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Structural characterization and evolutionary analyses of the *Coccidioides immitis* and *Coccidioides posadasii* mitochondrial genomes

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17   **Keywords: Coccidioides, coccidioidomycosis, mitochondrial, introns group I and II.**

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Abstract

Fungal mitochondrial genomes encode for genes involved in crucial cellular processes, such as oxidative phosphorylation and mitochondrial translation, and these genes have been used as molecular markers for population genetics studies. *Coccidioides immitis* and *C. posadasii* are endemic fungal pathogens that cause coccidioidomycosis in arid regions across both American continents. To date, almost one hundred *Coccidioides* strains have been sequenced. The focus of these studies has been exclusively to infer patterns of variation of nuclear genomes (nucDNA). However, their mitochondrial genomes (mtDNA) have not been studied. In this report, we describe the assembly and annotation of mitochondrial reference genomes for two representative strains of *C. posadasii* and *C. immitis*, as well as assess population variation among 77 published genomes. The circular-mapping mtDNA molecules are 68.2 Kb in *C. immitis* and 75.1 Kb in *C. posadasii*. We identified the fourteen mitochondrial protein-coding genes common to most fungal mitochondria, including genes encoding the small and large ribosomal RNAs (*rns* and *rnl*), the RNA subunit of RNAse P (*rnp*B), and 26 tRNAs organized in polycistronic transcription units, which are mostly syntenic across different populations and species of *Coccidioides*. Both *Coccidioides* species are characterized by a large number of group I and II introns, harboring twice the number of elements as compared to closely related Onygenales. The introns contain complete or truncated ORFs with high similarity to homing endonucleases of the LAGLIDADG and GIY-YIG families. Phylogenetic comparison of the mtDNA and nucDNA genomes shows discordance, possibly due to differences in patterns of inheritance. In summary, this work represents the first complete assessment of mitochondrial genomes among several isolates of both species of *Coccidioides*, and provides a foundation for future functional work.

Introduction

47   Fungal mitochondrial genomes exist as either linear or circular-mapping molecules and range from

49   ~17.6 kb (e.g. *Schizosaccharomyces pombe* Genbank ID MK618090.1) to well over 200 kb (e.g.

50   272,238 bp in *Morchella importuna* (1)). Fungal mitochondrial genomes usually encode proteins

51   involved in oxidative phosphorylation - the main source of ATP production of the cell - as well as

52   two ribosomal RNA subunits, and a set of tRNAs involved in mitochondrial ribosome translation.

53   More specifically, fungal mitochondrial protein-coding genes fall into several classes: seven subunits

54   of ubiquinone oxidoreductase (*nad;* not present in a number of Saccharomycotina and in fission

55   yeasts, (2)), cytochrome b (*cob*), three subunits of cytochrome oxidase (*cox*) and up to three ATP

56   synthase subunits (*atp*; the presence of *atp8* and *atp9* varies among fungal taxa) (3). Also, a gene

57   encoding a ribosomal protein subunit (*rps3*) is present in most fungal mitochondrial genomes.

58   Mitochondrial protein-coding genes are frequently intercalated with genes that encode structural

59   RNAs: ribosomal RNAs (small and large subunit rRNAs *rns* and *rnl*), the RNA subunit of RNase P

60   (*rnpB*) with infrequent occurrence across fungi, and variable numbers of tRNAs. Notable exceptions

61   are the *nad* genes, which tend to be organized in operon-like structures, with some of the genes

62   overlapping without discernable intergenic regions (e.g., *nad4L* situated upstream of *nad5*,

63   overlapping by one to a dozen or more nucleotides) (3).

64   Mitochondrial genes in fungi contain highly variable numbers of group I and II introns that

65   are inserted in protein-coding as well as rRNA genes (4). For instance, *Endoconidiophora* species

66   seem to contain more than 80 mitochondrial introns (5), which can create gene annotation challenges

3

67 especially when transcriptome data are not available. Both intron groups may contain complete or

68 truncated ORFs that encode either homing endonucleases of the LAGLIDADG and GIY-YIG

69 families, or reverse transcriptases/maturases (6). If present, these proteins direct an intron transfer

70 within mitochondrial genomes of genetically compatible fungal isolates, or less frequently across

71 genera, and even kingdom boundaries (7). Mitochondrial DNA (mtDNA)-encoded genes are

72 particularly prone to crossing species boundaries. As intron transfer *via* homing endonucleases

73 involves genetic co-conversion of flanking exon sequences, phylogenetic inferences using mtDNA—

74 especially genes with high intron numbers (e.g., *cox1*, *cob* and *rnl* (3, 8))— may reveal replacement

75 of coding regions, related to ongoing intron invasion.

