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Molecular mechanisms of extensive mitochondrial gene rearrangement in plethodontid salamanders

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Abstract

Extensive gene rearrangement is reported in the mitochondrial genomes of lungless salamanders (Plethodontidae). In each genome with a novel gene order, there is evidence that the rearrangement was mediated by duplication of part of the mitochondrial genome, including the presence of both pseudogenes and additional, presumably functional, copies of duplicated genes. All rearrangement-mediating duplications include either the origin of light strand replication and the nearby tRNA genes or the regions flanking the origin of heavy strand replication. The latter regions comprise *nad6*, *trnE*, *cob*, *trnT*, an intergenic spacer between *trnT* and *trnP* and, in some genomes, *trnP*, the control region, *trnF*, *rrnS*, *trnV*, *rrnL*, *trnL1*, and *nad1*. In some cases, two copies of duplicated genes, presumptive regulatory regions, and/or sequences with no assignable function have been retained in the genome following the initial duplication; in other genomes, only one of the duplicated copies has been retained. Both tandem and non-tandem duplications are present in these genomes, suggesting different duplication mechanisms. In some of these mtDNAs, up to 25% of the total length is composed of tandem duplications of non-coding sequence that includes putative regulatory regions and/or pseudogenes of tRNAs and protein-coding genes along with otherwise unassignable sequences. These data indicate that imprecise initiation and termination of replication, slipped-strand mispairing, and intra-molecular recombination may all have played a role in generating repeats during the evolutionary history of plethodontid mitochondrial genomes.

Introduction

In contrast to the conclusions drawn from early and limited sampling, animal mitochondrial genomes possess unexpected diversity both in gene order and in the presence, extent, and distribution of non-coding sequence (Inoue et al. 2003; Boore, Medina and Rosenberg 2004; Miller et al. 2004; Yokobori et al. 2004). Groups of organisms with highly differing gene orders are appropriate model systems for studying the factors that effect mitochondrial gene rearrangement (Dowton and Campbell 2001). Such groups have been identified among invertebrates (Yamazaki et al. 1997; Dowton and Austin 1999; Shao et al. 2001), and testable hypotheses for the causes of frequent gene rearrangement have been generated; for example, highly-rearranged parasitic wasp mitochondrial genomes may result from oxidative stress imposed by the host immune response (Dowton and Campbell 2001). Within the largest salamander family, Plethodontidae, six of the 22 mitochondrial genomes examined have rearrangements of independent phylogenetic origin, and 12 of these 22, plus one from the family Rhyacotritonidae, have tandem repeats in non-coding sequences. This high instance of gene rearrangement makes plethodontid salamanders an excellent model system for examining the mechanisms of such vertebrate mitochondrial genome instability.

In the most commonly invoked model of mitochondrial gene rearrangement, a region of the genome is duplicated and the supernumerary genes, no longer maintained by selection, are eliminated; the original gene arrangement is either restored or altered, depending on the pattern of gene loss (Moritz, Dowling and Brown 1987; Boore 2000). Genomic evidence for this model of rearrangement includes: (1) repeated motifs, which can cause duplication via slipped-strand mispairing; (2) stem-loop structures, which can

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cause duplication via intra-molecular recombination (Stanton et al. 1994; Moore, Gudikote and Van Tuyle 1998); and (3) pseudogenes, which may be ancestral duplications in the process of being eliminated (Arndt and Smith 1998; Macey et al. 1998). This mechanism cannot explain gene inversions, which have also been reported (Smith et al. 1989; Boore 1999; Dowton et al. 2003; Miller et al. 2004). In each of the six rearranged genomes reported here, evidence of at least one historical duplication event has been retained. Plethodontid rearrangements involve regions of the genome that are independently rearranged or duplicated in other vertebrate taxa (Moritz and Brown 1986; Desjardins and Morais 1990; Moritz 1991; Pääbo et al. 1991; Quinn and Mindell 1996). The molecular mechanisms of gene rearrangement at work in plethodontids may therefore be the same mechanisms acting across vertebrates.

Organismal, cellular, and genomic level properties may be linked to mitochondrial genome instability, both in these salamanders and more generally. In this paper, we (1) describe mitochondrial genome rearrangements and expansions of non-coding DNA in plethodontid and related salamanders, (2) infer possible molecular mechanisms by which these rearrangements and expansions originated, and (3) outline possible explanations for the extensive mitochondrial genome instability in this clade.

