its shortened length.

**rRNA genes**

Both ribosomal RNA genes were included in the A. hardii–specific duplication. Duplicate copies of both rRNAs, as well as the intervening tRNA-V, have been almost completely deleted from genomes with the A. hardii–specific duplication. The functional copies of these three genes together total 2520 bp of contiguous sequence; however, only a 111-bp-long segment of unrecognizable sequence remains of the duplicates.

**tRNA genes**

tRNA-E, -P, -F, -V, and -L were included in the A. hardii–specific duplication. With the exception of one tRNA, we were able to identify all A. hardii–specific duplicated tRNA gene copies (except tRNA-V, located between the two rRNAs), which appear to largely remain functional based on intact stem-loop structures and the anticodon sequences. Both individuals with the A. hardii–specific duplication retain identical copies of tRNA-P and likely tRNA-E as well (although we were unable to identify a second copy in RAC20). tRNA-L paralog sequences differed by two single-basepair substitutions (one shared, one unique) in both individuals and share 97.7% sequence identity, even though each tRNA sequence is unique. For individuals with the A. hardii–specific duplication, tRNA-F sequences upstream of the intact rRNA genes are identical, while tRNA-F sequences adjacent to the pseudogenized rRNA genes are more variable and likely nonfunctional. The latter tRNA-F differs from the former by three bases (in RAC20) and five bases (in RAC25), with only two differences being shared by both individuals. The relatively short length of tRNA genes (~70 bp) limits the likelihood of accumulating substitutions and indels within these genes, which would allow for the conservation and functional persistence of tRNAs over other duplicated genes of greater length.

**Non-coding sequences**

Two regions of non-coding, repetitive DNA remain in all Aneides mitochondrial genomes from the ancestral duplication at the base of the clade. These regions are predicted to be the remnants of (1) a copy of CYTB + tRNA-T + IGS and (2) a copy of IGS + tRNA-P. Both were included in the A. hardii–specific duplication, and at least a portion of both sequences is retained in duplicate following the A. hardii duplication. We were unable to fully resolve these regions of the genome because they are repetitive and could not be assembled with confidence. Average sequencing read depth of these regions is at least double that of the rest of the genome, suggesting that these regions occur at least twice as often.

Taken together, our results show that, following the A. hardii–specific duplication event, different duplicate sequences have had dramatically different fates. Ribosomal gene copies have been almost completely deleted from the genome, and the sequence remaining bears no identity to the functional rRNA copies. In contrast, duplicate protein-coding sequences have not been deleted; specifically, ψND6 is now longer than the functional paralog, and ψND1 does not show evidence of substantial deletion. Both duplicated protein-coding sequences have decayed to pseudogenes and now encode short, presumably non-functional ORFs. Duplicate tRNAs (except trn-V) also show no evidence of deletion; tRNA-E, -P, and -L remain apparently functional, retaining 98–100% sequence similarity between the two paralogs.