76   In this study, we focus on describing the mitogenomes of *Coccidioides immitis* and *C.*

77 *posadasii* (Ascomycota, Onygenales), which are fungal species endemic to both American

78 continents, and the causative agents of coccidioidomycosis (9). This disease is most frequently

79 reported in the "Lower Sonoran Life Zone" in California, Arizona, Texas, and northwestern Mexico

80 (10). However, the disease is also reported in arid and semi-arid areas throughout the American

81 continents (11). The two species have a complex evolutionary history dominated by biogeographic

82 distribution patterns (12, 13). *Coccidioides immitis* has been found in California and Baja Mexico as

83 well in eastern Washington state, and each region harbors unique genotypes (14-16). *Coccidioides*

84 *posadasii* is present throughout Arizona, Texas, Central, and South America, and population

85 structure has been described as containing an Arizona population, a Texas/Mexico/South America

86 (TX/MX/SA) population, and a distinct Caribbean population (13).

4

87   Notably, nucDNA studies have found extensive differentiation between species of

88   *Coccidioides* with some evidence for gene flow between species (17, 18). The two species, *C.*

89   *immitis* and *C. posadasii,* can be discriminated based on polymorphisms found at the first intron of

90   the *cox1* gene (19). Yet, no studies have addressed whether or not mtDNA reflects the divergence of

91   ncDNA, or if mtDNA has moved between *Coccidioides* species or among populations. In this study

92   we: *i*) describe the full circular-mapping mitogenomes of *C. posadasii* and *C. immitis*, *ii*) compare

93   their core genes, structural RNAs and introns of group I and II with other Onygenales fungal species,

94   and *iii*) compare the evolutionary trajectories between the mtDNA and nucDNA genomes among

95   publicly available genomes of this medically important fungal pathogen.

Materials and Methods

Mitochondrial genome assembly and annotation

98   Paired end Illumina sequence reads from 20 *Coccidioides immitis* and 57 *C. posadasii* were retrieved

99   from the Sequence Read Archive (SRA) and accessions and details are listed in **Table S1**. Following

100   cleaning and quality-clipping of reads with Trimmomatic v0.35, we assembled the genomes of *C.*

101   *posadasii* Tucson-2 and *C. immitis* WA221 using the SPAdes Genome Assembler v3.14.0 (20) with

102   a kmer sizes 61, 91, and 127. We identified mitochondrial contigs in this initial assembly using

103   similarity searches with expected fungal genes. To minimize assembly error we (i) used Rcorrector

104   [Song, L., Florea, L. Rcorrector: efficient and accurate error correction for Illumina RNA-seq reads.

105   GigaSci 4, 48 (2015).] for read correction, (ii) reduced the number of Illumina reads to a target kmer

106   coverage of the mtDNA between 30-50x, (iii) reads mapping against the identified mitochondrial

107   contigs were identified with Bowtie2 (21), which were then (iii) reassembled with Spades, resulting

108   in preliminary (uncorrected) mitogenome assemblies. In a final step, all reads of the reduced 30-50x

109   read set were aligned back to the preliminary assembly with Bowtie2 and analyzed for kmer coverage

110   with Bedtools v2.29.2 (22). We identified incorrectly-assembled reads, defined by kmer frequency

111   values of two or lower (likely the result of hybrid reads, originating from ligation of unrelated

112   genomic DNA fragments during library construction), and removed them from the final assemblies.

113   For both species, we obtained single circular-mapping closed contigs that carry the expected full set

114   of fungal mitochondrial genes.

115   To compare the *Coccidioides* mtDNA assembles with other fungi, we retrieved full mitochondrial

116   assemblies from other Onygenales available in the NCBI GenBank database: *Histoplasma*

117   *capsulatum* H143 (GG692467.1, direct submission) *Paracoccidioides brasiliensis* Pb18

118   (AY955840.1, (23)), *Blastomyces dermatitidis* ATCC 18188 (GG753566.1, direct submission),

119   *Epidermophyton flocossum* ATCC 26072 (AY916130.1, (24)), *Trichophyton rubrum* BMU 01672

120   (FJ385026.1, (25)) and *Ascosphaera apis* ARSEF 7405 (AZGZ01000045.1, (26)). Other close

121   relatives of *Coccidioides* (e.g. *Uncinocarpus*) had incomplete mitogenomes (27).