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Materials and Methods

Twenty-four complete mitochondrial genomes of plethodontid and related salamanders were sequenced, assembled, and annotated as described elsewhere (Mueller et al. 2004) (GenBank accession numbers AY728212–AY728235). Annotated genomes were examined for novel gene orders and the presence of unassignable sequences. Putative pseudogene sequences in each rearranged genome were identified by position and aligned with the corresponding functional gene sequences using ClustalW (gap parameters set to default: open=10, extend=5) and manual adjustment. Two other non-coding regions of the genome were also examined: the control region (CR hereafter), and an intergenic spacer between *trnT* and *trnP* (IGS hereafter) present in diverse salamander clades that may be a remnant of at least one older duplication (McKnight and Shaffer 1997; Zardoya et al. 2003; Zhang et al. 2003a; Zhang et al. 2003b). Because these regions are highly variable with only a few, or no, short conserved sequences (Shadel and Clayton 1997), we defined these two regions by their genome position. All non-coding regions were tested for the presence of tandem repeat elements and for shared repeats among the different regions by the construction of Pustell DNA-DNA matrices using MacVector (window size=30, minimum % score=80, hash=6) (Accelrys). The genome sequence of *Hydromantes italicus* is incomplete (*trnT*, the IGS, *trnP*, the CR, and *trnF* are missing) and therefore was not examined for repeats. Finally, each genome was tested for possible heteroplasmy in the numbers of repeated regions by examining the sequences of individual clones for their ability to produce assemblies with identical end sequences but different numbers of repeats from the primary assembly.

Results and Discussion

Duplications have mediated most rearrangements

Six of 22 plethodontid genomes have novel gene orders and arrangements of non-coding sequences that are consistent with the duplication-random loss model of gene rearrangement (Boore 2000). In this model, a portion of the genome containing at least two genes is duplicated. One of the two copies of each duplicated gene eventually loses function, becomes a pseudogene, and is excised from the genome by mutational processes because it is not maintained by selection. Which gene copy is ultimately lost is determined by the first loss-of-function mutation, with some patterns restoring the original order, and others leading to rearrangement. In this study, two such rearrangements include the origin of light strand replication (O_L), two include the IGS, and two include both the origin of heavy strand replication (O_H, contained within the CR) and the IGS. In some cases, there are evident pseudogenes that signal historical duplications. In others, these pseudogenes have decayed beyond ~60% identity to the functional copy and are inferred based solely on their genome position. In all cases, the exact boundaries of the duplicated fragments have likely been obscured by deletion, and the extent of the original duplication may therefore be underestimated. The phylogenetic positions of the taxa with these rearranged genomes indicate separate rearrangement events, with the possible exception of one rearrangement shared by *Aneides flavipunctatus* and *Aneides hardii*, although the genome of *A. hardii* also appears to have undergone a second duplication-mediated rearrangement (fig. 1). For each rearranged mitochondrial genome, we infer the minimum set of contiguous genes that, when

duplicated, create an intermediate sequence from which gene losses could have established the observed gene arrangement.

Rearrangements including the O_L

Batrachoseps attenuatus — The hypothesized duplicated region in this species' mtDNA includes a small fragment of *trnW* and all of *trnA*, *trnN*, O_L, *trnC*, and *trnY* (~300 bp total) (fig. 2). For the purposes of these analyses, O_L refers to the entire non-coding region normally found between *trnN* and *trnC* that has the potential to form a large stem-loop structure known to function as an origin of replication in some systems. We refer to a copy of O_L by pseudogene annotation if it is greatly deficient in potential for forming this structure relative to the other copy, although no experimental data is available to infer which, if either, actually functions as an origin of replication. Deletions in the pseudogenes have reduced their sizes to the following lengths and percentages of original length: partial *ψtrnW* + *ψtrnA*, 77 bp (the percentage of original length cannot be determined, because the amount of *trnW* included in the initial duplication is unknown); *ψtrnN*, 65 bp (93%); ψO_L, 8 bp (27%); *ψtrnC*, 56 bp (86%); and *ψtrnY*, 28 bp (amount of *trnY* duplicated is unknown).

