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A Unique Mitochondrial Gene Block Inversion in Antarctic Trematomin Fishes: A Cautionary Tale

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Abstract

Many Antarctic notothenioid fishes have major rearrangements in their mitochondrial (mt) genomes. Here, we report the complete mt genomes of 3 trematomin notothenioids: the bald notothen (*Trematomus* (*Pagothenia*) *borchgrevinki*), the spotted notothen (*T. nicolai*), and the emerald notothen (*T. bernacchii*). The 3 mt genomes were sequenced using next-generation Illumina technology, and the assemblies verified by Sanger sequencing. When compared with the canonical mt gene order of the Antarctic silverfish (*Pleuragramma antarctica*), we found a large gene inversion in the 3 trematomin mt genomes that included *tRNA^{Ile}*, *ND1*, *tRNA^{Leu2}*, *16S*, *tRNA^{Val}*, *12S*, *tRNA^{Phe}*, and the control region. The trematomin mt genomes contained 3 intergenic spacers, which are thought to be the remnants of previous gene and control region duplications. All control regions included the characteristic conserved regulatory sequence motifs. Although short-read next-generation DNA sequencing technology has allowed the rapid and cost-effective sequencing of a large number of complete mt genomes, it is essential in all cases to verify the assembly in order to prevent the publication and use of erroneous data.

Key words: mitochondrial genome, Nototheniodei, *Trematomus bernacchii*, *Trematomus* (*Pagothenia*) *borchgrevinki*, *Trematomus nicolai*

The teleost fauna of the Southern Ocean is dominated by a clade of perciform fishes belonging to the suborder Notothenioidei (Eastman 1993). This suborder is comprised of 8 families, of which the 3 basal lineages (Bovichtidae, Pseudaphritidae, and Eleginopsidae) are predominantly non-Antarctic in distribution. Most species in the remaining 5 families (Nototheniidae, Harpagiferidae, Artedidraconidae, Bathydraconidae, and Channichthyidae) are endemic to the Southern Ocean (Eastman 2005).

The phylogenetic relationships of the notothenioids have been studied for more than 2 decades. Recently, the phylogeny of this group has been reanalyzed and updated using a combination of mitochondrial (mt) and nuclear DNA markers, including 15 new complete and 2 partial mt genomes (Papetti et al. 2021). The study identified a number of novel rearrangements in the mt genomes, including an extremely rare inversion event in the Trematominae. Papetti et al. (2021) generated a new phylogeny which showed that the mt evolution of the notothenioids has been characterized by multiple, relatively rapid changes in mt gene order.

With the advent of next-generation DNA sequencing, complete mt genomes have become much more cost effective and feasible to collect. However, the assembly of these genomes often relies on using a scaffold from a closely related species. This assumes the gene order between these genomes is the same or very similar, and ignores the possibility of major gene rearrangements. Furthermore, mt genomes obtained from the assembly of short reads from next-generation DNA sequencing are seldom verified by targeted PCR amplification, potentially resulting in the publication of incorrectly assembled genomes.

The vertebrate mt genome is highly conserved, consisting of 13 protein-coding genes, 2 ribosomal RNA (rRNA) genes, and 22 transfer RNA (tRNA) genes (Wolstenholme 1992). In addition, there are a number of noncoding regions, the most significant of which is the control region (CR) that contains transcriptional promoters for both the heavy (H) and light (L) strands. Apart from the presence of the D-loop, the CR can be divided into 3 domains: a domain associated with the termination-associated sequences, a conserved central domain (CCD), and conserved sequence block (CSB) domains (Anderson et al. 1981; Brown et al. 1986). The order of the 37 genes and noncoding regions in the mt genome tends to be conserved among most vertebrate species studied to date, although deviations from the canonical order have been identified in various groups, including fishes (Satoh et al. 2016).

