

Atypical Evolution of the F₁F_o Adenosine Triphosphate Synthase Regulatory ATP6 subunit in Glacier Ice Worms (Annelida: Clitellata: *Mesenchytraeus*)

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Shirley A Lang¹ and Daniel H Shain²

¹Graduate School of Biomedical Sciences, Rowan University, Stratford, NJ, USA. ²Department of Biology, Rutgers, The State University of New Jersey, Camden, NJ, USA.

ABSTRACT: The glacier ice worm, *Mesenchytraeus solifugus*, is among a few animals that reside permanently in glacier ice. Their adaptation to cold temperature has been linked to relatively high intracellular adenosine triphosphate (ATP) levels, which compensate for reductions in molecular motion at low physiological temperatures. Here, we show that ATP6—the critical regulatory subunit of the F₁F_o-ATP synthase and primary target of mitochondrial disease—acquired an unprecedented histidine-rich, 18-amino acid carboxy-terminal extension, which counters the strong evolutionary trend of mitochondrial genome compaction. Furthermore, sequence analysis suggests that this insertion is not of metazoan origin, but rather is a product of horizontal gene transfer from a microbial dietary source, and may act as a proton shuttle to accelerate the rate of ATP synthesis.

KEYWORDS: energy, mitochondria, *atp6*, ATP6 subunit, horizontal gene transfer

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CORRESPONDING AUTHOR: Daniel H Shain, Department of Biology, Rutgers, The State University of New Jersey, 315 Penn Street, Camden, NJ 08102, USA. Email: dshain@camden.rutgers.edu

Introduction

Numerous microbial organisms (eg, bacteria, algae, fungi) but relatively few animal taxa (eg, representatives of Annelida, Arthropoda, Rotifera) have adapted to the permanently cold and harsh conditions of glacier ice.^{1–3} Common to all obligatory glacial species that have been examined are paradoxically high intracellular adenosine triphosphate (ATP) levels in direct comparisons with temperate (ie, mesophilic) species.^{4–6} This has been interpreted as a compensatory mechanism that offsets reductions in molecular motion at cold temperatures by increasing the probability of molecular collisions with ATP, the universal currency of energy that drives most biochemical reactions.⁷

The glacier ice worm, *Mesenchytraeus solifugus*, was the first organism in which this paradoxical energetic profile was observed⁴ and subsequent efforts were aimed at determining the underlying metabolic mechanism. Our collective analyses suggest that enhanced ATP synthesis (with respect to ATP consumption) can explain the observed gains in ATP levels at cold physiological temperatures, and previous investigations targeted adenosine monophosphate (AMP) degradative machinery. Components of this pathway regulate intracellular AMP levels and their abrogation leads to gains in ATP and cold tolerance across disparate cell types^{8–10}; however, genetic analyses found no significant divergence within this pathway in glacier ice worms.

To expand our investigation, we focused here on the F₁F_o-ATP synthase complex, the primary source of ATP production under aerobic conditions¹¹ and a primary target of mitochondrial disease.¹² Gene sequence data, encoding 11 core subunits of ATP synthase, were extracted from the ice worm, 4 closely related congener species and 2 con-familial worms.¹³ Comparative analyses revealed an unprecedented ~50 bp (base

pairs), 3'-extension of the mitochondrial-encoded *atp6* gene in ice worms, corresponding to a polar carboxy-terminal tail of ~18 amino acids. ATP6, a membrane-intrinsic component of the F_o domain, plays a key role in formation of the proton half-channels that provide H⁺ translocation across the inner mitochondrial membrane driving ATP production.^{14–16} Considering the functional importance of the ATP6 subunit and strong evolutionary trend of mitochondrial genome compaction,^{17,18} an insertion of this magnitude is unlikely to be maintained in the genome without providing a significant fitness advantage.

Materials and Methods

Specimens

Mesenchytraeus specimens were collected from field sites in Alaska (*M solifugus*-Byron), Washington (*Mesenchytraeus gelidus*, *Mesenchytraeus hydrius*), California (*Mesenchytraeus pedatus*), and British Columbia (*Mesenchytraeus antaeus*, *M solifugus*-Mariner Mountain) between 2011 and 2015, express-shipped live to Rutgers University, and maintained according to their endogenous habitat/temperature (eg, soil, refrigerated). *Enchytraeus crypticus* species were a laboratory-maintained stock and *M solifugus*-Davidson species were ethanol-preserved samples (see Lang et al¹³ for details about collection sites).