122   Mitochondrial genes as well as introns of group I and group II, tRNAs, RNase P RNA (*rnpB*), and

123   the small and large subunit rRNAs (*rns* and *rnl*) for *Coccidioides* and other related Onygenalean

124   fungi were annotated using the MFannot pipeline (https://megasun.bch.umontreal.ca/cgi-

125   bin/dev_mfa/mfannotInterface.pl; https://github.com/BFL-lab/Mfannot). *Coccidioides* annotations

126   were manually inspected and intron boundaries were checked and adjusted by aligning available

127   RNAseq data (27) with respective mitochondrial assemblies using Bowtie 2 (21). The assemblies and

128   annotations were deposited in GenBank (accession numbers TBD) and were visualized with the

129   OGDRAW pipeline (28).

130   **Single nucleotide polymorphism assessment and phylogenetic analysis**

131   SNPs from 77 *Coccidioides* isolates were identified among the mitochondrial genomes. We mapped

132   Illumina paired-end reads into individual mitochondrial coding-genes using Burrows-Wheeler

133   Aligner (BWA) v 0.7.7 (29) to assembled mitochondrial references *C. posadasii* strain Tucson2 or *C.*

134   *immitis* strain WA221. Indels were realigned to its reference genomes using GATK

135   RealignerTargetCreator and IndelRealigner tools (GATK toolkit v 3.3-0 (30)). To call SNPs, we used

136   the UnifiedGenotyper package. We only included SNPs not located in potentially duplicated loci (as

137   identified by NUCmer, (31)), with more than 10X coverage, and with a minor allele frequency of at

138   least 10% . We used the same approach to call SNPs for the nucDNA genomes (13). We generated

139   Maximum Likelihood (ML) concatenated trees for mtDNA and nucDNA using methods

140   implemented in IQ-TREE software (32) using -m MFP option (ModelFinder - (33)) for model

141   selection and 1,000 ultrafast bootstraps coupled with Shimodaira–Hasegawa-like approximate

142   likelihood ratio test (SH-aLRT) were performed for branch confidence test (34).Finally, we

143   compared the topology of the two trees using FigTree v1.4.2 -

144   http://tree.bio.ed.ac.uk/software/figtree/, and scored the disagreements the two topologies were using

145   TOPD/FMTS v 4.6 (35).

146   **Results**

147   **The *Coccidioides* spp. mitogenome**

148   We assembled complete circular mtDNA molecules for each of the two species of *Coccidioides*. The

149   two assemblies differ in size: the mtDNA genome is 68.6 Kb in *C. immitis* and 75.1 Kb in *C.*

150   *posadasii* (Figure 1). There is variation in mtDNA genome size among Onygenales (Table 1). The

151   mtDNA of both species of *Coccidioides* are on the larger end of the continuum. The mitogenomes of

152   *Coccidioides* harbor 14 protein-coding genes responsible for the formation of ubiquinone

153   oxidoreductase, cytochrome b, cytochrome oxidase and ATP synthase protein complexes (Figure 1,

154   Figure 2). The two ribosomal small and large subunit rRNA genes (*rns* and *rnl*), RNase P RNA

7

155   (*rnpB*) and 26 tRNAs organized in polycistronic transcription units are all present. The gene

156   composition and synteny are conserved between *Coccidioides* (Onygenaceae), *Blastomyces*,

157   *Histoplasma,* and *Paracoccidioides* (all Ajellomycetaceae) (Figure 1, (36)). The position of the gene

158   *atp8* differs between the Onygenaceae/Ajellomycetaceae species and other species of the

159   Onygenales, such as dermatophytes (*Trichophyton rubrum and Epidermophyton floccosum* -

160   Arthrodermataceae), and the bee-pathogenic fungus *Ascosphaera apis* (Ascosphaeraceae, Figure 2).

161   We observed no gene gain and losses of core mitochondrial genes within Onygenalean fungi (Figure

162   2).