Changes in gene order can be misinterpreted as gene loss unless detailed analyses are undertaken, as shown in the supposed loss of the mt NADH dehydrogenase subunit 6 (*ND6*) and *tRNA^{Glu}* genes in notothenioids (Papetti et al. 2007). In fact, in the 5 Antarctic notothenioid families studied, these

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2 genes were not lost but simply translocated from their canonical location between the NADH dehydrogenase subunit 5 (*ND5*) and cytochrome b (*Cytb*) genes to the CR, and subsequently overlooked (Zhuang and Cheng 2010). Papetti et al. (2021) have recently shown that whole mt genomes of Antarctic notothenioids vary greatly with respect to gene order. Their study was the first to report novel gene orders from representative species of all Nototheniidae families, including the trematomins.

In the present study, we identified a unique gene order when assembling the complete mt genomes from sequences of 3 Antarctic fish species: the bald notothen (*Trematomus* (*Pagothenia*) *borchgrevinki*), the spotted notothen (*T. nicolai*), and the emerald notothen (*T. bernacchii*). The gene order was verified using PCR and Sanger sequencing. We also characterized the CR domains in these 3 species.

Methods

Sample Collection and DNA Extraction

Adult specimens of the 3 trematomins (*T. borchgrevinki*, *T. nicolai*, and *T. bernacchii*) were collected from the vicinity of Ross Island, McMurdo Sound, Antarctica using routine fishing methods. Each specimen was identified using morphological features and specimen identification was confirmed using standard DNA barcoding methods (Ratnasingham and Hebert 2007). Additional specimen details including collection date and location can be found in Table 1.

Cells were scraped from the gill tissue and re-suspended in STE (50 mM NaCl, 50 mM Tris-HCl, 100 mM EDTA, pH 8.0) buffer. Genomic DNA was isolated from the suspension using Proteinase K digestion followed by a phenol: chloroform: isoamyl extraction (Sambrook et al. 1989). DNA was then digested with 20 µg/µL RNase at 37 °C for 4 h.

Library Construction and Assembly

Libraries were constructed using the Illumina TruSeq Nano kit (2 × 250 bp reads) or the Affymetrix Prep2Seq kit (2 × 300 bp reads) and sequenced on the Illumina MiSeq (San Diego, CA) platform. For *T. borchgrevinki*, an additional library was constructed using Rubicon Thruplex DNA-seq (2 × 125 bp paired-end) and sequenced on the Illumina HiSeq 2500 (San Diego, CA) platform using v4 chemistry.

Sequencing data were quality checked with FastQC (Andrews 2010) to ensure that there were no issues with the sequencing process or the resulting data. The data were then mapped against 2 reference genomes: the black notothen (*Notothenia coriiceps*) full nuclear genome and the *N. coriiceps* mt genome (accession numbers: AZAD00000000, NC_015653) using the tool bbsplit from the bbmap package to isolate fish-only reads (Bushnell 2014). To map to a reference, a candidate sequence needed to share at least 97% identity. The mapping was repeated with a minimum of 90% identity, using the output of the previous step. Raw reads were then mapped against the mt genomes of *T. borchgrevinki* (accession number KU951144.1) and 3 closely related species: the Patagonian toothfish (*Dissostichus eleginoides*), *N. coriiceps*, and the Antarctic silverfish (*Pleuragramma antarctica*) (accession numbers NC_018135.1, NC_015653, and JF933905, respectively) in order to extract only the mt DNA reads. The mt DNA sequence pools were assembled into one contig which, when annotated with the MITOS web server (Bernt et al. 2013), contained the *COI* gene. This gene was used as a seed for MITObim (Hahn et al. 2013) to assemble the mt genomes. Each of the assembled mt genomes of *T. borchgrevinki, T. bernacchii*, and *T. nicolai* showed a large gene block inversion.

To verify the inversion, a ~9 kb fragment of the mt was amplified using primers designed to *Cytb* (forward primer) and *ND2* (reverse primer). A long-range PCR was carried out using the TaKaRa LA *Taq* kit according to the manufacturer's instructions. Once the gene orientation was verified at either ends of this ~9 kb fragment through Sanger sequencing, smaller overlapping fragments were then amplified for the remaining region (see Supplementary Table S1 for primer details). PCRs were run in 25 µL volume reactions and included 10–30 ng template DNA, 2 mM $MgCl_2$, 0.4 µM forward and reverse primers, and 0.1 U *Taq* (Life Technologies). In some instances, betaine and dimethyl sulfoxide (DMSO) were used in both the PCR and cycle sequencing reactions for areas containing repetitive sequences to inhibit secondary structure formation. The thermal cycling conditions were as follows: 2 min at 94 °C followed by 30 cycles of 30 s at 94 °C, 45 s at the annealing temperature (Supplementary Table S1) and 60 s at 72 °C, and a final extension of 5 min at 72 °C.