**Loss of duplicate sequences in A. hardii occurred over a shallow evolutionary timescale**

We measured the number of substitutions between the A. hardii mitochondrial genomes that (1) retained the ancestral Aneides gene order, and (2) underwent the A. hardii–specific duplication. The average number of nucleotide substitutions per site between the two different mitochondrial gene orders found within A. hardii was low ($D_{XY} = 0.024$). This result is congruent with our nuclear gene phylogenetic analysis, which shows very low genetic divergence among A. hardii individuals (Fig. 1). Thus, all of the duplicate gene sequence decay/loss we identify in the A. hardii–specific duplication has occurred in the time taken to accumulate only 2.4% sequence divergence from the ancestral Aneides mitochondrial genomes.
Additionally, the accumulation of noncoding DNA would favor the elimination of extraneous sequence. The efficiency of mitochondrial replication and transcription emerge as an essential organelar genome, includes from its inception as a bacterial endosymbiont to its 56\textsuperscript{th} mutation gradient 54–56. Consistent with this overall trend, it has been hypothesized that selection for efficiency of mitochondrial replication and transcription would favor the elimination of extraneous sequence. Additionally, the accumulation of noncoding DNA can pose a mutational hazard [3–5, 57]. In Aneides, we do not see evidence that deletions of extraneous sequence are frequent targets of strong positive selection. Rather, we see (1) non-coding sequences persisting/expanding in length in all species of Aneides that originated from a duplication event tens of millions of years ago, (2) non-coding sequences (i.e. repeat sequences and pseudo-protein-coding genes) persisting in the A. hardii genomes that underwent a duplication event much more recently, and (3) full-length duplicate tRNAs persisting in A. hardii genomes, all of which suggest that these genomes do not rapidly purge duplicated regions. These patterns have at least two possible (somewhat overlapping) interpretations. First, it is possible that duplicate sequences do not strongly negatively impact mitochondrial function through their effects on rates of duplication and transcription or their mutational hazard. Accordingly, insertions and deletions in these sequences reflect the underlying mutational spectrum, and large deletions are relatively rare. Second, it is possible that large deletions may have enough of an impact on duplication/transcription/harmful mutation rate to be targets of positive selection, but that such large deletions are sufficiently rare, relative to substitutions and smaller insertions and deletions, that duplicate sequences persist in Aneides genomes, despite some negative fitness consequences.

In contrast to the persistence of duplicate tRNAs and pseudogenized protein-coding genes, as well as the persistence/expansion of repetitive sequence, duplicate ribosomal RNA genes have been almost completely deleted since the recent A. hardii duplication event. This suggests that selection may have favored deletion of these sequences. Although the presence of additional identical rRNA gene copies in the mitochondrial genome may be unlikely to have deleterious fitness consequences, the presence of pseudo-rRNA genes that compromise protein translation could negatively impact fitness. Thus, we hypothesize that (1) duplicate rRNA genes sustained mutations that compromised translational capacity, and (2) deletions eliminating such sequences were then favored by selection. With our data, however, we cannot exclude the alternate possibilities that a large deletion eliminating the duplicate rRNA genes was fixed by genetic drift or by selection acting on duplication/transcription. Characterizing the evolutionary fate of other recent rRNA gene duplications would aid in discriminating among these hypotheses.

Conclusions
Although metazoan mitochondrial genomes have conserved gene content, gene order can vary across taxa, reflecting partial genome duplication and subsequent paralog loss. We identified two duplication events in the history of the salamander genus Aneides: one that occurred tens of millions of years ago at the base of the clade, and the other that occurred recently within a single species (A. hardii). Our results suggest that some extraneous sequences have remained in Aneides mitochondrial genomes for tens of millions of years, while duplicate rRNA genes have been eliminated over much shorter timescales. Together with previous work showing that (1) increased metabolic demand is not correlated with mitochondrial genome streamlining in amphibians [21], and (2) genome expansion does not place genes in more vulnerable positions along a strong mutation gradient in salamanders [23], our results suggest that overall DNA loss is not likely to be a strong target of selection in salamander mitochondrial genomes. This parallels the pattern seen in salamander nuclear genomes, which also show slow rates of DNA loss [47]. Further studies identifying recent rRNA duplications in other taxa are required to test whether additional rRNA genes are functionally disadvantageous and targeted by purifying selection.

Additional file

Additional file 1: ClustalW-created multiple sequence alignments for each of the mitochondrial protein-coding genes including two individuals representing each A. hardii gene order. (TXT 45 kb)

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Availability of data and materials
The datasets supporting the conclusions of this article are available in the GenBank repository (https://www.ncbi.nlm.nih.gov/genbank/), and we have provided a table listing corresponding accession numbers. Datasets supporting the conclusions of this article are also included within the article and its additional file.

Authors’ contributions
RAC and RLM contributed to the conception and design of the study, RAC collected and analyzed the data, RAC and RLM wrote the manuscript. Both authors read and approved the final manuscript.

Ethics approval
All work was completed in compliance with Colorado State University’s Institutional Animal Care and Use Committee (Protocol 08-184A-02).

Consent for publication
Not applicable

Competing interests
The authors declare that they have no competing interests.

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