163   The large size of the mtDNA genome in *Coccidioides* is due to the presence of introns and

164   intron-encoded open reading frames (ORFs) in both *Coccidioides* species, resulting in a dramatic

165   increase of intron type I and intron type II (Table 1, Figure 1) compared to other Ajellomycetaceae

166   fungi. In fact, *Coccidioides* harbors twice the number of elements found in *B. dermatitidis*. The

167   dermatophyte genera, *Epidermophyton* and *Trichophyton,* have only six and two intron elements

168   respectively, whereas *C. immitis* and *C. posadasii* contain 39 elements respectively (Table 1). The

169   introns found in the *Coccidioides* mitogenomes contain complete or truncated ORFs with high

170   similarity to homing endonucleases of the LAGLIDADG and GIY-YIG families (Table 1). Both

171   species contain 15 complete copies of Intron IB (Table 1). *Ascopharaceae apis* also has a large

172   mtDNA genome (118.65Kb) and a high number of intron-type I, specifically the intron I - derived,

173   B1 element (Table 1). The frequency and distribution of intron-types I and II in the genes *nadh5*, *cob*

174   and *cox1* differ between *C. immitis* and *C. posadasii,* but these features are not differentiated within

175   the species-complexes (Figure 1, Figure 2).

176   **Comparing mtDNA and nucDNA whole-genome trees**

8

177   Finally, we compared the mitochondrial phylogeny with the whole genome species phylogeny. To

178   score the differences in partitions produced between mtDNA and nucDNA trees, we used TOPD

179   (35). If two trees are completely congruent the split distance score is 0, whereas with complete tree

180   disagreement the score is 1. Differences are due to dissimilarity between the topologies as well as the

181   number of overlapping taxa. The split distance is the ratio of (different/possible) between the

182   *Coccidioides* mtDNA and nucDNA phylogenetic tree is 0.89 (132 /148), thus indicating that overall

183   topologies are consistent. Both topologies support species divergence between *C. immitis* and *C.*

184   *posadasii* (Figure 3). The mtDNA tree topology, shows a clear distinction between *C. immitis* and *C.*

185   *posadasii* with no isolates being assigned to a different species. This results suggest no mtDNA gene

186   exchange.

187   Those populations have low intraspecific genetic variation, suggesting either strict clonal dispersion,

188   or a recent founder effect (13).

189   Even though the main shape of the two topologies were consistent with each other. We also

190   observed differences. Consistent with previous results, the nucDNA tree topology revealed three *C.*

191   *posadasii* monophyletic populations: Arizona, Mexico/Texas/South America (MX/TX/SA) and

192   Caribbean (13). Based on mtDNA among the three main *C. posadasii* populations that have been

193   previously defined by markers differ substantially (Figure 3). Specifically, the mtDNA phylogeny

194   shows *C. posadasii Arizona*, *Texas/Mexico/South America*, and *Caribbean* clades are paraphyletic,

195   and individuals from these previously defined populations are dispersed into multiple clades in the

196   mitogenome tree (Figure 3). First, the clade *Mito 1* harbors 16 isolates in which 13 concordantly

197   belong to the Arizona population using nucDNA genomes. The isolates B10813, Tucson2 and

198   Tucson20 (*AZ clade I*) have conflicting phylogenetic distributions, previously these placed within

199   *Texas/Mexico/SouthAmerica* and *AZ clade I* (Figure 3). Second, the clade *Mito II* contains the

200   Venezuela group and three strains from the Arizona population (Tucson3, Tucson4 and Tucson14),

201   although the Venezuela mitochondrial and nucDNA topologies are congruent. The next clade of *C.*

202   *posadasii* is composed of four strains from Guatemala, and is perfectly concordant with the nucDNA

203   tree. The clade *Mito III* is composed of two isolates from the *AZ Clade I* (Tucson6 and Tucson16)

204   and two others from the *Caribbean* clade, and this lineage also shows conflicting phylogenetic

205   placement (Figure 3). Finally, the clade *Mito IV* is composed primarily of isolates from the

206   *Texas/Mexico/South America* clade.

207   Both nucDNA and mtDNA phylogenies revealed a clade composed of strains from

208   Washington (16) which is genetically distinct from the rest of *C. immitis* (Figure 3). No other

209   consistent pattern of clustering was observed for the remaining *C. immitis* individuals comparing the

210   two phylogenies (Figure 3).

211   In general, our results suggest that nucDNA and mtDNA have similar evolutionary

212   trajectories with no evidence of interspecific mtDNA exchange, but also the existence of

213   phylogenetic incongruence at recent scales possibly due to within-species recombination.