All PCR products were purified, cycle sequenced using Big Dye 3.1 chemistry, and subsequently analyzed on an ABI Prism 3130xl genetic analyzer (Applied Biosystems). PCR products were sequenced in both directions. Sequences were edited manually using GENEIOUS (http://www.geneious.com/) and then aligned to the assembled complete mt genomes. The mt genomes of all 3 species had a ~9 kb fragment verified by PCR. An exception to this was a ~500 bp region of the *T. bernacchii* CR which proved difficult to amplify due to the presence of repetitive sequences.

Results and Discussion

Illumina Sequencing

The Illumina TruSeq Nano libraries produced 33 million barcoded reads for each of the 3 samples (*T. borchgrevinki*, *T. nicolai*, and *T. bernacchii*). The Affymetrix Prep2Seq libraries

Table 1. Sample identifiers and collection details for the bald notothen (*Trematomus borchgrevinki*), spotted notothen (*T. nicolai*), and emerald notothen (*T. bernacchii*)

Species	Sample ID	Collection date	Collection coordinates
T. borchgrevinki	11/134	December 2011	Between 77.635 and 77.885°S and 166.311 and 166.770°E
T. nicolai	11-10	December 2011	
T. bernacchii	11/145	December 2011	

produced 28.8 million barcoded reads for *T. borchgrevinki*, 28.8 million barcoded reads for *T. nicolai*, and 14.4 million barcoded reads for *T. bernacchii*. The *T. borchgrevinki* library from the Rubicon Thruplex DNA-seq kit produced 440 million barcoded paired-end reads.

Mitochondrial Genome Organization

The lengths of the mt genomes of the 3 trematomins under study were 18 981 bp (*T. borchgrevinki*), 19 358 bp (*T. nicolai*), and 19 795 bp (*T. bernacchii*) (Figure 1). They are registered in GenBank under accession numbers MZ779011, MZ779013, and MZ779012, respectively. Each mt genome contained 13 protein-coding genes, 22 tRNA genes, 1 large (16S) and 1 small (12S) rRNA gene, as well as 1 CR and 3 intergenic spacers (Table 2). The gene order is described in Table 2 starting with *ND4L* and ending with $tRNA^{Arg}$. The start and stop codons were divergent in several genes. *COI* had a GTG start codon, while 6 genes, *ND2*, *COII*, *COIII*, *ND3*, *ND4*, and *Cytb*, had incomplete stop codons that require the post-transcriptional addition of A bases. This was found to be the case for all 3 trematomins. There were no frameshifts, stop-in-frame codons, and deviations from the vertebrate mt genetic code found within any of the protein-coding genes, indicating that nuclear mitochondrial DNA (numts) were not included in the assemblies (Antunes and Ramos 2005). The base composition of the *T. borchgrevinki* mt genome was 24.5% A, 30.8% T, 21.8% G, and 22.8% C, that of the *T. nicolai* mt genome was 24.1% A, 31.6% T, 21.8% G, and 22.5% C, and that of the *T. bernacchii* mt genome was 24% A, 31.6% T, 21.9% G, and 22% C.

A number of studies have published the complete mt genomes of trematomin species (*T. loennbergii*, *T. borchgrevinki*, *T. bernacchii*, and *T. pennellii*), but surprisingly none has reported any differences in the canonical gene order (Liu et al. 2016; Song et al. 2016; Alam et al. 2019; Choi et al. 2021). Additionally, Song et al. (2016) published the *T. bernacchii* mt genome with an incomplete *ND6* and an incomplete 12S sequence. Furthermore, 2 of the above studies do not provide any information on how the sequence data were obtained, their methods of assembly or data verification. The GenBank submission for these 2 studies also lacks any accompanying metadata. In order to evaluate the assemblies of complete mt genomes, we strongly recommend that the sequencing data be made available to other researchers on request or deposited in an appropriate database.

