nrDNA:

Goodall-Copestake, William Paul

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Title

nrDNA:mtDNA copy number ratios as a comparative metric for evolutionary and conservation genetics

Author and Corresponding Author

William Paul Goodall-Copestake

Address and Contact Details

British Antarctic Survey (Natural Environment Research Council, UK Research and Innovation), High Cross, Madingley Road, Cambridge, CB3 0ET, UK.

Email wgco@bas.ac.uk

Telephone +44 (0)1223 221653

Fax +44 (0)1223 362616

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Abstract

Identifying genetic cues of functional relevance is key to understanding the drivers of evolution and increasingly important for the conservation of biodiversity. This study introduces nuclear ribosomal DNA (nrDNA) to mitochondrial DNA (mtDNA) copy number ratios as a metric with which to screen for this functional genetic variation prior to more extensive omics analyses. To illustrate the metric, quantitative PCR was used to estimate nrDNA(*18S*) to mtDNA(*16S*) copy number ratios in muscle tissue from samples of two zooplankton species: *Salpa thompsoni* caught near Elephant Island (Southern Ocean) and *S. fusiformis* sampled off Gough Island (South Atlantic). Average 18S:16S ratios in these samples were 9:1 and 3:1, respectively. nrDNA 45S arrays and mitochondrial genomes were then deep sequenced to uncover the sources of intra-individual genetic variation underlying these 18S:16S copy number differences. The deep sequencing profiles obtained were consistent with genetic changes resulting from adaptive processes, including an expansion of nrDNA and damage to mtDNA in *S. thompsoni*, potentially in response to the polar environment. Beyond this example from zooplankton, nrDNA:mtDNA copy number ratios offer a promising metric to help identify genetic variation of functional relevance in animals more broadly.

Keywords

Adaptation, Antarctica, Biodiversity, Environment, Omics, Tunicate

Introduction

The use of genetic data in evolutionary and conservation biology has expanded beyond the inference of population structure, species identity, and phylogenetic relationships to incorporate inferences about function and adaptation to the environment, e.g. with the advent of ecological genomics (Ungerer *et al.*, 2008). However, identifying genetic cues of functional relevance is not trivial because extensive omics analyses are often required - reviewed by Porcelli *et al.* (2015). Screening for variation at candidate loci putatively associated with function can yield valuable insights to complement subsequent and more extensive omics investigations. Nuclear ribosomal DNA (nrDNA) and mitochondrial DNA (mtDNA) are of considerable functional significance but have been under-exploited as markers within this context (Weider *et al.*, 2005; Ballard and Pichaud, 2014). In this study, I propose that nrDNA and mtDNA could be assessed for functionally relevant variation through the comparison of their copy number ratios.

nrDNA and mtDNA vary in copy number between cells, tissues, individuals and species. Classic examples of the former come from studies on toad oocytes that are known to accumulate both nrDNA and mtDNA (Dawid, 1966; Gall, 1968). By contrast, nrDNA content is reduced during the development of copepods' somatic tissue (Zagoskin *et al.*, 2010). Levels of human mtDNA show more variation among tissues within individuals than between individuals (Wachsmuth *et al.*, 2016). Nevertheless, tissue-specific inter-individual variation can be considerable. Gibbons *et al.* (2014) found mtDNA content to vary c.4-fold, and nrDNA more than an order of magnitude, among the human individuals included in the 1000

Genomes Project Consortium (2012) dataset. Inter-individual copy number comparisons are dominated by studies involving human pathological states, such as neurodegeneration and cancer, with which differences in nrDNA and mtDNA abundance have been associated (Hallgren *et al.*, 2014; Pyle *et al.*, 2016; Reznik *et al.*, 2016; Wang and Lemos, 2017). Between-species comparisons of copy number have revealed substantial variation in nrDNA content and an association with genome size (Prokopowich *et al.* 2003; Vierna *et al.,* 2013). There have yet to be any such multi-species comparisons of mtDNA content.

The most obvious functions of nrDNA and mtDNA relate to the structural and catalytic products these DNA regions encode, which are essential for protein synthesis and energy production (Weider *et al.*, 2005; Ballard and Pichaud, 2014 and references therein). Associations between the physiological demand for these products and copy numbers are clear in certain tissue-specific instances (e.g. toad oocytes: Dawid, 1966; Gall, 1968) and they have been proposed to be involved in pathological conditions (e.g. human cancers: Reznik *et al.*, 2016; Wang and Lemos, 2017). Some intriguing examples of associations between copy number and physiological demand have also emerged at the organismal level. Elser *et al.* (2000) hypothesised that nrDNA abundance is positively associated with growth rates, citing several examples from plants and animals (see also Weider *et al.*, 2005). More recently, Cheng *et al.* (2013) and Li *et al.* (2016) have explored the contribution of mtDNA levels to physiological adaptation in human populations and reported associations between copy numbers and environmental parameters.