Hydromantes brunus — The hypothesized duplicated region in this species' mtDNA is slightly larger than in *B. attenuatus*, encompassing the end of *nad2* and all of *trnW* in addition to *trnA*, *trnN*, O_L, *trnC*, and *trnY* (~525 bp) (fig. 3). Deletions in the pseudogenes have reduced their sizes to the following lengths and percentages of original length: partial *ψnad2*, ~127 bp (amount of *nad2* duplicated is unknown); *ψtrnW*, ~60 bp (88%); *ψtrnA*, 55 bp (81%); *ψtrnN*, 65 bp (94%); ψO_L, 27 bp (73%); *ψtrnC*, 57 bp

(86%); and *ψtrnY*, 39bp (58%). Although the initial duplications of *B. attenuatus* and *H. brunus* involved similar regions of the genome, different copies of the redundant genes were subsequently lost; this is consistent with random loss, in which the copy of the gene that sustains the first disabling mutation continues to decay.

Rearrangements including the CR and/or the IGS

Stereochilus marginatus — The hypothesized duplication in this species' mtDNA includes *nad6*, *trnE*, *cob*, *trnT*, and the IGS for a total of 1,790 bp plus the unknown length of the IGS (fig. 4). Deletions in the pseudogenes have reduced their sizes to the following lengths and percentages of original lengths: *ψnad6*, 186 bp (36%); *ψtrnE*, 66 bp (98.5%); and *ψcob*, 42 bp (3.7%). *ψtrnT*, if it still exists, cannot be identified. Two different sets of repeats exist in the two regions of the genome inferred to be copies of the IGS.

Plethodon elongatus — This species' mitochondrial genome has a gene order and pattern of non-coding sequence consistent with two separate duplications. The initial hypothesized duplication spans the region of the genome including *nad6*, *trnE*, *cob*, *trnT*, the IGS, *trnP*, and the CR for a total of 2,811 bp plus the unknown pre-duplication length of the IGS (fig. 5). Different copies of the redundant genes subsequently decayed to pseudogenes in *P. elongatus* than decayed in *S. marginatus.* Deletions in the inferred redundant genes have reduced their sizes to the following lengths and percentages of original lengths: *ψnad6* + *ψtrnE* + *ψcob*, 47 bp (2.7%); *ψtrnP*, length unknown because the boundary between the IGS and *ψtrnP* is unassignable; and *ψtrnT* + IGS, 107 bp (original length of the IGS is unknown). Notably, two 959-bp, 97% identical copies of

the putative CR are retained in the genome, indicating that the two may be undergoing concerted evolution as has been reported for several other taxa (Arndt and Smith 1998; Kumazawa et al. 1998; Lee et al. 2001; Inoue et al. 2003).

Following this initial rearrangement by duplication-random loss, we hypothesize that a second duplication gave rise to two copies of the region including the last 57 bp of *nad5*, *ψnad6* + *ψtrnE* + *ψcob*, *trnT*, the IGS, *ψtrnP*, and the first 761 bp of one CR (1,150 bp total). These two copies are not adjacent to one another; rather, they are separated by 3,054 bp. The two copies are >99% identical in sequence, implying either (1) very recent duplication, or (2) concerted evolution, which is also operating to maintain the sequence identity of the two full-length CRs.

Aneides flavipunctatus — The hypothesized duplication in this species' mtDNA includes *nad6*, *trnE*, *cob*, *trnT*, the IGS, and *trnP* for a total of 1,860 bp plus the unknown length of the IGS (fig. 6). The same copies of the redundant genes subsequently decayed to pseudogenes in *A. flavipunctatus* as decayed in *S. marginatus.* Deletions in *ψnad6* and *ψtrnE* have completely excised them from the genome. *ψcob* and *ψtrnT*, if they still exist, cannot be identified. As seen in the *S. marginatus* genome, two different sets of repeats exist in the two regions of the genome inferred to contain copies of the IGS.

Aneides hardii — This mitochondrial genome has a gene order and pattern of non-coding sequence consistent with two separate tandem duplications (fig. 7). The initial hypothesized duplication includes a near-identical region of the genome duplicated in *A. flavipunctatus* (from *nad6* through the IGS), suggesting that this duplication may be a synapomorphy of the *Aneides* clade; however, unlike in *A. flavipunctatus*, no evidence remains that *trnP* was duplicated in *A. hardii*. The absence of an identifiable *ψtrnP* in this

duplicated region of the *A. hardii* genome suggests that (1) the *ψtrnP* identified in *A. flavipunctatus* is false, (2) *ψtrnP* has been excised or completely degraded from *A. hardii*, or (3) a different duplication-mediated rearrangement occurred independently in each lineage. The second hypothesized duplication in *A. hardii* includes *nad6* – the IGS, plus a second IGS, *trnP*, the CR, *trnF*, *rrnS*, *trnV*, *rrnL*, *trnL1*, and *nad1* for a total of 6,227 bp and the unknown lengths of the two IGS regions. The copies of *nad6* and *trnE* involved in the second round of duplication must have been functional at the time of re-duplication, based on the current position of functional copies in the genome. The *ψnad6* and *ψtrnE* between *nad5*and *cob*, which were not involved in the second round of duplication, have been reduced to 11 bp (2%).