Based on our results and the recent findings of Papetti et al. (2021), we believe that the gene order reported by Liu et al. (2016), Song et al. (2016), Alam et al. (2019), and Choi et al. (2021) is incorrect. Furthermore, due to the lack of information regarding the methods used, it is very difficult to identify the source(s) of the likely problems, for example, incorrect assembly; the use of closely related mt genomes as a scaffold; and reliance on only short read next-generation sequencing. As a result, these mt genome assemblies should not be used in any future analysis or at the very least viewed with caution.

Importantly, it is now possible to overcome many of the problems discussed above and the issue of inclusion of numts in the mt genome assemblies. Although, Illumina sequencing is readily available and cost effective, it generates large numbers of short sequences (100–300 bp), which require assembly. In contrast, long-read sequencing technology, such as PacBio and Nanopore, has the potential to sequence the entire mt genome in a single read. PacBio is relatively expensive and is therefore unlikely to be used to sequence novel mt genomes. However, Nanopore technology in combination with long-range PCR and pooling of individually barcoded samples allows fast and cost-effective sequencing of entire mt genomes (Formenti et al. 2021).

Mitochondrial Gene Rearrangements

We found a major rearrangement in the gene order between the 3 trematomins reported and that of other notothenioids such as *P. antarctica* and *N. coriiceps*. The most significant differences observed were a large gene block inversion of the mt genome region which falls between $tRNA^{Glu}$ and $tRNA^{Gln}$ of the *P. antarctica* mt genome, and the presence of 3 intergenic spacers. The gene block inversion contained 7 genes and 2 noncoding regions in the following order; an intergenic spacer (UN3), followed by $tRNA^{Ile}$, *ND1*, $tRNA^{Leu2}$, *16S*, $tRNA^{Val}$, *12S*, $tRNA^{Phe}$, and ended with the CR. All 3 trematomins showed the same general gene order and pattern except for the intergenic spacers and CRs being of differing lengths (Figure 1a–c; Table 2).

The gene complements of the 3 trematomin mt genomes reported here are the same as the basal non-Antarctic Bovichtus species (Satoh et al. 2016). However, the gene order is notably different between *ND5* and $tRNA^{Gln}$ (Figure 1). According to Zhuang and Cheng (2010), a tandem gene duplication event occurred between the basal non-Antarctic bovichtids and the common ancestor of the Antarctic clade. This duplication was followed by the early loss/degradation of *ND6*, $tRNA^{Glu}$, and *Cytb* leading to "Pattern I" as described by these authors and exemplified in the mt genome of the extant *P. antarctica*. For the trematomins described in this paper, a possible evolutionary pathway from this point to the current trematomin gene order has been detailed by Papetti et al. (2021), who now refer to this pathway as "TremaGo." This involves 1) partial random loss of CR1 in the *P. antarctica* mt genome as evidenced by the lack of characteristic CR conserved sequences (e.g., extended termination-associated [ETASs] and CSBs); 2) partial random loss of $tRNA^{Thr}$ and $tRNA^{Pro}$ between *ND6* and CR2; and 3) inversion of the gene block CR2, $tRNA^{Phe}$, *12S*, $tRNA^{Val}$, *16S*, $tRNA^{Leu}$, *ND1*, *and* $tRNA^{Ile}$. It is important to note that intergenic spacers are generated during this pathway which are thought to include the remnants of lost/duplicated genes. Overall, the results of the mt genome assembly of *T. borchgrevinki* are consistent with those shown by Papetti et al. (2021).

Control Region

The CR of mammalian mtDNA typically lies between the $tRNA^{Pro}$ and $tRNA^{Phe}$ genes, and this is reflected in the mt genome of the basal non-Antarctic notothenioid thornfish (*Bovichtus argentinus*) (Satoh et al. 2016). In the trematomins studied here, the position of the $tRNA^{Pro}$ gene which delineates one end of the canonical mammalian CR is now occupied by $tRNA^{Gln}$ due to a gene block inversion.