Associations between copy numbers and the physiological demand for proteins and energy are complicated by other factors including sample-specific differences in the regulation of DNA transcription and RNA translation, and the related issue of genetic and epigenetic variation affecting nrDNA and mtDNA functions. These functions have been suggested to extend beyond encoding products for protein synthesis and energy production to other roles, which in the case of yeast nrDNA, involve modulating genome integrity and adaptability (Kobayshi, 2011). The nrDNA within other organisms may have similar additional functions given reported instances of redundancy in nrDNA copy numbers which exceed cellular requirements for ribosomal RNA (Weider *et al.*, 2005). Indeed, changes in nrDNA levels that alter genome stability may have a functional role in human cancers (Wang and Lemos, 2017). Copy number redundancy has also been suggested to play a role in the maintenance of mitochondrial genome integrity in mammals by provisioning for the selective degradation of damaged molecules (Alexeyev *et al.*, 2013). Furthermore, mtDNA may have 'other functions' by encoding for products involved in processes other than energy production, such as the mitochondrial-derived peptide *humanin* (Capt *et al.*, 2016).

The extent to which these proposed functions of nrDNA and mtDNA ultimately drive copy number variation remains an understudied issue. So too is the impact of other potential drivers on copy numbers, that include mechanistic cellular processes (e.g. DNA recombination and replication) and underlying evolutionary processes (e.g. drift, gene flow). Accumulating evidence from recent work on humans suggests that it is worth exploring the potential of nrDNA and

mtDNA copy numbers as markers of functionally relevant genetic variation in animals more broadly. Obtaining nrDNA and mtDNA copy number data is straightforward because it can be readily estimated through quantitative PCR (qPCR) by taking advantage of archived sequences as a resource for primer design. Due to the typically high abundance of nrDNA and mtDNA in nucleic acid extractions, copy numbers can also be efficiently estimated from low coverage 1+nth generation sequencing surveys. mtDNA levels estimated in this way are less prone to bias arising from the inadvertent qPCR amplification of nuclear-mitochondrial DNA (NUMTS). Estimating relative amounts of nrDNA and mtDNA instead of their absolute quantities offers practical advantages. Deriving copy number ratios removes the need for internal references (e.g. single copy genes), which require additional background information for qPCR primer design and deep rather than low coverage sequencing. Furthermore, the use of ratios greatly simplifies quantitative comparisons within and between studies by removing the need for data on absolute template concentrations associated with qPCR/DNA sequencing.

To explore the use of nrDNA:mtDNA copy number ratios within an evolutionary and conservation genetics setting, a comparison was made between a pair of closely related zooplankton species that might be expected to show physiological adaptations to different environments. The salp (pelagic tunicate) *Salpa thompsoni* was selected as the first species because it occurs within the polar waters of the Southern Ocean (Foxton, 1961) and has interesting nrDNA-mtDNA properties: abundant nrDNA (Jue *et al.*, 2016), and mtDNA duplications and

heteroplasmy (Goodall-Copestake, 2017). The other species selected for comparison was *S. fusiformis*, which is non-polar and has a near-cosmopolitan distribution (Van Soest, 1975). Little is known of *S. fusiformis* genetics other than its nrDNA 18S sequence that confirms its close affinity to *S. thompsoni* (Govindarajan *et al.*, 2011). Species distributions (Van Soest, 1975) and phylogenetic relationships (Govindarajan *et al.*, 2011) are consistent with *S. thompsoni* as a polar lineage of the largely tropical-temperate genus *Salpa*. Adaptive genetic changes in response to the polar environment might thus be expected in *S. thompsoni* relative to *S. fusiformis*, some of which may have driven differences in the nrDNA:mtDNA copy number ratio between these species. To this end, quantitative PCR (qPCR) was used to determine if the ratio of 18S nrDNA to 16S mtDNA differed between samples of *S. thompsoni* and *S. fusiformis*. Traditional cloning-sequencing and deep sequencing were then used to generate 45S rRNA array (from *18S* to *28S*) and mitochondrial genome sequences from both salp species, high-resolution copy number data, and intra-individual single nucleotide polymorphism (SNP) and structural variation data to place the qPCR results into a broader genomic background.