Nucleic acid processing and polymerase chain reaction

For verification of RNA-Seq data, genomic DNA extractions were performed using E.Z.N.A. Tissue DNA Isolation Kit (Omega Bio-tek, Inc., Norcross, GA, USA) following the tissue protocol. Multiple sequence alignments (MEGA



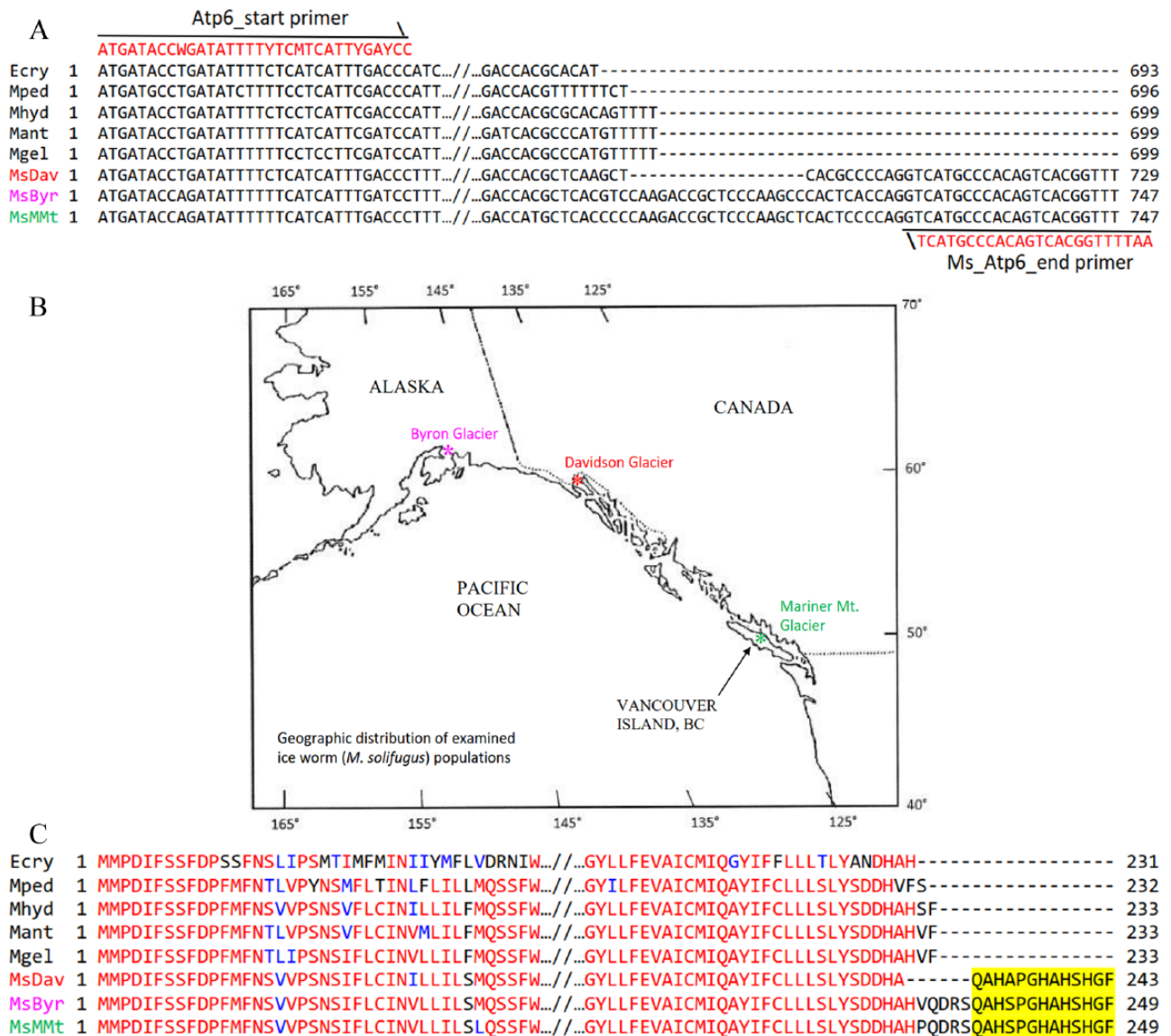


Figure 1. Verification of *Mesenchytraeus solifugus atp6* 3'-extension in different ice worm populations. (A) Full-length *atp6* sequence was polymerase chain reaction amplified from genomic DNA using an enchytraeid-specific forward primer (red) and the ice worm-specific 3'-extension primer and/or a species specific end primer (highlighted). (B) In addition to the Northern clade (Byron Glacier, MsByr) ice worms used for RNA-Seq analyses, 2 additional ice worm populations, Davidson Glacier (MsDav), considered ancestral,²⁰ and Mariner Mountain Glacier (MsMMt) were also examined (species color-coordinated to map). The 3'-extension primer amplified only ice worm samples, but the forward primer amplified all when paired with species-specific end primers. The translated protein alignment (C) shows the conserved block of amino acids (highlighted) found in all 3 ice worm populations, but not found in any other species. The 3'-extension is 6 amino acids shorter in the Davidson population. The alignment in (A) is shown with stop codons removed.