The two large, duplicated fragments resulting from the second hypothesized round of duplication (Copy I and Copy II; see Fig. 7) share high levels of sequence similarity over much of their length, although *ψnad6*, much of *ψrrnS*, and all of *ψtrnV* and *ψrrnL* have been excised from the genome and *ψnad1* has accumulated multiple stop codons. In contrast, two very similar, presumably functional copies of *trnP*, *trnF*, *trnL1*, the CR, and possibly *trnE* have been retained in this genome. There are several possible explanations for this pattern. Consistent with the duplication-random loss model, this duplication event may be sufficiently recent that mutations have not yet disrupted *trnP*, *trnF*, *trnL1*, the CR, or *trnE*. Alternatively, these copies may have been maintained by selection, or may have had their mutations corrected by gene conversion (Eberhard, Wright and Bermingham 2001). Selection to retain duplicate copies of these genes and the CR may act either on the production of their gene products or their ability to form secondary structures necessary for message processing (Kumazawa et al. 1998), or on

regulatory function, respectively. However, the presence of tandem repeats highly conserved between Copy I and Copy II is difficult to explain by selection in the absence of a clear function assignable to these sequences. Rather, it suggests either a recent duplication event or a purely mechanistic cause of concerted evolution between portions of these two fragments (Moritz and Brown 1987).

Possible mechanisms yielding tandem duplications

Several mechanisms may produce duplications in a circular molecule: recombination, slipped-strand mispairing, errors in synchronizing the points of initiation and termination of replication, or some combination of these processes (Macey et al. 1997a; Macey et al. 1997b; Boore and Brown 1998; Boore 2000; Dowton and Campbell 2001). At least two such mechanisms are plausible explanations for the patterns observed in the order of genes and non-coding DNA in most plethodontid rearrangements. In the genomes of *H. brunus*, *B. attenuatus*, and *A. hardii* (second duplication), the replication origin is in the center of the duplicated region, indicating that both initiation and termination of replication occurred at alternative sites; alternate initiation or termination alone would duplicate only the region of the genome upstream or downstream, respectively, of the replication origin. A presumably functional O_L with viable potential secondary structure (Pääbo et al. 1991; Macey, Schulte and Larson 2000) exists in both *H. brunus* and *B. attenuatus*. This suggests that light-strand replication initiation has not been completely transferred to alternate structures in these genomes and that alternate initiation may be causally linked to imprecise termination. In the genomes of *S. marginatus*, *A. flavipunctatus*, and *A. hardii* (first duplication), no evidence remains that

a replication origin was involved in the duplication. The patterns in these genomes are still consistent with initiation and termination of replication at alternate sites, but both sites are located downstream of the O_H. The IGS, which is the putative alternative initiation site for *S. marginatus* and *A. hardii*, may be an ancient duplicated CR based on its position in the genome (McKnight and Shaffer 1997) and thus may retain limited initiation capability. Imprecise termination alone is also a plausible duplication mechanism for *S. marginatus*, *A. flavipunctatus*, and *A. hardii* based on the proximity of these duplicated regions to the CR and the possible erosion of the ends of the duplicated region. The first duplication in the *P. elongatus* genome is bounded by the CR; depending on the location of the O_H within the CR, imprecise termination alone, or imprecise termination with initiation at an alternate site, are possible duplication mechanisms. We cannot eliminate the alternative possibility that either intra-molecular recombination or slipped-strand mispairing caused any of these duplications, nor can we rule them out as mechanisms for excision of redundant genes following these duplications (Holt, Dunbar and Jacobs 1997; Lunt and Hyman 1997; Kajander et al. 2000; Tang et al. 2000a; Miller et al. 2004).