Methods

Ten *S. thompsoni* samples (five oozooids and five blastozooids) were obtained from the archives of British Antarctic Survey cruise JR26, sampling event 184 near Elephant Island (Southern Ocean). Ten *S. fusiformis* (three oozooids, seven blastozooids) were sampled near Gough Island (South Atlantic) during cruise JR287

event 46. Morphological determinations followed descriptions in Foxton (1961). Body muscles were dissected out for molecular genetic analysis.

Procedures for DNA extraction, long-range PCR, amplicon visualization, cloning, Sanger sequencing - editing - assembly and annotation followed those previously described in Goodall-Copestake (2017). Novel PCR primers were designed from salp *18S* (Govindarajan *et al.*, 2011) and mtDNA (Goodall-Copestake WP, unpublished data) sequences using the Oligo Analysis Tool by Eurofins Genomics (Ebersberg, Germany). Other procedures and methodological variations were as follows.

qPCR was carried out in triplicate 10µL reactions using a KiCqStart SYBR Green qPCR ReadyMix (Sigma-Aldrich, Staffordshire, UK), 5ng of template, and the novel *18S* primers (F 5'- CAAAGATTAAGCCATGCAAGTGTAAG -3', R 5'- TCACGCATGTATTAGCTCTAGAATTG -3') and *16S* primers (F 5'- CCTTATGCAATTGGTGTTTTACGAC -3', R 5'- GAGATAAAATCCGGTCTAATTCTCACC -3') on an Eco 48 machine (PCRmax Limited, Staffordshire, UK). The bundled PCRmax Eco Study software was used to determine average Cq fluorescence values from which nrDNA *18S* to mtDNA *16S* abundance ratios were generated.

Near complete fragments of *45S* nrDNA were amplified using the primer *18S* F paired with '11_RC' from Machida and Knowlton (2012). Mitochondrial genomes were amplified as two overlapping fragments using the *16S* qPCR primers paired with the novel *trnR* primers (F 5'- GAACAGGTGATTTCGGGTTACC -3', R 5'- GGTAACCCGAAATCACCTGTTC -3'). Amplicons were single pass Sanger sequenced by Eurofins Genomics following a primer walking strategy. However, repetitive

194 *nad3-nad4-trnM-cox1* mtDNA in *S. thompsoni* (see Goodall-Copestake, 2017) could
195 not be sequenced using this method. Instead, the longest cloned amplicon obtained
196 with the novel flanking primers nd2 F (5'- GGAGACTGGGTGTTGGTTACT -3') and cx3
197 R (5'- TCGTGTGTGATCCCTAACTAGTC -3') was sequenced as nested deletions
198 following TOPO TA Cloning Kit (Life Technologies, Paisley, UK) instructions; Sbf I
199 then Pme I were selected to make linear plasmids with blunt 5' ends and 3'
200 overhangs and a 37°C exonuclease III digestion sampled ten times every 90 seconds
201 was used to generate successive 5' end deletions (enzymes from New England
202 BioLabs, Ipswich, USA).

203 TruSeq nano DNA library preparation and Illumina HiSeq 100bp paired-end
204 sequencing were outsourced to the University of Bristol Genomics Service (Bristol,
205 UK). Geneious v10.2 (Biomatters, Auckland, New Zealand) was used to trim
206 overrepresented and low-quality sequences identified with FastQC v0.11.5
207 (Andrews, 2010). Majority (most common base call) consensus sequences for *45S*
208 nrDNA and mtDNA genomes were generated by mapping the trimmed HiSeq reads
209 to the Sanger sequence derived templates using Geneious with a medium
210 sensitivity and paired reads only option. MITOS (Bernt *et al.*, 2013) was used to
211 guide mtDNA sequence annotation.

212 Repeated HiSeq read mapping to the majority consensus sequences was
213 used to estimate relative copy numbers, the *tbx1* gene was applied as a single copy
214 reference following Jue *et al.* (2016). A relative measure of SNP prevalence
215 (proportion of mapped reads) was inferred by identifying sites where >10% of reads
216 contained a base call differing from the consensus. Geneious structural variant

mapper was used to identify putative deletions, small insertions (limited by read length), and inversions. Relative measures of these variants were estimated by identifying sites where >0.1% of mapped reads contained a variant junction. The lower cut-off used for structural variants compared to SNPs was based on the lower frequency of such errors in HiSeq datasets (Schirmer *et al.*, 2016).