version 6; Tamura et al¹⁹) of full-length *atp6* transcripts derived from RNA-Seq data were used to design primers. A degenerate forward primer (ATGATACCWGATATTTTYTCMTCATTYGAYCC) was paired with species-specific reverse primers (Figure 1A, highlighted). No sequence data downstream of *atp6* were available or retrievable to enable amplification from outside the *atp6* coding region. Polymerase chain reaction (PCR) reactions contained 34.5 μ L water, 200 μ M dNTPs, 600 nM primers, 10 μ L of 5 \times Phusion HF buffer, 1 U Phusion Hot Start II DNA Polymerase (Thermo Scientific, Waltham, MA, USA), and 50 to 200 ng total genomic DNA. Reaction conditions were as follows: denaturation at 98°C for 30", followed by 35 cycles of 98°C for 10 seconds, 47 to 60°C for 20 seconds, and 72°C for 0.5

to 2 minutes; final extensions were 5 minutes at 72°C. Single-band PCR products were purified using DNA Clean and Concentrator (Zymo Research, Irvine, CA, USA); multiband products were electrophoresed on 0.8% agarose gels and desired bands excised. Gel-excised DNA was purified using a Zymoclean Gel DNA Recovery Kit (Zymo Research) according to the manufacturer's protocol. Fragments were cloned into pMiniT vectors (New England Biolabs, Ipswich, MA, USA), transformed into NEB 10-beta chemically competent *Escherichia coli*, and 3 to 5 clones/fragment/species were selected for purification using Zippy Plasmid Miniprep (Zymo Research). Transcriptomes and RNA-Seq data were generated and processed as described in Lang et al.¹³

M_solifugus_ATP6	HAHVQDRSQAHSPGHAHSH--G	ATP6 carboxy terminal extension
Malus_domestica	HAHHHDHGHGHSSGHSLSH--G	Transmembrane transporter (70% pos.)
Marinomonas_sp.	HSHSHDHSLSHSDHSHLSH--G	Sirohydrochlorin cobaltochelataase (70% pos.)
Aureobasidium_sub.	HAHSHDHSLSHSDHSHLSH---	Hypothetical protein (78% pos.)
Thalassospira	HDHGHSDHLSHSHGHSHLSH---	Siroheme synthase (73% pos.)
Burkholderia_sp.	HGHGHSDHLSHSHGHLSH--G	ABC transporter ATP-binding prot. (70% pos.)
Oscillatoria_sp.	HSHEQDRSHDHSLSHSHLSHSHG	Hydrogenase accessory protein (68% pos.)
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Figure 2. Protein alignment of NCBI nr-pBLAST results using *Mesenchytraeus solifugus* ATP6 C-extension as query. Histidine-rich domains are involved in numerous cellular processes, but membrane transport is a well-represented function. *Malus domestica* (apple), *Marinomonas* (marine bacteria), *Aureobasidium subglaciale* (glacier fungus), *Thalassospira* (marine bacterioplankton), *Burkholderia* (Betaproteobacteria), *Oscillatoria* (filamentous cyanobacteria).

Mitochondrial sequence alignment and annotation

DNA sequence chromatograms were evaluated and trimmed using Chromas 2.4.3 (Technelysium, South Brisbane, QLD, Australia). Overlapping sequence fragments were aligned in MEGA version 6 and assembled into consensus sequence contigs using Mesquite v.3.04.^{19,21} Protein-coding genes were identified using National Center for Biotechnology Information's (NCBI) BLASTx and BLASTn algorithms. Extracted transcripts were subjected to GenBank searches to verify gene identity, translated to ensure that they were not pseudogenes, and aligned to each other to assess relatedness. Extracts with questionable or incomplete sequence were re-BLAST using genus-specific queries and/or subjected to raw read mapping to rule out assembly error. In effort to determine the origin of the *atp6* extension, the translated protein fragment corresponding to the last 18 amino acids was used as a query for local BLAST searches against our RNA-Seq databases as well as NCBI's nonredundant protein sequence database.