Comparative sequence analysis of the CR between the 3 trematomins and other notothenioids allowed us to infer the presence of 2 extended termination-associated sequences (ETAS1 and ETAS2) within the CR domain, each 31 nucleotides long (Figure 2). These are significantly shorter than the ~60 bp sequences originally identified by Sbisà et al. (1997), but are largely consistent with the sequences

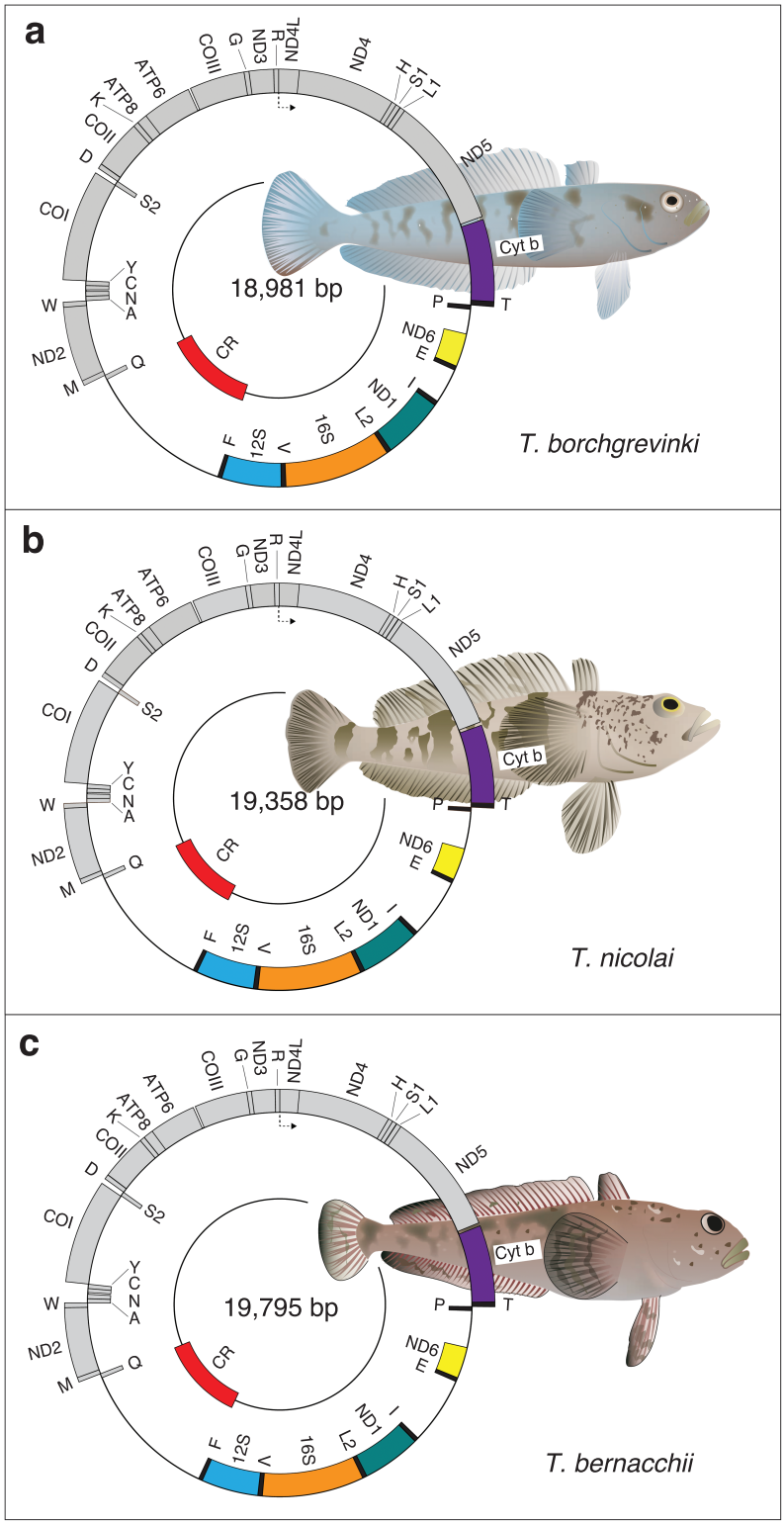


Figure 1. Complete mt genomes of 3 trematomin fishes: (**a**) bald notothen (*Trematomus borchgrevinki*), (**b**) spotted notothen (*T. nicolai*), and (**c**) emerald notothen (*T. bernacchii*). The lengths of the boxed genes are to scale starting with *ND4L*, and strand affiliations are signaled by boxed regions lying either inside (L strand) or outside (H strand) the circle. Genes are indicated in their abbreviated form. tRNA genes are shown as single-letter amino acid codes. Colored areas signify a region of interest relating to gene duplication, loss, and rearrangement in notothenioids.

