Long read sequencing reveals atypical mitochondrial genome structure in a New Zealand marine isopod

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Abstract

Most animal mitochondrial genomes are small, circular, and structurally conserved. However, recent work indicates that diverse taxa possess unusual mitochondrial genomes. In Isopoda, species in multiple lineages have atypical and rearranged mitochondrial genomes. However, more species of this speciose taxon need to be evaluated to understand the evolutionary origins of atypical mitochondrial genomes in this group. In this study, we report the presence of an atypical mitochondrial structure in the New Zealand endemic marine isopod, *Isocladus armatus.* Data from long and short read DNA sequencing, suggests that *I. armatus* has two mitochondrial chromosomes. The first chromosome consists of two mitochondrial genomes that have been inverted and fused together in a circular form, and the second chromosome consists of a single mitochondrial genome in a linearized form. This atypical mitochondrial structure has been detected in other isopod lineages, and our data from an additional divergent isopod lineage (Sphaeromatidae) lends support to the hypothesis that atypical structure evolved early in the evolution of Isopoda. Additionally, we find that a heteroplasmic site previously observed across many species within Isopoda is absent in *I. armatus*, but confirm the presence of two heteroplasmic sites recently reported in two other isopod species.

Introduction

Mitochondrial genomes display a diversity of structure across Eukaryotes (reviewed in Burger et al., 2003), varying from multiple circular chromosomes to single linear chromosomes. However, within Bilateria, mitochondrial genomes tend to be circular in structure and contain 37 genes (13 protein-coding, two rRNAs, and 22 tRNAs), with a conserved arrangement (Lavrov & Pett, 2016). Here, we refer to this structure as 'typical'. However, this structure and arrangement is not ubiquitous, as atypical mitochondrial arrangements have been found in some taxa. For example, booklice (Psocoptera) possess a multipartite mitochondrial genome consisting of two circular chromosomes (Wei et al., 2012). Thrips (Thysanoptera) also possess a multipartite mitochondrial genome with massive size asymmetry (0.9 kb and 14 kb chromosomes) (Liu et al., 2017). Tuatara, *Sphenodon punctatus*, the basally diverging lepidosaur reptile endemic to New Zealand, possesses a duplicated mitochondrial genome with a high degree of divergence between the two mitochondrial chromosomes (Macey et al., 2021).

Recent research suggests that some isopods have an atypical multipartite mitochondrial genome structure (Fig. 1) consisting of both a linear and a circular chromosome (Fig. 2) (Peccoud et al., 2017; Raimond et al., 1999). This structure is particularly common across Isopoda. The circular chromosome consists of two mitochondrial genome copies fused together in palindrome or as inverted repeats (Fig. 2A) (Peccoud et al., 2017). The second, linear, chromosome (Fig. 2B) is hypothesized to be the result of linearization and self-renaturation of a single strand of the circular chromosome during replication. Self-renaturation is possible, because the circular chromosome is made of two copies that are inverted and thus self-complementary (Peccoud et al., 2017). Aside from the presence of telomeric hairpins, this linear chromosome would be considered at a nucleotide level to be 'typical'. Throughout this paper we will refer to the circular chromosome as the "dimer" and the linear chromosome as the "monomer". We primarily refer to either the dimer, or the "unit" which represents the fundamental repeated unit across both mitochondrial chromosomes, alongside any unique sequence between the repeats.

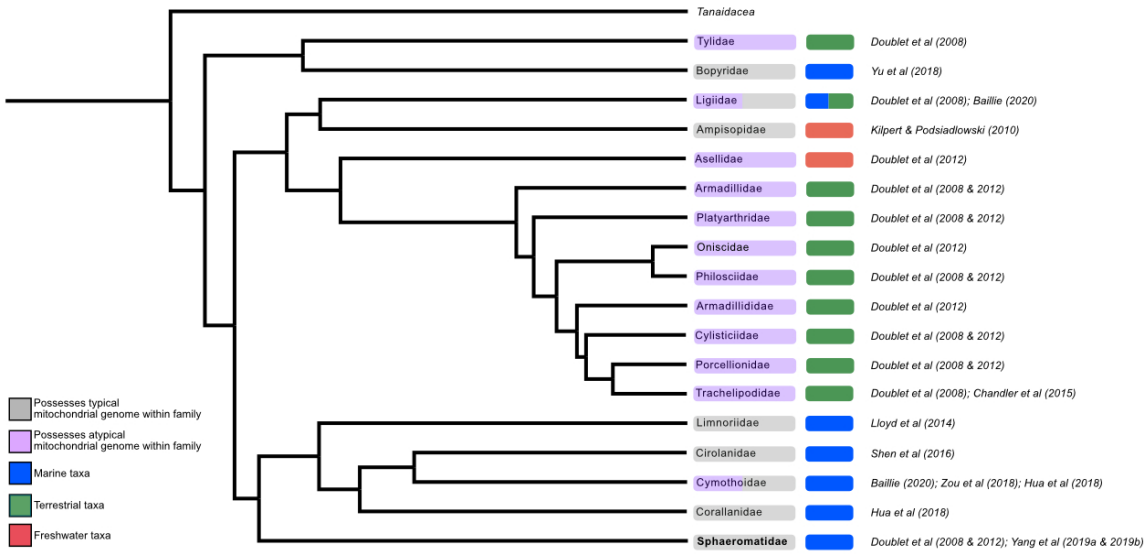


Figure 1 The currently known distribution of atypical mitochondrial (purple) across Isopoda at the family level. Purple indicates that for all species for which information is available, mitochondrial genomes are atypical in structure, while grey indicates they are typical. Families with purple and grey (in Cymothoidae and Ligiidae) indicate reports of both types of structure within the family. Green indicates that the taxa with the associated mitochondrial structure is terrestrial, red indicates freshwater, and blue indicates marine. This tree reflects the relationships based on Fig. S4 from (Lins et al., 2017), based on the nuclear 18S, 28S and mitochondrial COI genes. Sphaeromatidae, in bold, includes *Isocladus armatus* – which is the focus of this investigation.

These different copies or structural units of the mitochondrial genome in some isopod species are not entirely identical. Peccoud et al. (2017) have shown that there are single nucleotide differences in mirrored loci at tRNA sites, each encoding three different tRNAs. We refer to these types as either SNPs (when in reference to the mitochondrial unit) or as heteroplasmic sites (when in reference to the dimer), following the convention of Doublet et al., (2012).

This atypical mitochondrial genome is thought to have evolved prior to the divergence of suborders, such as Asellota and Oniscidea (Doublet et al., 2012). However, this hypothesis is complicated by the presence of 'typical' mitochondrial structure patchily dispersed in suborders such as Sphaeromatidea (family: Sphaeromatidae), Phreatoicidea (family: Amphisopodidae) , and Asellota (family: Asellidae) (Fig. 1) (Doublet et al., 2012; Kilpert et al., 2012; Yang, Gao, Hui, et al., 2019; Yang, Gao, Yan, et al., 2019).