Results

Computational alignments of transcriptome-derived F₁F₀-ATP synthase subunits from glacier ice worms and congener species identified a novel, ice worm-specific sequence extending the 3' end of *atp6* by ~50bp. Raw read mapping showed this sequence was present in >100 raw reads of 2 independent ice worm transcriptomes (and absent in all other transcriptomes) and therefore not an assembly error. To confirm its authenticity, PCR amplifications of full-length *atp6* were performed from genomic DNA templates using a forward primer designed to amplify all considered species and an ice worm extension-specific reverse primer (Figure 1A). In addition to the 6 considered species, 2 additional ice worms from widely separated populations (Davidson Glacier, Mariner Mountain Glacier) were evaluated to determine whether the *atp6* extension was common to ice worms or an isolated population anomaly (Figure 1B). Robust amplification occurred in all ice worm samples but not in other worms. Mariner Mountain ice worms displayed a 3'-end extension of the same length as the Byron Glacier population (equivalent to 18 amino acids), differing only in a single amino acid (V/P), whereas Davidson worms, members of the putative ancestral clade,²⁰ displayed a truncated version of the extension—the final 13 amino acids, with a single conserved change (S/A).

The translated amino acid sequence—(V/P)QDRSQAHS(S/A)PGHAHSHSGF (Figure 1C)—was not represented in any of our transcriptomes, nor was it analogous to any known metazoan sequence deposited in GenBank. Surprisingly, it shared strong sequence identity (≥68%) to functionally diverse histidine-rich protein motifs from single-celled microbes (Figure 2).

Discussion

Confirmation of the ice worm *atp6* ~50bp 3'-extension has multiple implications. First, an insertion of this magnitude is unusual due to the strong evolutionary trend in metazoans toward compaction of the mitochondrial genome^{17,18}; thus, sequences are not maintained unless they offer some evolutionary advantage. Davidson and Byron Glacier ice worms diverged from each other during the mid-Miocene (5-10 million years ago²⁰), and the observation that both lineages maintained *atp6* extensions indicates strong Darwinian selection.

Second, the structural and functional roles of the ATP6 subunit in the F₁F₀-ATP synthase are not only critically important but are also highly sensitive to modification. Specifically, most of the human mitochondrial diseases resulting from ATP synthase dysfunction are a consequence of *atp6* single-nucleotide polymorphisms.²² Indeed, of the 30 currently described *atp6* disease-associated mutations, 28 are SNPs and 2 are dinucleotide deletions in *atp6* (MITOMAP¹²). In addition, a single amino acid change in the *atp6*-equivalent "A" subunit of an extreme alkaliphilic *Bacillus* bacterium is necessary and sufficient to confer high-pH adaptation.²³ Thus, the comparatively large change of an additional 18 amino acids to ATP6 is likely to have a major impact on its functional activity.

Third, the protein domain encoded by the ice worm ATP6 extension is clearly not random, but rather contains an ordered arrangement of alternating histidine residues, an infrequently used amino acid which can function as both proton donor and acceptor within a physiological pH range. Its origin does not appear to be metazoan, based on GenBank searches, instead it shares striking similarity to microbial sequences (up to 78%), particularly a glacier-dwelling yeast, *Aureobasidium subglaciale* (see Figure 2). Horizontal gene transfer (HGT), once thought to only occur in bacteria, is now recognized as an ancient and continuing process across all domains of life.^{24,25} Furthermore, the most common sources of HGT in eukaryotes are endosymbionts

and diet.^{26–28} Notably, glacier-obligate microorganisms are the primary food source for ice worms,²⁹ and recent work identified a novel bacterial species living as an endosymbiont in the ice worm gut.³⁰ Note also that organisms occupying extreme environments are enriched in foreign genes, and these play important roles in ecological adaptation.^{31,32} It follows that the ice worm *atp6* extension was likely acquired by HGT from a single-celled dietary source, representing only the second mitochondrial transfer event reported in Metazoa.^{33,34}

The putative function of the ice worm ATP6 extension remains unknown, but a number of microbial genes containing similar His-rich domains function in ion/membrane transport, and histidine residues play a well-described role in proton shuttling.³⁵ Due to the known structural position of the ATP6 carboxy terminal at the matrix edge of the ATP synthase proton channel, we propose that the ice worm ATP6 extension enhances ATP synthesis via His-dependent shuttling of protons away from the matrix-inner membrane interface so as to increase the local pH, thereby increasing H⁺ ion flow. Genetic manipulations are inherently challenging in mitochondria; nonetheless, our collective data provide a logical mechanism associated with elevated ATP levels in glacier ice worms, as well as a high potential target for treating energy-related, mitochondrial disease.

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Author Contributions

SAL conducted experiments, analyses, and contributed to writing the manuscript. DHS collected field specimens and contributed to writing the manuscript.

Data Accessibility

DNA sequences: GenBank accessions KU728746–KU728800.

Ethical Statement

No vertebrate animals were used in these experiments.

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