Possible mechanisms for yielding non-tandem duplications

The second duplication event in *Plethodon elongatus* produced non-tandem repeat fragments separated from one another by 3,054 bp, which nonetheless have 99.5% sequence identity. This pattern cannot be easily explained by slipped-strand mispairing, errors in initiation or termination of replication, or any combination of these processes. However, this pattern is consistent with the action of intra-molecular recombination. One

copy of the region comprising the putative IGS, *ψtrnP*, and one of the two CRs may have been excised from one genome, forming a separate mini-circle that was then re-integrated into another genome between the second CR and *trnF*. Such intra-molecular recombination has been reported in the mitochondrial genomes of both healthy and diseased tissue in several taxa (Holt, Dunbar and Jacobs 1997; Lunt and Hyman 1997; Kajander et al. 2000; Tang et al. 2000a; Miller et al. 2004; Yokobori et al. 2004). In addition to this non-tandem repeat in *P. elongatus*, the pattern of widely separated repeat units in *S. marginatus* is consistent with the action of intra-molecular recombination. However, the pattern in *S. marginatus* is also consistent with retention of these widely-separated repeat units since the original duplication event, coupled with slower accumulation of mutations in the repeat units relative to the remainder of the duplication fragment.

Tandem repeats in the control region and IGS that do not effect gene rearrangement

Only four of the 24 genomes analyzed for this study contain a duplication-mediated rearrangement involving the CR or nearby regions of the genome. Thirteen of the remaining 20 genomes contain tandem-repeats of non-coding sequence in the CR and/or the IGS, as reported in other taxa (Wallis 1987; Delarbre et al. 2001). These results are summarized in Table 1. The length, number, and sequence of the repeat units vary within and among genomes, although in two cases, the same repeats are present in both the IGS and the CR. In the case of non-tandem repeats, intra-molecular recombination is a more plausible generation mechanism. In the case of tandem repeats, we cannot discriminate between slipped-strand mispairing and intra-molecular

recombination. The tandem repeats in the *Rhyacotriton variegatus* genome are unusual and are discussed in more detail below.

In the *R. variegatus* genome, the 5,527-bp IGS contains six copies of an ~880-bp tandem repeat. The first ~820 bp of this repeat are a *ψcob*, and the last 62 bp are an additional copy of *trnT*. *ψcob* is missing the first 260 bp of the gene, but each of the six copies is >80% identical to the corresponding portion of the functional copy, not including ~20 bp of deletions. All copies of the pseudogene contain numerous stop codons when translated in all three reading frames. The copies of *trnT* are ~90% identical to the inferred original copy but, in contrast to *ψcob*, all but one may remain functional; their secondary structures appear viable. Following the sixth complete repeat unit, there are 247 bp which appear unrelated to the repeat sequence. In contrast, there are no repeats in the 755-bp CR, nor do the CR and IGS regions share any common sequence.

Heteroplasmy has been reported in some individuals whose mitochondrial genomes contain tandem repeats (Densmore, Wright and Brown 1985; Wallis 1987; Townsend and Rand 2004). Similarly, patterns in the assembly of individual clone sequences into contigs may suggest heteroplasmy in seven plethodontid genome sequences: *E. bislineata*, "Bolitoglossa sp. nov.," *B. wrightorum*, *H. scutatum*, *P. petraeus*, *E. eschscholtzii*, and *Aneides flavipunctatus*. However, we note that PCR-based evidence for presence or absence of heteroplasmy is imperfect. A non-heteroplasmic sequence may indicate that multiple mitochondrial genome haplotypes are absent or that, although present, the additional copy or copies were not amplified by PCR. Furthermore, products that appear heteroplasmic may be generated by strand switching during PCR, analogous to slipped-strand mispairing. Finally, heteroplasmy is predicted in genomes

with high numbers of tandem repeats, and the assembly of sequences of individual clones into one contig may prove problematic for such genomes even in the absence of heteroplasmy.

Possible explanations for extensive gene rearrangement in plethodontids

A high incidence of rearranged mitochondrial genomes may result from any or some combination of four factors: (1) a high rate of mitochondrial genome partial duplication; (2) a low rate of duplication excision; (3) a low mitochondrial genome effective population size, particularly a bottleneck in the number of mitochondrial genomes transmitted to offspring via maternal oocytes; and (4) selection for, or absence of selection against, rearranged/expanded mitochondrial genomes at either the cell or population level. No data addressing any of these four factors are currently available for plethodontids. However, studies of selection on mitochondrial genomes containing duplications have been carried out in other systems. Here, we briefly discuss their possible relevance to the high levels of plethodontid mitochondrial instability. We note, however, that inferences drawn from such studies should be considered as hypotheses directing further research, and that studies explicitly measuring all four of these factors in plethodontids and other clades with high levels of mitochondrial genome rearrangement are required to draw any firm conclusions.