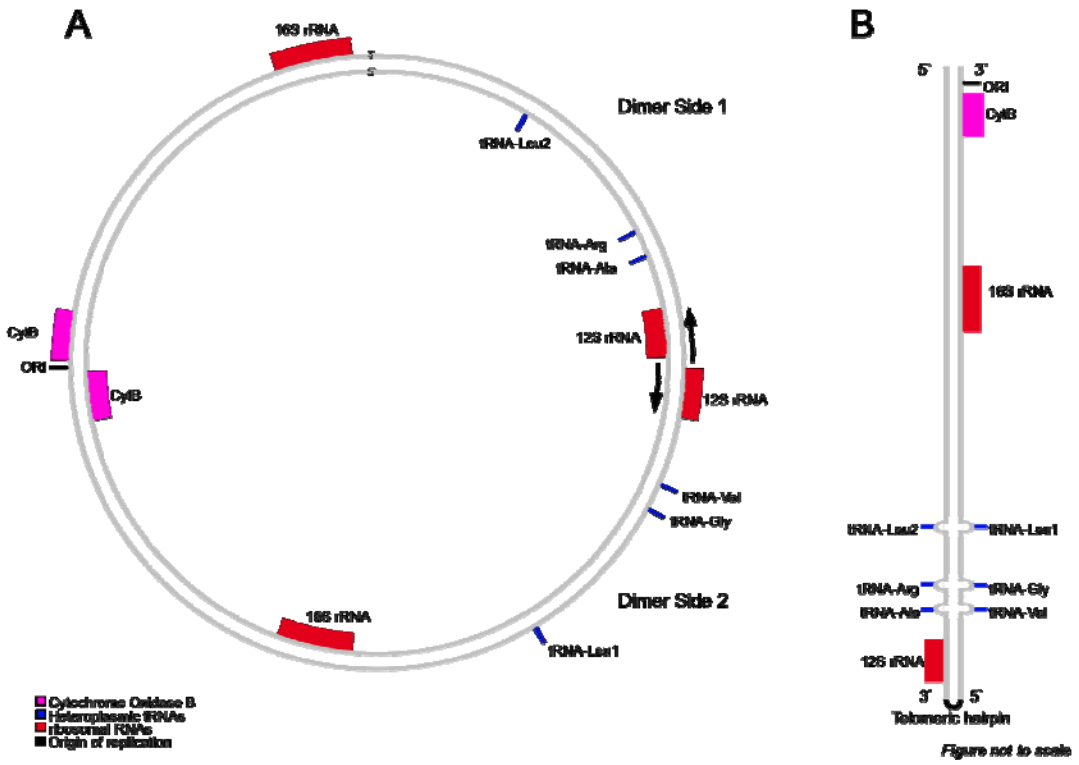


Figure 2 A. The proposed structure of the dimer. The black arrows indicate the direction of transcription, and are paired with the 12S rRNA hairpin in 2B. Putatively heteroplasmic tRNA loci are show in blue. **B.** The proposed structure of the monomer, as outlined by Peccoud et al. (2017) and Doublet et al. (2013). The monomer is a linearized copy of the dimer containing a telomeric hairpin. Importantly, there appear to be heteroplasmic sites with mismatched bases in the monomer (shown in blue, and with loops at these sites), as indicated by the presence of mirrored loci coding for different tRNAs.

A second hypothesis has recently been proposed that relates the dominant occurrence of positive GC skews within Isopoda to the patchy distribution of mitochondrial structures across Isopoda (Baillie, 2020). This hypothesis supports the early origin of atypical structure, and proposes that an early duplication event of the mitochondrial genome resulted in an inversion of the control region (CR) and a reversal of strand skews from the ancestral negative GC skew to a positive GC skew. Occasional reversions to a 'typical' structure in some lineages then explain the positive GC skews seen in these species, because they necessarily only retained the single, functional, CR during the reversion event. Whilst this hypothesis necessitates regaining function of the tRNAs encoded for by heteroplasmy, there are multiple avenues for this, such as tRNA recruitment or post-transcriptional modification (Doublet et al., 2015; Sahyoun et al., 2015).

In this study, we use long read and short read DNA sequencing to investigate the structure and arrangement of the mitochondrial genome of *Isocladus armatus*, a marine isopod of the Sphaeromatidae family, which is endemic to New Zealand. We show that the *I. armatus* mitochondrial genome is atypical in structure, possessing a 28 kb circular mitochondrial genome, similar to that found in other species of isopods. Because *Isocladus armatus* is highly diverged from other known lineages to possess atypical structure, it can help to resolve the evolutionary history of isopod mitochondrial genome structure. In addition, we observe two heteroplasmic tRNA sites that have been observed previously (Chandler et al., 2015). However, we find no

evidence of a third more widely studied heteroplasmy which causes a change from tRNA-Val to tRNA-Ala (Chandler et al., 2015).

Methods

DNA Extraction

We extracted DNA from one individual for Nanopore sequencing using a modified Qiagen DNEasy Blood and Tissue protocol, developed for *I. armatus* (Pearman et al., 2020). We extracted DNA from a second individual for Illumina sequencing using a modified Promega Wizard protocol. This protocol consisted of crushing the cephala of a specimen in a solution of chilled lysis buffer (120 µl of 0.5M EDTA and 500 µl of the provided Nuclei Lysis solution), alongside 100 µl of 1M DTT, and 30 µl of Proteinase K. The crushed sample in solution was then incubated at 65°C overnight. After overnight lysis, the sample was cooled to room temperature and 10 µl of RNAse A was added, and the sample incubated at 37°C for 30 minutes. Following this, 250 µl of protein precipitation solution was added, and the protocol was completed according to manufacturer's instructions (page 11, Promega #TM050).

Sequencing and Quality Control

DNA from both individuals was sequenced using both Oxford Nanopore and Illumina sequencing. For Nanopore sequencing, we followed the manufacturers protocol for native barcoding of genomic DNA for the SQK-LSK109 kit (protocol version: NBE_9065_v109_revV_14Aug2019) with a R9.4 RevD flow cell. Nanopore reads were basecalled using Guppy 3.4.3, and demultiplexed and adapters removed using PoreChop (https://github.com/rrwick/Porechop).

Illumina sequencing was carried out on an Illumina NovaSeq using 150 bp paired end reads, with an insert size of 150 bp. Potential contaminant reads from bacteria, human, or viruses were identified and discarded using Kraken2 with the maxikraken2 database (maxikraken2_1903_140GB, https://lomanlab.github.io/mockcommunity/mc_databases.html)

Assembly

Nanopore reads were assembled into a draft genome using *Flye* (Kolmogorov et al., 2019) under default parameters with an estimated genome size of 1 GB. The mitochondrial contigs were identified by mapping all contigs to the mitochondrial genome of *Sphaeroma serratum* (Kilpert et al., 2012), resulting in the identification of a single mitochondrial contig. We then mapped all Nanopore reads to this contig, and performed a re-assembly using only the reads that mapped to the initial mitochondrial contig. For this assembly, the default settings were used with an estimated genome size of 28 kb (this size was selected based on the concordance between assembly size of the first mitochondrial contig, the size of other isopod mitochondrial genomes, and the length of the longest Nanopore reads of mitochondrial origin.).