Discussion

215   Analysis of mitochondrial genomes as molecular markers confirms that the *Coccidioides* genus

216   contains two species: *C. immitis* and *C. posadasii*. Mitochondrial markers are extensively used as

217   molecular markers in speciation studies, including for *Coccidioides* (19, 37, 38). However molecular

218   systematics of *Coccidioides* based on mitochondrial genes may lead to ambiguous conclusions at an

219   intraspecies population level. Interestingly, both the mtDNA phylogeny and admixture plots of two

220   distinct and divergent populations *C. immitis* Washington and *C. posadasii* Venezuela clearly reveal

221   monophyly, as they are both reciprocally homozygous and no mixed genotypes are found (Figure 3).

222   Both of these populations have emerged within the last 6,000 years according to estimates (13, 16).

223   This suggests a strong founder effect followed by asexual reproduction in two endemic areas of the

224   disease (39).

225   The evolutionary trajectories of both mtDNA and nucDNA genomes have been investigated

226   using next generation sequencing data. Certainly, mtDNA and nucDNA genotypic incompatibilities

227   may exist, as well as undetermined effects of cross-species hybridization and introgression (40). This

228   is due in part to the fact that mitochondrial replication and division is not synchronized with nuclear

229   division, and cells can contain numerous mitochondria, which may not undergo genetic

230   recombination and may increase in number without cell division (41). Conflicting phylogenetic and

231   population distributions have been observed in other pathogenic fungi, and our results indicate shared

232   ancestry among recently diverged *C. immitis* and *C. posadasii* populations. For example,

233   *Paracoccidioides brasiliensis* and *P. restrepiensis* appear to be polyphyletic using mtDNA markers,

234   and the tree topologies differ from those obtained from nucDNA markers (42). Moreover, it is

235   suggested that mitochondrial interspecific hybridization and introgression occurs in *Paracoccidioides*

236   (42). Mitochondrial genomes can be difficult to assemble if high heterozygosity exists, as observed

237   for the opportunistic pathogen *Candida metapsilosis*, which is part of the *C. parapsilosis* complex

238   (43). This novel pathogen is a result of a hybridization event, which was detected in part by

239   analyzing the mitochondrial genome. Within the primary pathogen *Cryptococcus gattii* complex,

240   incongruences between mitochondrial and nuclear genes have been also reported (8). Mitochondrial

241   genotypic and consequent phenotypic variation among these pathogen complexes is associated with

242   virulence traits (44). These patterns are not restricted to human fungal pathogens. Some strains of the

243   plant fungal pathogen *Verticillium longisporum* present a mosaic mitochondrial genome structure due

244   to bi-parental inheritance impacting niche adaptation (45). Population genomic analyses of the

245   lichen-forming fungi *Rhizoplaca melanophthalma* species complex suggest that hybridization and

246   recombination in mitochondria might play a role in the speciation process of these symbiotic fungi

247   (46).

248   The pathogenic lifestyles of *C. immitis* and *C. posadasii* necessitate potentially endozoan

249   lifestyles (47) leading us to hypothesize that virulence, thermo-adaptation and oxidative stress could

250   be driving genetic differentiation in mtDNA in *Coccidioides* species and populations. For example,

251   in *Saccharomyces*, specific mutations the *cox1* gene in the mtDNA are associated with adaptation to

252   variable temperatures. The authors suggest that the yeast mitochondrial genome is a hotspot in the

253   evolution of thermal adaptation in *Saccharomyces* species (48, 49). *C. posadasii* is more heat tolerant

254   than *C. immitis* and private alleles found in the mitochondrial genome might be responsible for this

255   interspecific phenotypic variation. Importantly, in this manuscript we provided high-quality

256   assemblies and annotations for the *Coccidioides* mitogenomes, which will facilitate deeper

257   investigations into the impact of mitochondrial evolution in *Coccidioides'* niche adaptation, with

258   particular emphasis on mammalian host co-evolution and oxidative stress responses.