The fixation of structurally altered mitochondrial genomes in a population depends in part on whether they are selectively advantageous, neutral, or disadvantageous to the organism. Unlike deletions, large duplications of portions of the mitochondrial genome are generally not pathogenic in humans (Tang et al. 2000b; DiMauro and Schon

2003), suggesting that there may not be strong organism-level selection against the duplications in plethodontid mitochondrial genomes. However, a link between compact mitochondrial genomes and metabolic efficiency has been proposed (Selosse, Albert and Godelle 2001), and high levels of mitochondrial duplications such as those seen in plethodontids lead to measurable reduction in respiratory chain efficiency in human cell lines (Holt, Dunbar and Jacobs 1997). Salamanders have extremely low aerobic metabolic requirements compared to other ectotherms (Feder 1976). Organism-level selection against the reduction in aerobic efficiency that may result from a mitochondrial duplication is therefore unlikely to be as strong in plethodontids as in other, more highly aerobic tetrapods. Low energetic costs are also broadly correlated with the accumulation of non-coding DNA in the nuclear genome (Gregory 2003). If relatively weak organism-level selection against mitochondrial duplications is contributing to their high incidence in plethodontids, we would predict (1) high mitochondrial genome instability in other organisms with similarly low aerobic metabolic requirements, and (2) small fitness differences between plethodontids with and without mitochondrial duplications relative to differences between other, more highly aerobic animals with and without mitochondrial duplications.

Phylogenetic applications

The analyses presented in this study describe major genome-level mitochondrial mutations for one representative individual from each included lineage. Therefore, they cannot address the extent to which these rearrangements characterize populations, species, or more inclusive phylogenetic groups, with the possible exception of the two

Aneides (Moritz and Brown 1987). *Aneides flavipunctatus* and *A. hardii* may share a synapomorphic rearrangement, although high levels of such rearrangements within plethodontids necessitate further sampling from this clade to eliminate the possibility of homoplasy. Additional sampling within all rearranged lineages and their close relatives will enable dating of the appearance and persistence of individual rearrangements, thereby addressing the rate at which pseudogenes decay and/or are excised from the genome. Similarly, it will allow dating of the appearance and persistence of tandem duplications within and around the CR and IGS. Finally, it will enable more accurate identification of the ends of duplicated fragments, allowing characterization of secondary structures, repeats, or other genomic sequences that may facilitate duplication both in plethodontids and more generally. In addition to providing further insight into the evolutionary history of vertebrate mitochondrial genomes, this understanding of molecular- and population-level dynamics of mitochondrial genome instability is critical for evaluating the strength of using mitochondrial genome rearrangements as a genome-level character for phylogenetic analysis (Sankoff et al. 1992; Macey, Schulte and Larson 2000).

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Table 1

Repeat elements in the intergenic spacer (IGS) between *trnT* and *trnP* and the control region (CR) between *trnP* and *trnF* in thirteen species' genomes.

Taxon	IGS Length (bp)	IGS Repeat Motifs, # x Length (bp)	CR Length (bp)	CR Motifs, # and Length (bp)	Sequence in Common Between IGS and CR?
Gyrinophilus porphyriticus	666	2 x 86, 9 x 7	766	None	No
Eurycea bislineata	1,113	3 x 60	729	None	No
Nototriton abscondens	> 1,198[a]	27 x 12	1,173	22 x 10	No
"Bolitoglossa sp. nov."	11	None	6,373	12 x 325, 6 x 200	No
"Thorius sp. nov."	262	None	3,539	3-36 x ~10[b]	No
Batrachoseps attenuatus	652	21 x 6-9	1,336	None	No
Batrachoseps wrightorum	183	None	4,297	13 x 225	No
Hemidactylium	1,211	8 x 26, 21 x	930	16 x 11	No

scutatum		15			
Plethodon cinereus	798	1 x 798[c]	3,694	2 x 1,250	Yes[d]
Plethodon petraeus	754	none	3,251	6 x 54, 9 x 200	No
Desmognathus fuscus	564	2 x 77, 8 x 9, 6 x 10	735	None	No
Ensatina eschscholtzii	1,119	1 x 525[e], 2 x 71	5,004	3 x 1,500	Yes[d]
Rhyacotriton variegatus	5,527	6 x 880[f]	755	None	No

[a] The IGS is incompletely sequenced in *N. abscondens*.