The atypical mitochondrial structure we hypothesised (shown in Fig. 2) precluded complete polished assemblies of this mitochondrial genome, as Illumina reads were too short to be able to orient on either side of the dimer, as only reads containing the junctions can be accurately oriented. Thus, we used Geneious 9.1 (Drummond et al., 2011) to extract the 'monomer' from the assembly, and manually identified the primary repeat based on a self-self dotplot of the full length mitochondrial genome using YASS (Baillie, 2020; Noé & Kucherov, 2005) (Supp. Fig. 1). This was manually extracted alongside any unique sequence either side of the monomer, and this

was polished three times with Illumina reads using racon (Vaser et al., 2017), and BWA (Li & Durbin, 2009) as a mapper. This contig was then visualized in IGV (Robinson et al., 2011) and three putative single base pair indels (insertions/deletions) were removed based on having coverage of less than 5% of the adjacent sites.

Annotation

This monomer was then annotated using MITOS2 (Bernt et al., 2013), with the Al Arab protein prediction method (Al Arab et al., 2017), a non-circular assembly, a final maximum overlap of 150 bp (the number of bases that genes of different types [i.e. tRNA and rRNA] can overlap), and fragment overlap of 40 % (the fraction of the shorter sequence that can overlap with the larger sequence). These overlap values were based on existing research indicating high levels of gene overlap within isopod mitochondrial genomes (Doublet et al., 2015; Zou et al., 2018). MITOS2 also highlights potential gene duplicates and the modification of the overlap settings may increase the likelihood of erroneously identifying gene duplication. Thus, we removed annotations for potential duplicates where there was an order of magnitude difference in quality value (analogous to a BLAST e-value, low quality values are frequently spurious [http://mitos.bioinf.uni-leipzig.de/help.py]) between annotations of potential duplicates, retaining the annotation with the highest quality factor. GC-skew was calculated to confirm the position of the origin of replication using GenSkew (https://genskew.csb.univie.ac.at/), using a step size of 20 bp and window size of 100 bp (Doublet et al., 2013).

Heteroplasmic sites were identified by treating them as SNPs (Peccoud et al., 2017). These variants were identified using BWA (Li & Durbin, 2009) and bcftools (Li, 2011) with a minimum depth of 1000 X and a maximum depth of 8000 X.

Results

Assemblies

Nanopore sequencing on the genomic DNA from one individual was performed, and after assembly with *Flye* we identified a single mitochondrial contig. 1,817 of the Nanopore reads could be mapped back to this contig (median length 1405 bp, max length 27,851 bp). The distribution of read lengths was roughly trimodal, with peaks at approximately 1400 bp, 14,000 bp, and 28,000 bp. The peak at 1400 bp is likely the result of shearing and incorporating of low molecular weight DNA in the library, while the smaller peaks observed at 14 kb and 28 kb may result from the presence of two full length chromosomes (Supp. Fig. 2). Using *Flye* we performed a re-assembly using the 1,817 reads, resulting in a single 28,745 bp circular contig (Fig. 3), with a mean coverage of 194 X.

The assembled mitochondrial genome for *Isocladus armatus* thus consists of a 28.7 kb circular chromosome consisting of two inverted repeats of a ‘typical’ mitochondrial genome. The mitochondrial unit is 14,382 bp long, and the junctions between copies of the unit comprises a total of 186 bp (Supp. Fig. 2). These junctions are located between each copy of the 12S gene, and each copy of the tRNA-Glu gene. The junction between copies of the 12S gene is 155 bp in length, while the junction between the tRNA-Glu loci is 591 bp in length.

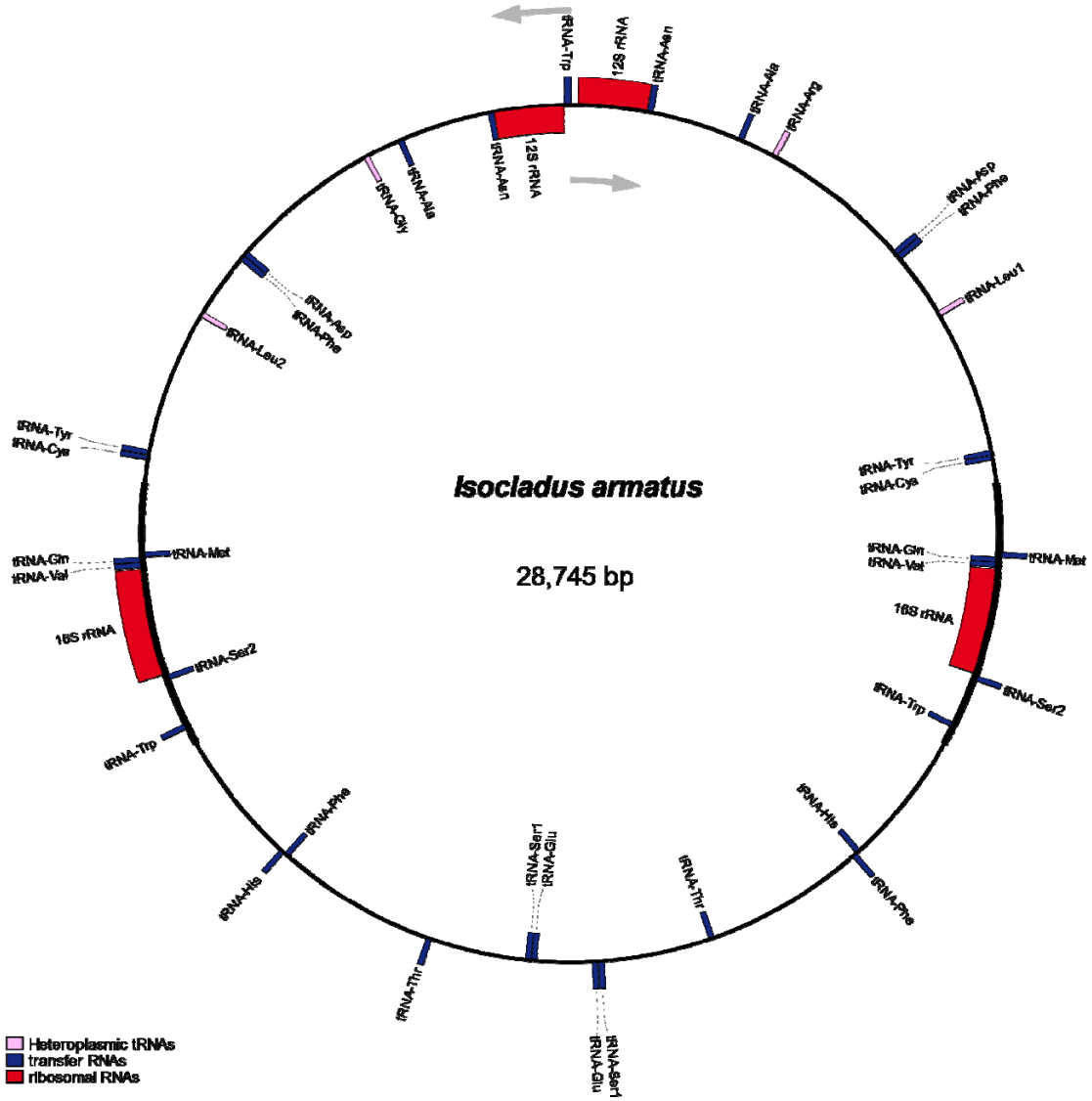


Figure 3 Nanopore read only assembled mitochondrial genome for *Isocladus armatus*. Grey arrows indicate the direction of transcription, blue sites indicate transfer tRNAs, pink sites indicate tRNA loci whose function varies depending on side of the dimer (heteroplasmic sites), while red sites indicate rRNAs. Annotations were created using MITOS2, and figure created using OGDRAW2.