identified by Zhuang and Cheng (2010). Both ETAS domains in all 3 trematomins contain the sequence 5′-ATGA-3′ (with reference to the L-strand) as the complementary termination-associated sequence (cTAS). This sequence is at the 5′ end of a 15 bp sequence referred to as the coreTAS on the L-strand in humans (Jemt et al. 2015).

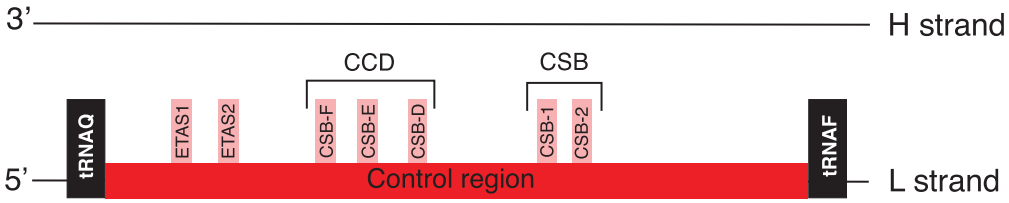
In most marine teleosts, the CCD contains 3 CSBs (CSB-F, CSB-E, and CSB-D), and we confirmed their presence in

Table 2. Mitogenome organization of the bald notothen (*Trematomus borchgrevinki*), spotted notothen (*T. nicolai*), and emerald notothen (*T. bernacchii*)

		T. borchgrevinki			*T. nicolai*			*T. bernacchii*			
Gene	Abbreviation	Start	End	Length	Start	End	Length	Start	End	Length	Strand
ND4L		1	297	297	1	297	297	1	297	297	H
ND4		291	1671	1381	291	1671	1381	291	1671	1381	H
tRNA^{His}	H	1672	1740	69	1672	1740	69	1672	1740	69	H
tRNA^{Ser1}	S1	1741	1807	67	1741	1807	67	1741	1807	67	H
tRNA^{Leu1}	L1	1812	1884	73	1812	1884	73	1812	1884	73	H
ND5		1885	3723	1839	1885	3723	1839	1885	3723	1839	H
Intergenic spacer	UN1	3724	3765	43	3724	3771	49	3724	3770	48	
Cyt b		3766	4906	1141	3772	4912	1141	3771	4911	1141	H
tRNA^{Thr}	T	4907	4978	72	4913	4984	72	4912	4983	72	H
tRNA^{Pro}	P	4978	5047	70	4984	5053	70	4983	5052	70	L
Intergenic spacer	UN2	5048	5429	383	5054	5661	609	5053	5662	611	
ND6		5430	5948	519	5662	6180	519	5663	6181	519	L
tRNA^{Glu}	E	5949	6016	68	6181	6248	68	6182	6249	68	L
Intergenic spacer	UN3	6017	6563	547	6249	7207	960	6250	7368	1120	
tRNA^{Ile}	I	6564	6633	70	7208	7277	70	7369	7438	70	L
ND1		6638	7612	975	7282	8256	975	7443	8417	975	L
tRNA^{Leu2}	L2	7613	7686	74	8257	8330	74	8418	8491	74	L
16S		7687	9375	1689	8331	10 019	1689	8492	10 183	1692	L
tRNA^{Val}	V	9377	9448	72	10 021	10 092	72	10 185	10 256	72	L
12S		9452	10 396	945	10 096	11 040	945	10 260	11 203	944	L
tRNA^{Phe}	F	10 397	10 464	68	11 041	11 108	68	11 204	11 271	68	L
Control region		10 465	12 808	2344	11 109	13 187	2079	11 272	13 612	2341	L
tRNA^{Gln}	Q	12 809	12 880	72	13 188	13 259	72	13 613	13 684	72	L
tRNA^{Met}	M	12 880	12 948	69	13 259	13 327	69	13 684	13 752	69	H
ND2		12 949	13 994	1046	13 328	14 373	1046	13 753	14 798	1046	H
tRNA^{Trp}	W	13 995	14 065	71	14 374	14 444	71	14 799	14 869	71	H
tRNA^{Ala}	A	14 067	14 135	69	14 446	14 514	69	14 871	14 939	69	L
tRNA^{Asn}	N	14 137	14 209	73	14 516	14 588	73	14 941	15 013	73	L
tRNA^{Cys}	C	14 235	14 300	66	14 612	14 677	66	15 042	15 107	66	L
tRNA^{Tyr}	Y	14 301	14 371	71	14 678	14 748	71	15 108	15 178	71	L
COI		14 373	15 923	1551	14 750	16 300	1551	15 180	16 730	1551	H
tRNA^{Ser2}	S2	15 924	15 994	71	16 301	16 371	71	16 738	16 808	71	L
tRNA^{Asp}	D	15 996	16 066	71	16 373	16 443	71	16 810	16 880	71	H
COII		16 069	16 759	691	16 446	17 136	691	16 883	17 573	691	H
tRNA^{Lys}	K	16 760	16 833	74	17 137	17 210	74	17 574	17 647	74	H
ATP8		16 835	17 002	168	17 212	17 379	168	17 649	17 816	168	H
ATP6		16 981	17 676	696	17 358	18 053	696	17 795	18 490	696	H
COIII		17 709	18 493	785	18 086	18 870	785	18 523	19 307	785	H
tRNA^{Gly}	G	18 494	18 563	70	18 871	18 940	70	19 308	19 377	70	H
ND3		18 564	18 912	349	18 941	19 289	349	19 378	19 726	349	H
tRNA^{Arg}	R	18 913	18 981	69	19 290	19 358	69	19 727	19 795	69	H