259

260   **Tables**

261   Table 1. Numbers of introns and classes among Onygenalean fungi

	C. immitis	*C. posadasii*	*H. capsulatum*	*P. brasiliensis*	*B. dermatitidis*	*E. flocossum*	*T. rubrum*	*A. apis*
Mitochondrial genome size (bp)	68,597	75,194	39,129	71,335	51,071	30,910	26,985	118,650
Intron IB (complete)	15	15	3	5	6	3	0	8
intron IB (extra insertion)	0	0	1	0	0	0	0	0
intron IB (5', partial)	0	1	0	0	0	0	0	1
intron IB (3', partial)	1	2	1	0	2	0	0	1
intron IA	3	3	1	0	1	1	2	1
intron IA (5', partial)	0	1	0	0	0	0	0	1
intron I (derived, A)	1	1	0	0	2	1	0	5
intron I (derived, B1)	2	2	0	4	0	0	0	11
intron ID	4	3	1	0	1	1	0	5

12

intron IC1	0	0	0	0	0	0	0	2
intron IC2	4	4	0	1	1	0	0	5
intron I (derived, B2)	1	1	1	0	1	0	0	1
intron II (domainV)	4	5	1	3	2	0	0	3
intron II, derived	1	1	0	0	0	0	0	0
Total	36	39	9	13	16	6	2	44

262

Figure Legends

264 **Figure 1** – Circular maps of *C. immitis* and *C. posadasii* mitogenomes. The assembled and annotated

265 genome features were converted into Genebank format and loaded into the OGDraw pipeline for

266 physical visualization of the coding and non-coding elements of the mitochondrial genomes.

267 **Figure 2** – Mitochondrial gene content and synteny among Onygenalean fungi. Genes as color-coded

268 (see legend) and displayed according positioning on the genome. The mitogenomes genomes are

269 highly syntenic but *atp8* gene positioning is divergent between those fungal families.

270 **Figure 3** – Tree topology comparisons of *Coccidioides* nucDNA (left panel) and mtDNA (right

271 panel) phylogenomic trees. Phylogenetic tree branches are proportional to the nucleotide divergence

272 (see scale) and the main clades are highlighted. Bootstrap support was calculated, and branch support

273 was added to the corresponding clade. The terminal taxa are color-coded according to their placement

274 on the nucDNA tree and taxa are connected between mtDNA and nucDNA phylogenomic trees in

275 order to visualize concordance (solid lines) vs discordance (dotted lines).

Conflict of Interest

277 *The authors declare that the research was conducted in the absence of any commercial or financial*
278 *relationships that could be construed as a potential conflict of interest.*

Author Contributions

280 The manuscript was written and edited by MT, JES, BFL, DRM and BMB. Data was analyzed by
281 MT and BFL. Funding provided by BMB.

Funding

294 Acknowledgments

298 Supplementary Material

299 Accessions and details are listed in **Table S1**.

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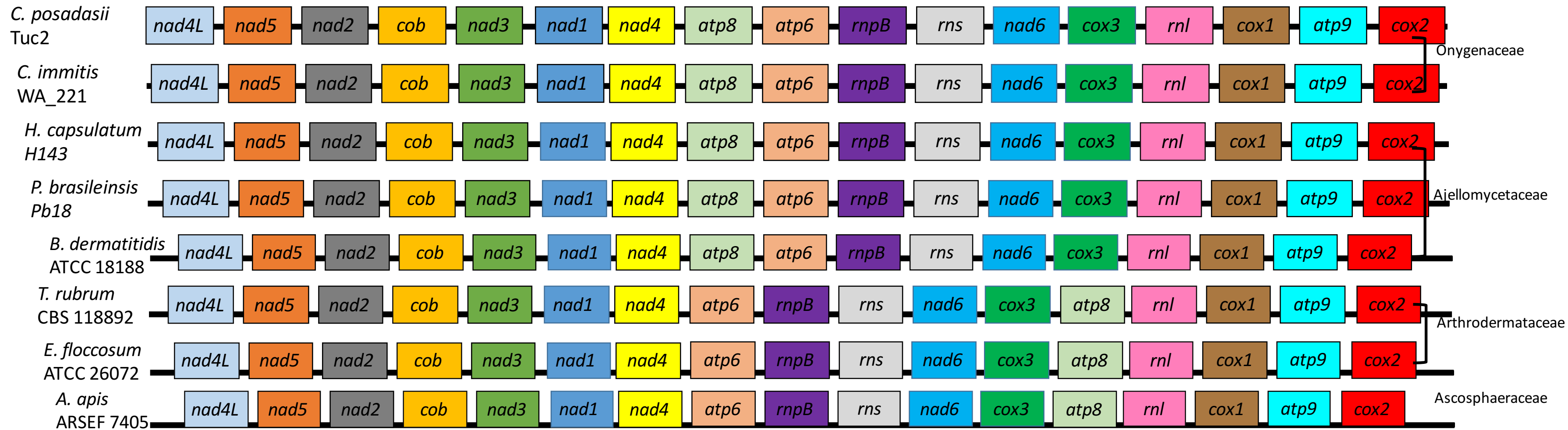
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433