Each unit is composed of the 13 protein-coding genes, 18 tRNAs, and 2 rRNAs. Of these tRNAs, two contain variants at mirrored sides of the dimer that enable coding for a different tRNA. Finally, another tRNA locus is found within the 16S junction, and is not duplicated. As a result, the complete dimer contains 13 duplicated protein-coding genes, 16 duplicated tRNAs, 5unique tRNAs, and 2 duplicated rRNAs (Fig. 3).

The unique component of the dimer (the 'unit' of the mitochondria, together with the junctions between the two copies on the dimer) was extracted, and polished with racon using Illumina data, producing a linear unit 14,569 bp long (Supp Fig. 3, GenBank: OK245257). This unit represents the repeated mitochondrial unit, alongside the unique sequences found in the junctions between

repeats, and has a positive GC skew. We selected this unit as it represents the complete unique mitochondrial genome for this species, with the exception of any single base pair heteroplasmies.

The mitochondrial unit had a mean coverage with Illumina reads of 6537x (Fig. 4), containing 13 protein coding genes, 19 tRNAs, and 2 rRNAs. Two of the tRNAs appeared heteroplasmic in nature.

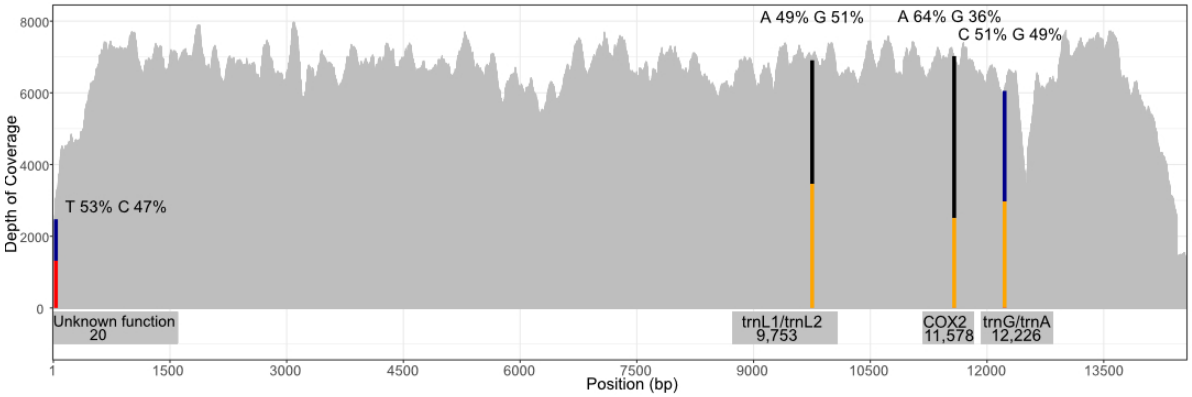


Figure 4 Illumina coverage of monomer, coloured bars indicate relative frequency of each base at the four SNP sites. Width of bars in these cases are not to scale. Captions within figure indicate function and position within the monomer.

Structure of the mitogenome

We identified four single base pair mitochondrial variants (Table 1, Fig. 5) using the Illumina reads and bcftools (see Methods) (Fig. 4). Two of these SNPs were at tRNA loci located either side of the dimer, resulting in a change in the tRNA encoded at that locus. A third variant was present at an approximately 50:50 ratio within the junction between sides of the dimer. This locus has no known function. Finally, the fourth variant was a synonymous substitution in the COX3 gene, present at a 2:1 ratio. This latter variant was also conspicuously absent in the Nanopore data.

*Table 1 'SNPs' identified in the monomer using bcftools. Four SNPs have been identified, three of which appear to be present in coding regions of the mitochondria. * indicates presence only in Illumina sequencing data.*

Position	Bases	Function	Frequency of variant (%)
20	T-C	Non-coding	1315:1158 (53:47)
9,753	A-G	tRNA-Leu1/tRNA-Leu2	3347:3460 (49:51)
11,578*	A-G	Unknown CAA-CAG Gln-Gln anticodon substitution in the COX3 gene	4517:2504 (64:36)
12,226	C-G	tRNA-Gly/tRNA-Arg	3094:2963 (51:49)

Overall, mitochondrial gene order in *I. armatus* was relatively consistent with other isopod species. As has been documented in other species of isopods, we found that the trnI locus was absent in *I. armatus* (Kilpert et al., 2012; Zou et al., 2018).

Discussion

Using a combination of long and short read DNA sequencing, we have shown that the marine isopod, *Isocladus armatus,* exhibits an atypical mitochondrial genome structure. This atypical structure consists of a circular 28.7 kb chromosome containing two copies of a 'typical' mitochondria fused together as inverted repeats. This circular structure is similar in size to the mitochondrial genomes found in other isopods with atypical structure (Chandler et al., 2015; Raimond et al., 1999). We identified three sites that represent differences between copies of these repeats (termed heteroplasmies). The first site is a novel single base pair substitution that occurs in a non-coding region near the junction of the repeats and appears to be unique to *I. armatus*. The other two heteroplasmies have been previously identified in other species of isopods (Chandler et al., 2015; Peccoud et al., 2017). These two sites (positions 9,753, and 12,226) are responsible for the change in tRNA function at these loci between copies of the dimer due to a single base change in the anticodon. We also provide evidence for a putative second chromosome, which is approximately 14 kb and is, in other isopods, a linearized copy of a single mitochondrial genome (non-duplicated). We term this chromosome the 'monomer'. While sequencing reads could not be assigned to the specific mitochondrial chromosome, read length distributions from nanopore sequence suggest the possible presence of this monomer.

The two heteroplasmic sites (tRNA-Gly-Arg, and tRNA-Leu1-Leu2) we observe were recently described in three species of Oniscid isopods (Chandler et al., 2015). The presence of these sites in *I. armatus* indicates that both the atypical structure, and heteroplasmic sites have been maintained over evolutionary time for hundreds of millions of years, as the most recent common ancestor between a Sphaeromatid and Oniscid isopod existed approximately 400 million years ago (Lins et al., 2012). Our data indicate that the heteroplasmic sites observed in *I. armatus*, have been conserved for approximately 150 million years longer than previous estimates (Chandler et al., 2015; Doublet et al., 2012). This is not particularly surprising as it is likely that this structure is maintained by balancing selection, as the loss of a tRNA would likely be lethal (Peccoud et al., 2017).