the CRs of the trematomins studied (Figure 2). CSB-F is positioned nearest the ETAS region and has a cTAS sequence at its 5′ end. The GTGGG box identified in the CSB-E domain of many teleost species was present as a modified GTGAG sequence in the trematomins. The CSB-D sequence was found within the CCD region as identified in groupers with minor variation (Zhuang et al. 2013). We also identified 2 conserved sequences in the CR CSB domain (CSB-1 and CSB-2). The CSB-1 region had the characteristic CATAA sequence at its 3′ end, while CSB-2 had the characteristic poly C stretch separated by TA (Zhuang et al. 2013).

A comparison between the mt genome sequences of *T. borchgrevinki* from our study and that of Papetti et al. (2021) nonetheless revealed several differences. The genome of *T. borchgrevinki* in our study was found to be a total of 656 bp longer. Much of the difference in length (644 bp) was found to be within the CR. This CR length difference could be further broken down into 3 regions, which contained extra



	ETAS1 containing cTAS and coreTAS	ETAS2 containing cTAS and coreTAS	cTAS and CSB-F
T. borchgrevinki	ATGTATTATCACCATAGATTTATATTAAACA (12614-12644)	ATGTATTATCACCATAGATTTATATTAAACA (12517-12547)	ATGTATTATCACCATAGATTTATATTAAACA (12191-12209)
T. nicolai	ATGTATTATCACCATAGATTTATATTAAACA (12982-13011)	ATGTATTATCACCATAAATTTATATTAAACA (12922-12952)	ATGTAGTAAGAACCGACCA (12596-12614)
T. bernacchii	ATGTATTATCACCATAGATTTATATTAACCA (13264-13294)	ATGTATTATCACCATAGATTTATATTAACCA (13203-13233)	ATGTAGTAAGAACCGACCA (12913-12931)