Results

Initial qPCR analysis revealed that the ratio of 18S nrDNA to 16S mtDNA in muscle tissue was consistently higher in the *S. thompsoni* samples than in *S. fusiformis* samples. For every copy of 16S, *S. thompsoni* contained on average 9 ±2.0 copies of *18S* (minimum 7, maximum 12, n=10). By contrast, in *S. fusiformis*, there were on average 3 ±0.5 copies of 18S for every copy of 16S (minimum 2, maximum 4, n=10). Although sample sizes were small, these inter-species differences in 18S:16S copy number ratios appear to be robust to the inclusion of both oozooid and blastozooid life history stages.

A total of 258,104,562 *S. thompsoni* and 279,916,278 *S. fusiformis* trimmed HiSeq reads were used for mapping to the long-range PCR derived Sanger sequenced templates. The resulting majority rule consensus sequences represent the first annotated *45S* nrDNA and mtDNA genome accessions for any salp species and as such, will provide a valuable reference for future comparative analysis (Figure 1 A, C). Average raw pairwise differences between the two species were 0.5% for *18S-5.8S-28S*, 6.9% for the ITS regions and 18.8% for mitochondrial coding DNA; the *cox1* DNA barcoding difference was 15.2%. Notable structural differences

involved the position of four tRNA genes, and in *S. thompsoni*, the presence of a near perfect sequence repeat associated with this tRNA rearrangement, and a region of tandemly repeated *nad3-nad4-trnM-cox1* coding DNA (Figure 1 C).

The average percentage of mapped reads per site was higher for *S. thompsoni* nrDNA than *S. fusiformis* nrDNA, and conversely, proportionately fewer reads mapped to *S. thompsoni* mtDNA than to *S. fusiformis* mtDNA (Figure 1 B). Copy number ratios derived from the average read depths were 9:1 for *S. thompsoni* and 2:1 for *S. fusiformis*. Considering only those sites amplified by the qPCR primers, there were 9 and 3 copies of 18S for every copy of 16S in *S. thompsoni* and *S. fusiformis*, respectively. These results were thus in accordance with those inferred using qPCR. When normalised to the read depth of the *tbx1* gene, the estimated haploid copy numbers were 484 for nrDNA and 54 for mtDNA in *S. thompsoni*, and 125 for nrDNA and 62 for mtDNA in *S. fusiformis*.

SNP and structural variant prevalence differed markedly between nrDNA and mtDNA, as well as between the salp samples (Figure 1 A, C). After correcting for sequence length, the *S. thompsoni* 45S HiSeq reads contained approximately one-third of the SNPs but twice as many structural variants as found in the *S. fusiformis* 45S reads. The *S. thompsoni* mtDNA HiSeq reads comprised greater numbers of both SNPs (3X more) and structural variants (33X more) than the *S. fusiformis* HiSeq reads.

Discussion

This study introduces nrDNA to mtDNA copy number ratios as a new metric with which to screen for functional genetic variation in evolutionary and conservation genetics studies. To investigate the potential of this metric, qPCR was used to estimate nrDNA:mtDNA copy number ratios in samples of two zooplankters, *S. thompsoni* and *S. fusiformis*, followed by deep sequencing to profile the underlying sources of genetic variation. Species-specific copy number ratios were found, as were different profiles of intra-individual nrDNA and mtDNA variation (Figure 1). Before discussing the potential functional significance of these nrDNA and mtDNA differences, it is important to consider the life-history characteristics of *S. thompsoni* and *S. fusiformis.* In particular, the capacity of salps for opportunistic population blooms and extensive asexual reproduction, which suggests that they may be prone to reductions in effective population size (Goodall-Copestake, 2017). Therefore, it is conceivable that genetic drift might have influenced, or even caused, the differences in species-specific nrDNA:mtDNA copy number ratios found in this study.