We identified heteroplasmies located on the tRNA-Arg/tRNA-Gly genes of the dimer. These sites are commonly 'heteroplasmic' in species with an 'atypical' mitochondrial genome, therefore enabling the expression of both tRNAs (Chandler et al., 2015). However, the tRNA-Arg gene is missing in some isopod species with a 'typical' mitochondrial genome structure (Kilpert et al., 2012; Yang, Gao, Hui, et al., 2019), while the tRNA-Gly gene is lacking in the Sphaeromatid isopod, *Sphaeroma terebrans* (M. Yang, Gao, Yan, et al., 2019). The lack of these specific tRNA loci in some isopods with 'typical' mitochondrial genome structures is puzzling because these lineages appear to have lost functionality of at least one tRNA locus. Despite being absent from mitochondrial gene annotations, the critical role that these tRNAs play in cellular function precludes the possibility that they are unexpressed. Instead, mechanisms such as multiple mitochondrial haplotypes or post-transcriptional modification may play a role in preserving the function of these genes. For example, Doublet et al. (2013) found evidence for multiple mitochondrial haplotypes that could ensure continued functioning of these genes in the genus *Armadillidium.* Additionally, post-transcriptional modification has been proposed as an

252 explanation for continued functionality of other tRNAs in *Ligia oceanica* (Kilpert et al., 2012). This
253 has also been directly observed in other isopods such as *Armadillidium vulgare,* where it has
254 been found to influence expression of the tRNA-His locus (Doublet et al., 2015).

255 In addition to heteroplasmies associated with tRNA function, we observed a fourth variable site
256 that we were unable to identify as heteroplasmic. This site was only present in the Illumina
257 sequencing data and was conspicuously absent in the Nanopore data. As a result, we are unable
258 to identify whether this site is variable between sides of the dimer, as short-read Illumina data
259 would not facilitate the determination of read orientation. However, we expect the variation at this
260 locus does not occur on the dimer (i.e. only one haplotype of the SNP is present on the dimer),
261 because when treating these variable sites as SNPs, heteroplasmic loci appear at a 1:1 ratio,
262 while this SNP occurs at a 2:1 ratio (with near 1:1 abundances for each SNP on the forward and
263 reverse strands). This SNP occurs in the COX3 gene and relates to a synonymous substitution in
264 a tRNA-Gln anticodon. We are unable to determine whether this variant is found universally in *I.*
265 *armatus*, because we only sequenced two individuals, using two different sequencing
266 approaches.

267 In *I. armatus*, there are two potential explanations for the presence of this SNP. Given the
268 occurrence of this SNP at a 2:1 ratio, one possibility is that this SNP occurs on the monomer
269 where the monomer represents c. 33% of copies of the mitochondrial unit. Peccoud et al (2017)
270 proposed that the monomer may simply be the result of self-renaturation of the dimer during
271 replication. Thus it would be non-functional due to the presence of mismatching bases at
272 heteroplasmic sites on reads originating from the monomer. Alternatively, the absence of this
273 SNP in Nanopore data, despite the presence of three other SNPs, could suggest the presence of
274 multiple mitochondrial haplotypes within an individual. We are unable to test either hypothesis
275 with the available data, because the sequences arise from two different individuals and may be
276 the result of rare or transient structures (Doublet et al., 2013).

277 Atypical mitochondrial structure, as described here, is patchily distributed across Isopoda (Fig. 1),
278 and sporadically present in various distantly-related aquatic and terrestrial lineages of isopods
279 (Baillie, 2020; Doublet et al., 2012) of both derived and ancestral origin. One hypothesis that has
280 been proposed to explain this distribution, is that atypical structure evolved early in the evolution
281 of Isopoda, prior to the division of sub-orders, and has been subsequently lost via reversion at
282 least three times across the order (Doublet et al., 2012). Our findings provide further support to
283 an early origin of atypical structure, because our research confirms its presence in a marine
284 isopod highly diverged from any other species hitherto known to possess this structure.

285 The patchy distribution of mitochondrial structures across Isopoda could be explained from a
286 functional perspective by a “terrestrial adaptation hypothesis”. Under this hypothesis, we suggest
287 that atypical structure may have provided an adaptive advantage to the ancestors of modern-day
288 terrestrial isopods in the transition out of a marine environment. Indeed, it has been posited that
289 mitochondrial heteroplasmy introduces additional genetic variation that may play a role in
290 increasing the ability of cells to withstand stressors (Giuliani et al., 2014; Hirose et al., 2018;
291 Leeuwen et al., 2008). Indeed, in the tuatara, mitochondrial duplication has been proposed as a
292 contributor to thermal adaptation, indicating a potential role for structural mitochondrial variants in
293 physiological adaptation (Macey et al., 2021).

294 The terrestrial adaptation hypothesis is supported by the presence of atypical structure for all
295 terrestrial isopods for which there are data (Fig. 1). A possible exception to this is *Ligia oceanica*,
296 which has contrasting reports of atypical mitochondrial structure (Baillie, 2020; Doublet et al.,

2012; Kilpert & Podsiadlowski, 2006). However, this littoral species is arguably semi-aquatic, possesses numerous transitional traits (Michel-Salzat & Bouchon, 2000; Raupach et al., 2014; Schmidt, 2008), and has recently been found to be more closely related to Valvifera or Sphaeromatidea than to the rest of Oniscidea (Dimitriou et al., 2019; Lins et al., 2017). An early origin of atypical structure does not, however, necessarily preclude the hypothesis that atypical structure is adaptive in the terrestrial environment. If this feature did indeed play an adaptive role in the transition to a terrestrial environment, then an early origin and subsequent retention of this feature through selection in the Oniscidea and some Ligiidae, with this trait being lost multiple times among the aquatic isopod suborders, could explain the current distribution of atypical structure. However, it remains to be determined why this trait has been maintained among several aquatic lineages, including *Isocladus*.

An alternative view however, is that the presence of atypical structure across the order is a result of convergent evolution, that is multiple independent origins of atypical structure. This hypothesis has recently gained traction from the recent phylogenetic evidence for multiple independent terrestrial transitions within Isopoda (Dimitriou et al., 2019; Lins et al., 2017). This hypothesis necessitates, however, the repeated duplication of the mitochondrial unit, as well as the evolution of tRNA heteroplasmies (which appear relatively conserved across Isopoda). This explanation is therefore less parsimonious relative to the hypothesis of early origins followed by multiple reversions to typical structure. To distinguish between these hypotheses, it is necessary to determine the mitochondrial arrangement of more species of marine, and transitional marine, species; in particular those within the predominantly supralittoral family Tylidae.

Conclusion

Isocladus armatus possesses an atypical mitochondrial genome consisting of two chromosomes – a 28.7 kb circular dimer, and likely also a 14 kb monomer. The 28.7 kb dimer consists of two copies of the monomer fused together as inverted repeats. Three variant sites occur that differentiate the, otherwise complementary, sides of the 28 kb dimer: the first of these is within a non-coding region, while the other two variants occur in two tRNA genes and cause non-synonymous changes in the tRNA anticodon between sides of the dimer. The presence of this atypical structure in a Sphaeromatid isopod, alongside the differences between sides of the dimer, supports the hypothesis that this structure originated early in the evolution of Isopoda, and the presence of the typical structure in some isopods may be the result of reversion to the ancestral metazoan form.