	CSB-E	CSB-D	CSB-1	CSB-2
T. borchgrevinki	AACGGTTAATGAAGGTGAGG (12150-12169)	TTATTCCTGACATTTGGTTCC (12098-12118)	TTCACGGGCATAA (11822-11834)	TTCCCCCTACCCCCTTA (11744-11760)
T. nicolai	AACGGTTAATGAGGGTGAGG (12555-12574)	TTATTCCTGGCATTTGGTTCC (12502-12522)	TTCACGGGCATAA (12228-12240)	TTCCCCCTACCCCCTTA (12150-12166)
T. bernacchii	AACGGTTAATGAGGGTGAGG (12872-12891)	TTATTCCTGGCATTTGGTTCC (12820-12840)	TTCACGGGCATAA (12546-12558)	TTCCCCCTACCCCCTTA (12468-12484)

Figure 2. Diagrammatic representation and sequence alignment of the conserved regulatory sequence motifs in the CR of 3 trematomins: bald notothen (*Trematomus borchgrevinki*), spotted notothen (*T. nicolai*), and emerald notothen (*T. bernacchii*). Numbers in brackets show the relative position of the conserved regulatory sequence motifs in reference to the start point at *ND4L*. The green highlighted bases indicate sequence differences among the 3 trematomins.

sequences of 404 bp, 46 bp, and 194 bp, respectively. Given that no differences were found in any of the coding genes, we believe these CR differences to be real rather than sequencing/assembly errors, and possibly the result of slippage replication of the repetitive regions in the CR. If verified, the CR variation among individuals of *T. borchgrevinki* and potentially other trematomins is an important novel finding.

With next-generation sequencing and indexing technologies, it is now practical to sequence the CR from a large number of individuals of a single species in order to investigate if population-level genetic variation is widespread. Complete CR sequences from multiple individuals and samples collected from different geographic regions of *T. borchgrevinki* would be necessary to establish the nature and extent of the CR variation. CR sequences are known to vary greatly within and between species, and have been used as valuable population genetic markers (Avise 2004; Jamandre et al. 2014). Based on the CR differences reported here, this may also be the case in *T. borchgrevinki* and other trematomins.

Complete mt genome data from notothenioids has typically been used for phylogenetic analyses. As a result there is, in many cases, complete mt genomic data available from only a single individual or at best a very small number of individuals for each species. In contrast, Lin et al. (2012) sequenced large regions of the mt genomes of the mackerel icefish (*Champsocephalus gunnari*) from 32 individuals and interestingly found variation in the number of CRs and genes among individuals. Similar studies of the mt genomes from *T. borchgrevinki* and other trematomins would be important, in order to establish if this finding occurs in other species within the family.

Conclusions

Each of the mt genomes of 3 trematomins species (*T. borchgrevinki*, *T. nicolai*, and *T. bernacchii*) was found to have a large, unique gene block inversion. These results provide evidence that many of the published genome assemblies for *T. borchgrevinki* and other trematomins are incorrect. There is significant length variation in the CR in *T. borchgrevinki* between the 2 individuals compared, one from this study and the other from Papetti et al. (2021). Recent advances in DNA sequencing technology and associated bioinformatic pipelines will lead to large numbers of high-quality, error-free mt genomes in the near future (Formenti et al. 2021). Furthermore, large-scale complete mt sequencing of a range of notothenioids species including the trematomins would provide valuable insights into the population genetics and the evolution of fish mt genomes.

Supplementary Material

Supplementary material is available at *Journal of Heredity* online.

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Authors' Contribution

Conceptualization: S.P., C.W.E., and C.D.M.; sampling: C.W.E.; data curation: S.P. and A.S.; formal analysis: S.P., C.W.E., A.S., N.J.M., and C.D.M.; funding acquisition: C.W.E., A.S., and C.D.M.; methodology: S.P., A.S., and N.J.M.; manuscript preparation: S.P., C.W.E., A.S., N.J.M., and C.D.M.

Data Availability

The sequence data are available in GenBank under accession numbers MZ779011, MZ779013, and MZ779012 for *T. borchgrevinki*, *T. nicolai*, and *T. bernacchii*, respectively. Raw Illumina data are available on Dryad Digital Repository https://datadryad.org/stash/share/cpEOPUfS6t6lkuIhwhsjKHhn_-j2GU5xpylpFc49vVY (Patel et al. 2022).

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