C immitis-WA221

mitochondrial genome

68,097 bp

C posadasii Tuc2

mitochondrial genome

75,117 bp
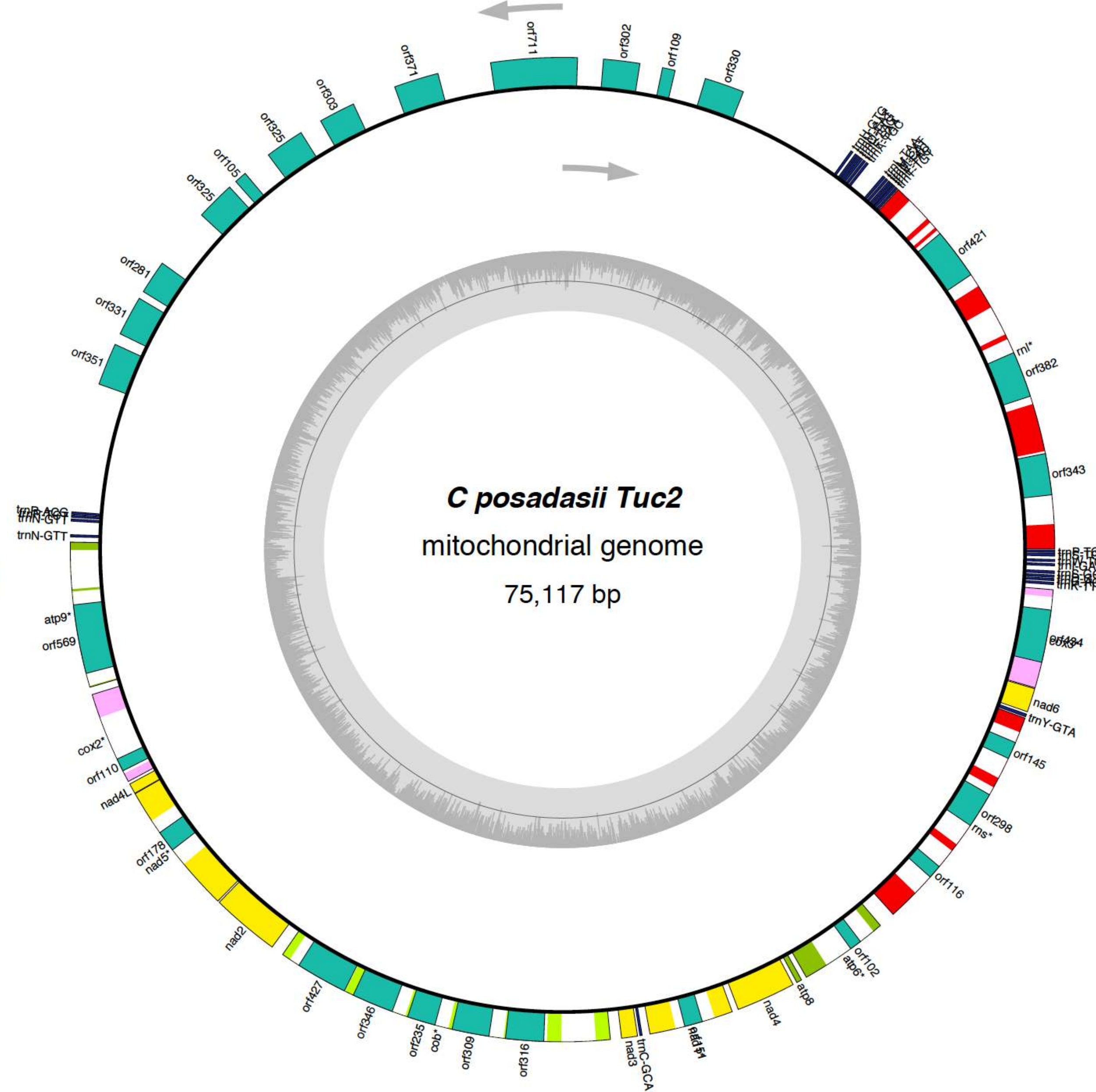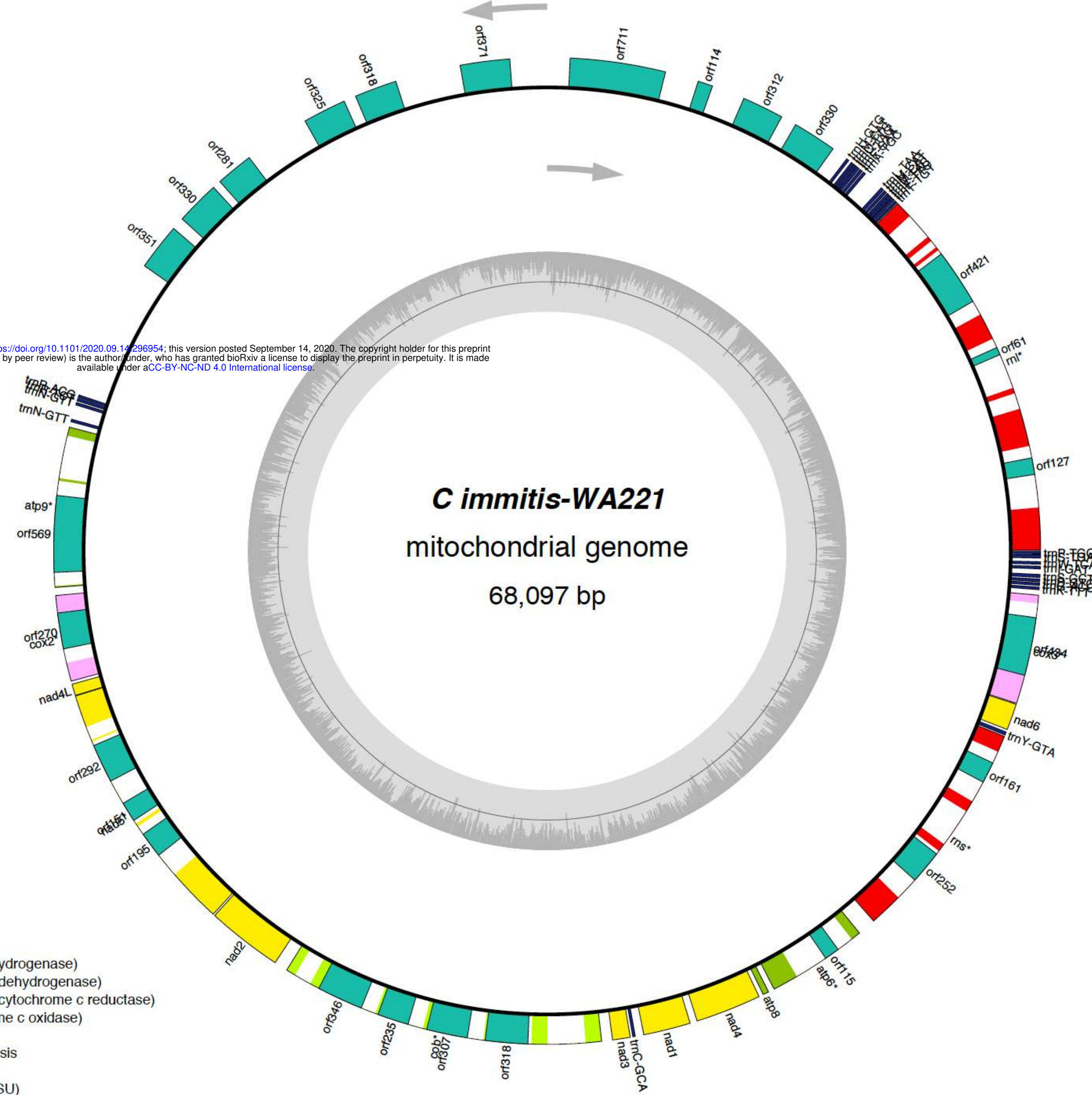
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- complex III (ubichinol cytochrome c reductase)
- complex IV (cytochrome c oxidase)
- ATP synthase
- cytochrome c biogenesis
- RNA polymerase
- ribosomal proteins (SSU)
- ribosomal proteins (LSU)
- maturases
- other genes
- ORFs
- transfer RNAs
- ribosomal RNAs
- origin of replication
- polycistronic transcripts
- introns