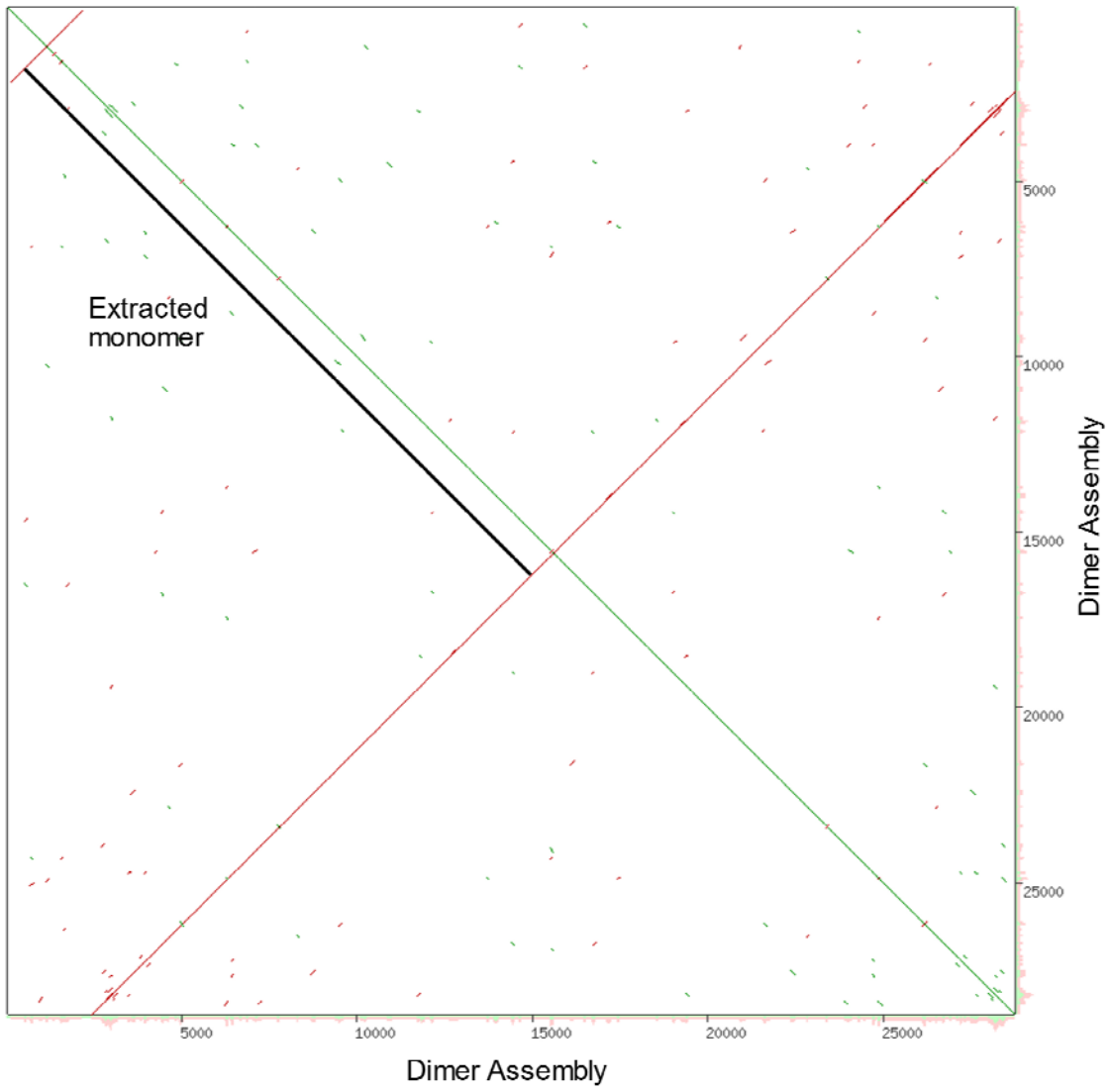
Data Accessibility Statement

The assembled mitochondrial unit has been uploaded to GenBank under the accession: OK245257.

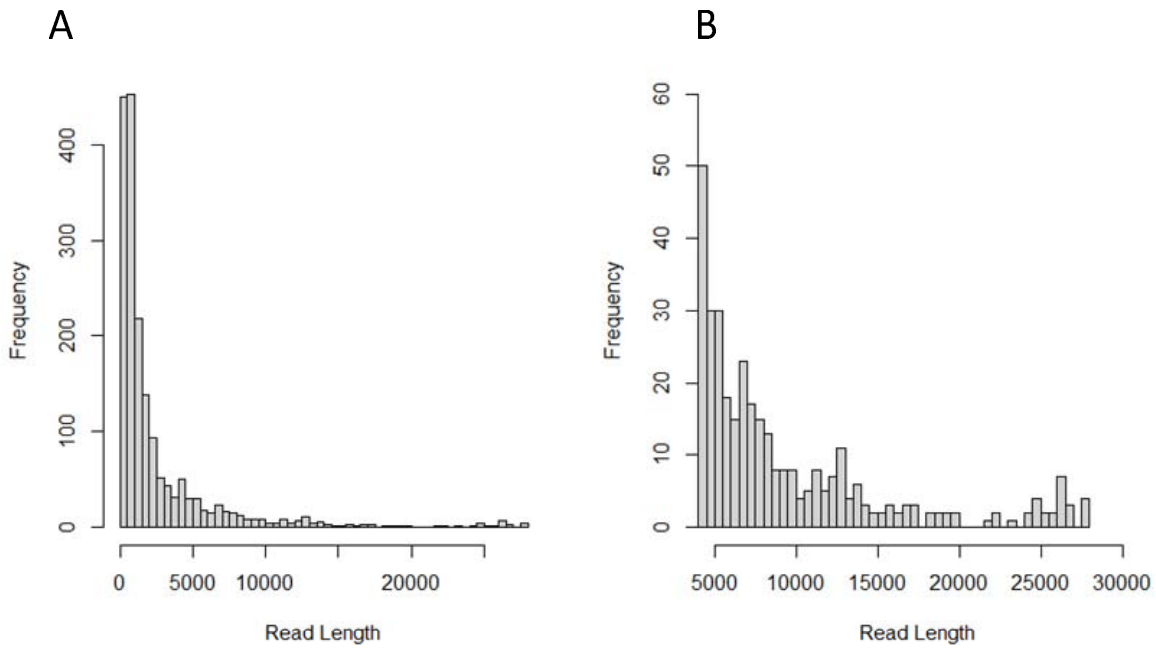
Acknowledgements

We would like to acknowledge the contributions of Stephen Shuster and Christopher Chandler, both of whom provided insight into the mechanisms behind this atypical structure.

Supplementary Figures



Supplementary Figure 1: The dotplot used to identify the primary mitochondrial unit, the green line indicates a 1:1 relationship between the forward strand and itself, while the red line indicates the relationship between forward strand and the reverse strand. The black line indicates the section that was extracted and considered the primary mitochondrial unit, inclusive of any unique sequences found within the genome – this is why the red lines do not equal the length of the green line.