Differences in nrDNA content between *S. thompsoni* and *S. fusiformis* account for most of the difference in nrDNA:mtDNA copy number ratios between these species (Figure 1B). The larger number of nrDNA copies found in *S. thompsoni,* associated with fewer SNPs but more structural variants (Figure 1A), suggests an expansion of a subset of similar nrDNAs in *S. thompsoni* relative to *S. fusiformis.* Jue *et al.* (2016) also inferred an expansion of nrDNA in their analysis of the *S. thompsoni* genome. By incorporating gene expression results from a

286 companion study (Batta-Lona *et al.*, 2017), Jue *et al.* (2016) suggested that nrDNA
287 content had increased in *S. thompsoni* to compensate for slow metabolism due to
288 the low temperatures of its polar environment. With the added benefit of
289 comparative data from non-polar *S. fusiformis*, the results obtained herein support
290 this possibility. Salps are ectotherms and an increase in the number of actively
291 transcribed nrDNA loci, and/or a locus advantageous in cold environments, may
292 help *S. thompsoni* to achieve growth rates at temperatures ranging from -1°C to 2°C
293 that "compare favourably" to growth rate estimates of *S. fusiformis* at 15°C (Loeb
294 and Santora, 2012). At a broader functional level, the difference in nrDNA content
295 between *S. thompsoni* and *S. fusiformis* may also reflect other environmentally
296 induced changes, such as those associated with the regulation of genome-level
297 integrity and adaptability (Kobayshi, 2011).

298 mtDNA content differed less considerably than nrDNA content between *S.*
299 *thompsoni* and *S. fusiformis* (Figure 1B), and accordingly, mtDNA had less of an
300 impact on nrDNA:mtDNA copy number ratios. Unlike nrDNA, levels of mtDNA do
301 not appear to have increased in *S. thompsoni* as part of a metabolic response to
302 low temperatures. On the contrary, mtDNA copy numbers were marginally lower
303 in *S. thompsoni* than in *S. fusiformis*. *Salpa thompsoni* may thus rely on different
304 mechanisms to ensure that energy production satisfies metabolic requirements in
305 its polar environment. There are a number of possible mechanisms by which this
306 may occur, prime among which are adjustments to mitochondrial membrane
307 composition that enhance respiratory activity (Guderley, 2004). However, these
308 membrane changes are at the cost of an increase in reactive oxygen species (ROS)

309 that are known to damage DNA (Guderley, 2004; Ballard and Pichaud, 2014). While
310 speculative, the mtDNA deep sequencing results suggest that *S. thompsoni* could
311 have undergone such a change in mitochondrial membrane composition as a
312 response to the cold. The rationale for this being that the mtDNA of polar *S.*
313 *thompsoni* contains abundant signatures of damage in the form of intra-individual
314 SNPs and structural rearrangements, while the mtDNA of non-polar *S. fusiformis*
315 does not (Figure 1C). It is worth noting that, in addition to ROS-mediated damage,
316 other factors might also have contributed to the signature of mtDNA damage in *S.*
317 *thompsoni*, including genome destabilization induced by mtDNA duplications, and
318 mutations impacting the efficacy of nuclear encoded genes involved in mtDNA
319 replication and repair. Two other issues warrant mention with regard to copy
320 numbers. The first is the possibility that mtDNA copy numbers in *S. thompsoni*
321 might be impacted through the selective degradation of damaged mtDNA
322 molecules (Alexeyev *et al.*, 2013). The other is the possibility that mitochondrial
323 ROS could influence nrDNA levels through retrograde signalling driven processes
324 (mitochondrion to nucleus signalling; see Ballard and Pichaud, 2014).

325 To conclude, this study reveals how nrDNA:mtDNA copy number ratios
326 obtained from the same tissue in different individuals and species can be used as a
327 metric of potentially functional genetic variation. In the case of *S. thompsoni* and *S.*
328 *fusiformis*, differences in nrDNA:mtDNA copy number ratios and the underlying
329 sources of sequence variation were consistent with adaptation and damage at the
330 DNA level in *S. thompsoni*, potentially in response to the polar environment. This
331 raises the intriguing possibility that *S. thompsoni* may be less adapted to polar

conditions than its distribution might suggest. More extensive omics analyses of salps are required to ascertain if this is the case. Data from a broad range of other animal species, sampled under different conditions, are also needed to further explore the circumstances under which levels of nrDNA and mtDNA vary. These data will provide much-needed insights about the drivers of copy number variation and the utility of nrDNA:mtDNA copy number ratios as a comparative metric.

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Data Archiving

Sequence data have been submitted to the DNA DataBank of Japan: accession numbers LC333178-LC333181.

Conflict of Interest

The author declares no conflict of interest.