[b] "Thorius sp. nov." CR contains ≥ 14 different short (9-11) repeat units repeated between three and 36 times.

[c] In the *P. cinereus* genome, the entire 798-bp IGS is repeated 1.85 times within the CR.

[d] In the mtDNAs of *P. cinereus* and *E. eschscholtzii*, the IGS and CR contain similar (~80-90% identical) sequences.

[e] In the *E. eschscholtzii* mtDNA, 525 bp of the IGS is repeated five times within the CR--once in each 1,500-bp CR repeat unit and two additional times. Each 1,500-bp CR repeat unit also contains a *ψtrnP* that is 91% identical to the functional copy but has a one-bp deletion in the anticodon.

[f] The IGS repeat unit in the *R. variegatus* genome is comprised of a partial *ψcob* and additional *trnT* and is described further in the text.

Figure Legends

FIG. 1.—Phylogenetic relationships among plethodontid salamanders and two outgroups from other families as indicated (from Mueller et al. 2004). Asterisks indicate lineages that have experienced a duplication-mediated rearrangement. *Aneides hardii* and *Aneides flavipunctatus* may share one synapomorphic rearrangement; however, *A. hardii* underwent a second duplication-mediated rearrangement not present in *A. flavipunctatus*. Numbers to the right of species names are mitochondrial genome sizes. The sequences of two species' genomes, *N. abscondens* and *H. italicus*, are incomplete.

FIG. 2.—Mitochondrial gene rearrangements in *Batrachoseps attenuatus*. O_L = origin of light strand replication. Single letters refer to tRNA genes for the corresponding amino acid. Shaded boxes represent recognizable pseudogenes and question marks indicate stretches of sequence that cannot be assigned based on sequence similarity. Lengths of genes and pseudogenes are not to scale. *(A)* Current gene order. *(B)* Hypothesized duplication-random loss model for deriving this gene order. *ψtrnN* retains 60% identity to the functional copy overall, not including two deletions (1 and 3 bp) in the pseudogene; the last 39 bp retain 74% to the functional copy. The remaining pseudogenes have decayed beyond recognition.

FIG. 3.— Mitochondrial gene rearrangements in *Hydromantes brunus*. Designations are as in Figure 2. *(A)* Current gene order. *(B)* Hypothesized duplication-random loss model for deriving this gene order. *ψtrnW* retains 69% identity to the functional copy, not

including two deletions (3 and 5 bp) in the pseudogene. *ψtrnA* retains 56% identity to the functional copy overall, not including two deletions (3 and 5 bp) in the pseudogene; the last 44 bp retain 66% identity. *ψtrnN* retains 82% identity to the functional copy, not including one 5-bp deletion in the pseudogene. ψO_L retains 70% identity to the functional copy overall, not including 10 bp missing from its end; the first 18 bp retain 94% identity. *ψtrnC* retains 87% identity to the functional copy, not including three deletions (2, 1, and 2 bp) in the pseudogene. *ψtrnY* and *ψnad2* have decayed beyond recognition.

FIG. 4.— Mitochondrial gene rearrangements in *Stereochilus marginatus*. Designations are as in Figure 2 except that IGS = intergenic spacer between *trnT* and *trnP* and CR = control region. *(A)* Current gene order. *(B)* Hypothesized duplication-random loss model for deriving this gene order. *ψnad6* retains 52% identity to the inferred corresponding 186-bp portion of the functional copy, not including two insertions (2 and 1 bp) in the pseudogene; base pairs 55-132 retain 74% identity. *ψtrnE* retains 85% identity to the functional copy, not including one 1-bp deletion in the pseudogene. The 42-bp *ψcob* retains 79% identity to the first 44 bp of the functional copy, not including one 2-bp deletion in the pseudogene. The first putative copy of the IGS, between *trnT* and *nad6*, is 1,982 bp in length and contains three complete, and one partial, >95% identical tandem repeats of 437 bp each. The sequence between *ψcob* and *trnP* contains five >99% identical copies of a different 110-bp tandem repeat sequence and a sixth 87% identical copy. Notably, following an intervening 102-bp stretch of non-repetitive sequence, there is a complete copy of the 437-bp repeat unit found in the putative IGS between *trnT* and

nad6. This repeat unit is ~85% identical to copies in the other putative IGS, despite being separated from them by 1,630 bp. No recognizable *ψtrnT* remains.