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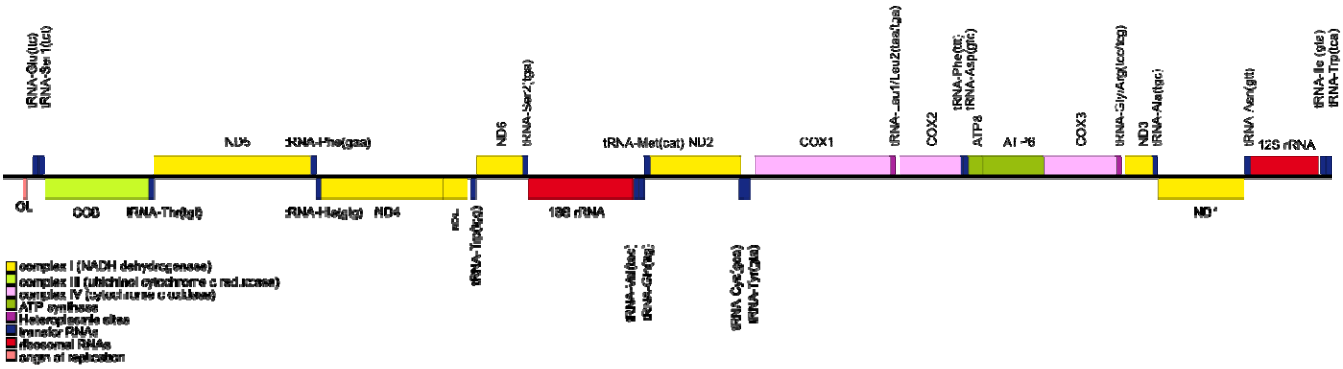
343 Supplementary Figure 2 Read length distributions for all mitochondrial originating reads (A) and all mitochondrial
344 originating reads greater than 5000 bp (B).



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346 Supplementary Figure 3 Annotated polished copy of the mitochondrial 'unit' for Isocladus armatus. This unit is a total of
347 14,569 bp, annotations were created using MITOS2, and figure created using OGDRAW2.

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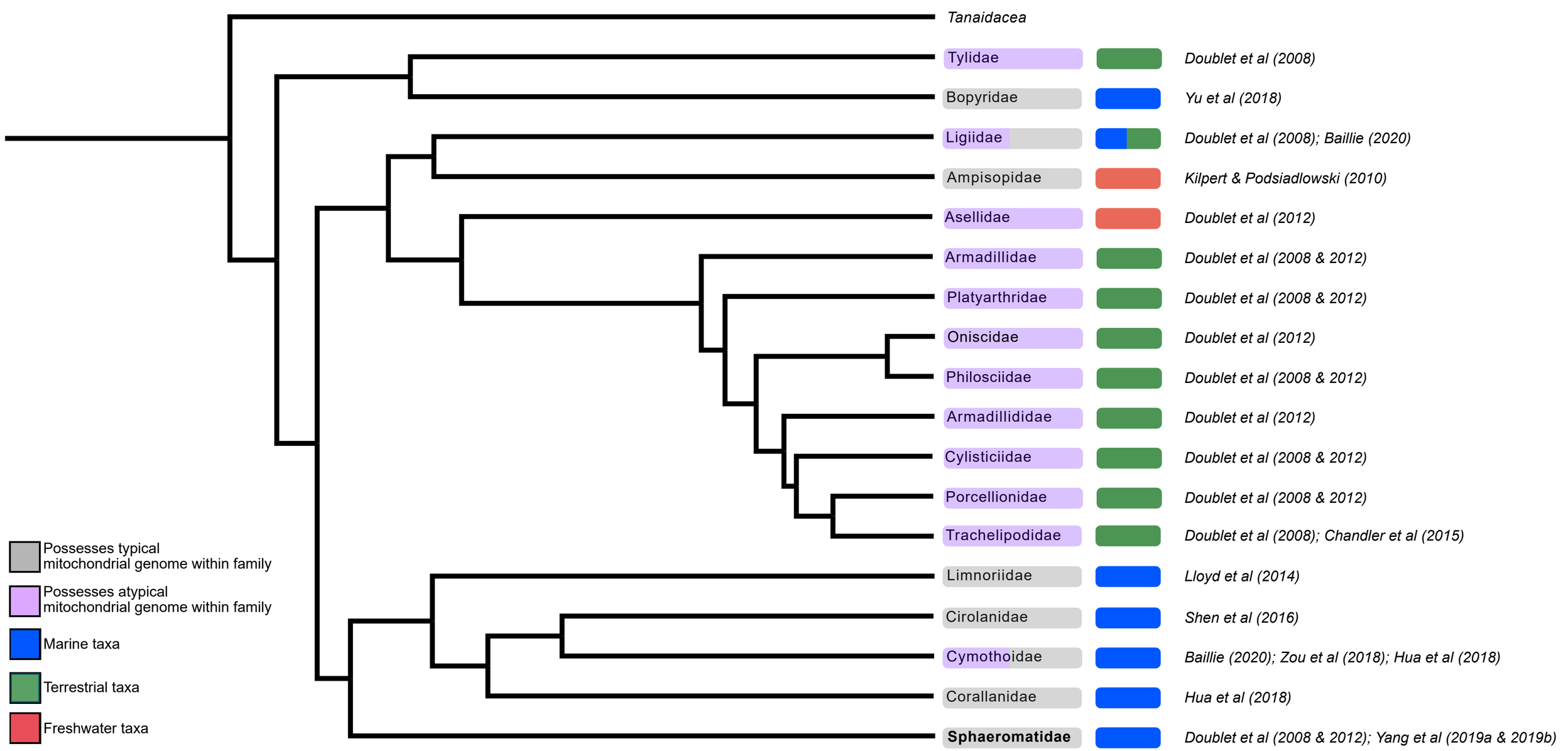

Tanaidacea
Tylidae
Doublet et al (2008)
Bopyridae
Yu et al (2018)
Ligiidae
Doublet et al (2008); Baillie (2020)
Ampisopidae
Kilpert & Podsiadlowski (2010)
Asellidae
Doublet et al (2012)
Armadillidae
Doublet et al (2008 & 2012)
Platyarthridae
Doublet et al (2008 & 2012)
Oniscidae
Doublet et al (2012)
Philosciidae
Doublet et al (2008 & 2012)
Armadillididae
Doublet et al (2012)
Cylisticiidae
Doublet et al (2008 & 2012)
Porcellionidae
Doublet et al (2008 & 2012)
Trachelipodidae
Doublet et al (2008); Chandler et al (2015)
Limnoriidae
Lloyd et al (2014)
Cirolanidae
Shen et al (2016)
Cymothoidae
Baillie (2020); Zou et al (2018); Hua et al (2018)
Corallanidae
Hua et al (2018)
Sphaeromatidae
Doublet et al (2008 & 2012); Yang et al (2019a & 2019b)
Possesses typical mitochondrial genome within family
Possesses atypical mitochondrial genome within family
Marine taxa
Terrestrial taxa
Freshwater taxa