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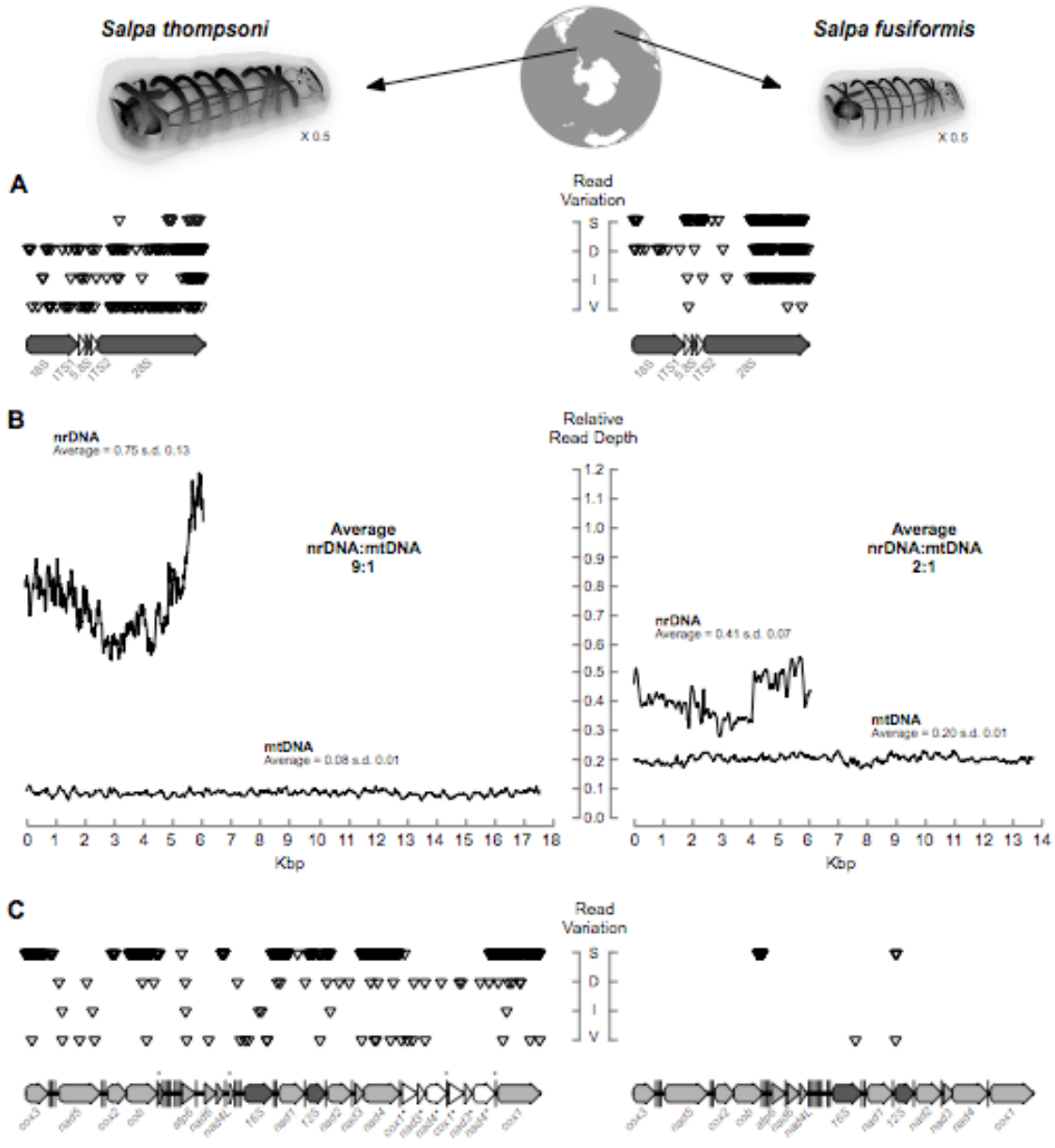
447

448 ## Figure 1 Legend

449 Variation (A, C) and relative abundance (B) along DNA sequences
450 comprising salp 45S nuclear ribosomal arrays and mitochondrial genomes. Relative
451 read depth is the proportion of total HiSeq reads mapped per nucleotide site
452 multiplied by 10,000. Read variation includes sites where >10% of mapped reads
453 contained a single nucleotide polymorphism (S); and sites where >0.1% mapped
454 reads contained deletions (D), insertions (I) or inversions (V); triangles mark the
455 mid-point of S, D, I, V feature junctions. Sampling locations and linear DNA
456 sequence maps are provided for reference; vertical bars indicate tRNA sites,
457 asterisks indicate duplicated DNA sequence features.
458

459 **Figure 1**



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