FIG. 5.— Mitochondrial gene rearrangements in *Plethodon elongatus*. Designations are as in Figure 4. *(A)* Current gene order. *(B)* Two hypothesized duplication events mediating rearrangement in the *P. elongatus* mitochondrial genome. The first duplication resulted in tandem repeats of the region spanning from *nad6* to the CR. *ψtrnP* retains 61% identity to the functional copy overall, not including two insertions (3 bp each) in the pseudogene; the last 44 bp retain 70% identity. *ψnad6, ψtrnE, ψcob,* and *ψtrnT* have all decayed beyond recognition. In contrast, the two copies of the CR are 97% identical. Later, a duplicate copy of the fragment comprising the end of *nad5, ψnad6, ψtrnE, ψcob, trnT,* the IGS, *ψtrnP*, and a portion of the CR was inserted between the second CR and *trnF*, likely by intra-molecular recombination. The two copies of this fragment are 99% identical.

FIG. 6.— Mitochondrial gene rearrangements in *Aneides flavipunctatus.* Designations are as in Figure 4. *(A)* Current gene order. *(B)* Hypothesized duplication-random loss model for deriving this gene order. *ψnad6* and *ψtrnE* have been excised from the genome. The region between *trnT* and *nad6*, which comprises a putative IGS and *ψtrnP*, is 3,050 bp in length and contains seven complete, and one partial, >96% identical 388-bp tandem repeats. Each tandem repeat contains a 72-bp *ψtrnP* with 54% overall sequence identity to *trnP*, not including two insertions in the pseudogene (2 and 1 bp); the last 51 bp are 63% identical to the functional *trnP*. The region between *trnE* and *trnP* contains ten

>92% identical copies of a 66-bp tandem repeat, a 357-bp stretch of variable numbers of short repeats (5-10 bp), and 71 bp of non-repetitive sequence. No recognizable *ψcob* or *ψtrnT* exist. The two repetitive regions of the genome resemble neither one another nor the CR.

FIG. 7.— Mitochondrial gene rearrangements in *Aneides hardii*. Designations are as in Figure 4. *(A)* Current gene order. *(B)* Two hypothesized duplication events in the history of the *A. hardii* mitochondrial genome. The first duplication comprised the region spanning from *nad6* to the IGS; this duplication may be a synapomorphy shared by *A. hardii* and *A. flavipunctatus*. The second duplication comprised the region spanning from IGS to *nad1*. The putative IGS (IGSa) that bounds the two copies of this duplication fragment (Copy I and Copy II) contains varying numbers of a 343-bp tandem repeat. Copy I has one complete and one partial tandem repeat units, and Copy II has four complete >95% identical tandem repeat units and one partial tandem repeat unit. Adjacent to these IGSa sequences, Copy II contains a functional *nad6*, whereas Copy I possesses neither a functional *nad6* nor a recognizable *ψnad6*. The two copies of *trnE*, adjacent to *nad6* (Copy II) and IGSa (Copy I), are 97% identical to one another excluding a 3-bp deletion in the D-stem of Copy II. *ψcob*, *ψtrnT*, and an additional IGS (IGSb), if retained in the genome, should be found in each fragment between *trnE* and *trnP*. There is no recognizable *ψcob* or *ψtrnT* in this region in either fragment, nor is there any sequence similarity with the 343-bp repeat unit found in IGSa. Rather, this region of each fragment contains 67-bp tandem repeats, followed by ~300 bp of non-repetitive sequence. Copy I has six complete tandem repeats; Copy II has four complete repeats and

a partial fifth. Not including this indel, the two copies of this region are 96% identical. Both Copy I and Copy II contain *trnP*, CR, and *trnF*; the two copies of this region are 96% identical overall, and both copies of both tRNAs have viable secondary structures. Adjacent to *trnF*, Copy I has a complete *rrnS*, *trnV*, *rrnL*, and *trnL1*. In contrast, Copy II contains 111 bp that are 86% identical to the first 111 bp of functional *rrnS*, followed by a *trnL1* that is 97% identical to the *trnL1* in Copy I; both *trnL1s* have viable secondary structure. Adjacent to *trnL1*, Copy I has a 672-bp *ψnad1* that is 92% identical to the first 672 bp of the functional copy but contains multiple stop codons; Copy II contains the functional *nad1*.