A

16S rRNA

3`

5`

Dimer Side 1

tRNA-Leu2

tRNA-Arg

tRNA-Ala

12S rRNA

CytB

ORI

CytB

12S rRNA

tRNA-Val

tRNA-Gly

Dimer Side 2

16S rRNA

tRNA-Leu1

Cytochrome Oxidase B

Heteroplasmic tRNAs

ribosomal RNAs

Origin of replication

B

5`

3`

ORI

CytB

16S rRNA

tRNA-Leu2

tRNA-Leu1

tRNA-Arg

tRNA-Gly

tRNA-Ala

tRNA-Val

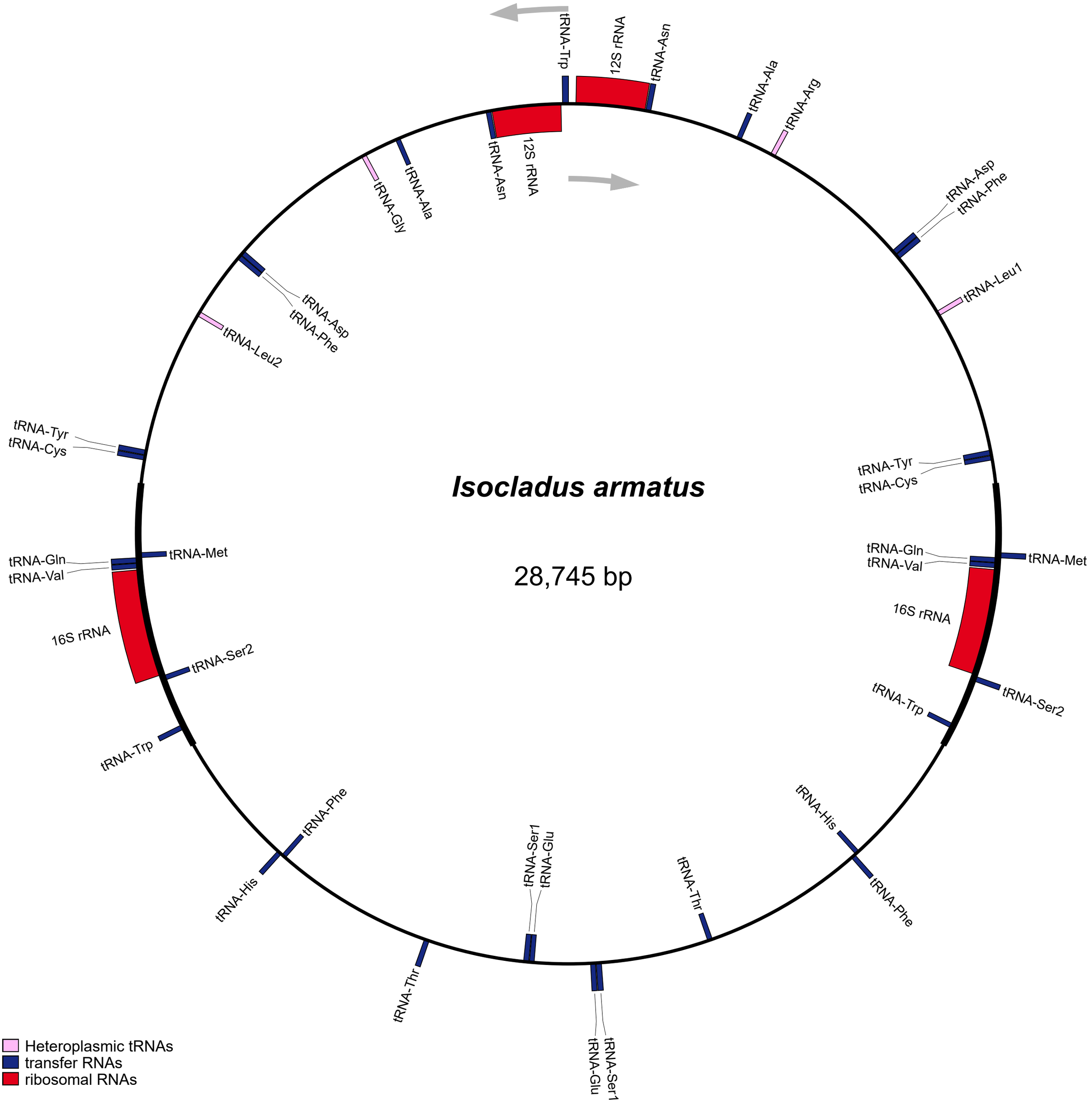
12S rRNA

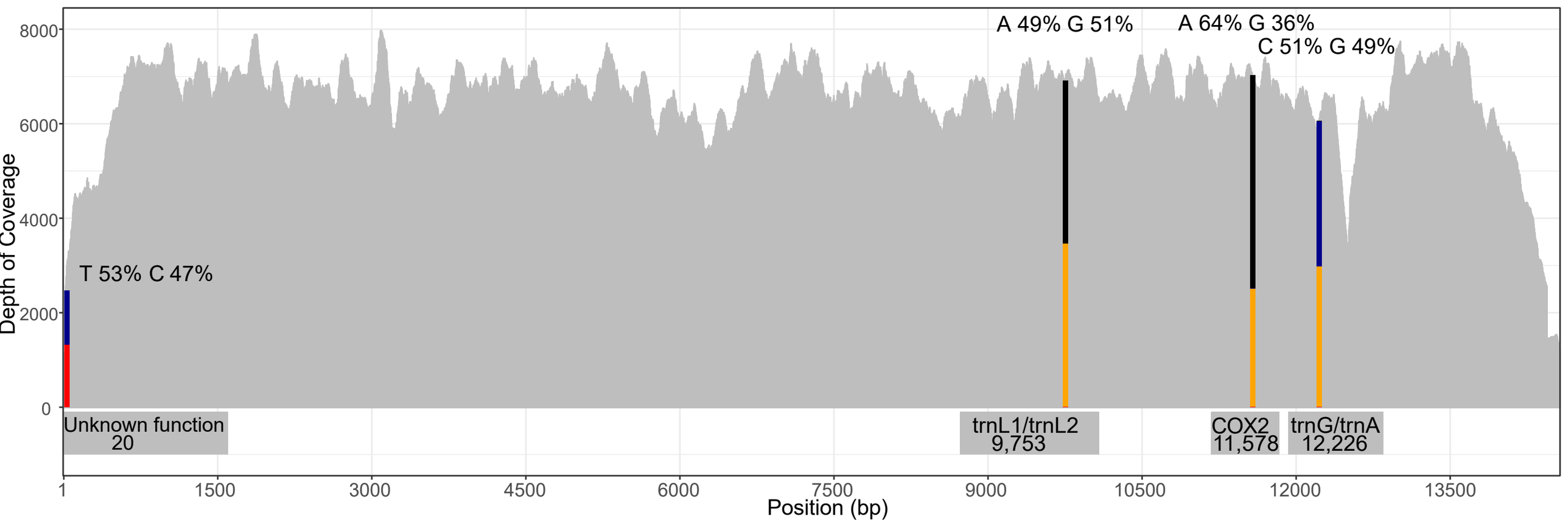
3`

5`

Telomeric hairpin

Figure not to scale


Isocladus armatus
28,745 bp
tRNA-Trp
12S rRNA
tRNA-Asn
tRNA-Ala
tRNA-Arg
tRNA-Asp
tRNA-Phe
tRNA-Leu1
tRNA-Tyr
tRNA-Cys
tRNA-Gln
tRNA-Val
tRNA-Met
16S rRNA
tRNA-Ser2
tRNA-Trp
tRNA-His
tRNA-Phe
tRNA-Thr
tRNA-Ser1
tRNA-Glu
tRNA-Gly
tRNA-Leu2
Heteroplasmic tRNAs
transfer RNAs
ribosomal RNAs


A 49% G 51%
A 64% G 36%
C 51% G 49%
T 53% C 47%
Depth of Coverage
8000
6000
4000
2000
0
Unknown function
20
trnL1/trnL2
9,753
COX2
11,578
trnG/trnA
12,226
1
1500
3000
4500
6000
7500
9000
10500
12000
13500
Position (bp)