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Comparison of whole mitochondrial genome sequences from two clades of the invasive ascidian, *Didemnum vexillum*

Kirsty F. Smith [a,b,*], Cathryn L. Abbott [c], Yasunori Saito [a], Andrew E. Fidler [b,d,e]

[a] Shimoda Marine Research Center, University of Tsukuba, 5-10-1, Shimoda City, Shizuoka 415-0025, Japan
[b] Cawthron Institute, Private Bag 2, Nelson 7042, New Zealand
[c] Biological Station, Fisheries and Oceans Canada, 3190 Hammond Bay Road, Nanaimo, British Columbia V9T 6N7, Canada
[d] Maurice Wilkins Centre for Molecular Biodiscovery, University of Auckland, Auckland 1142, New Zealand
[e] Institute of Marine Science, University of Auckland, Auckland 1142, New Zealand

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ABSTRACT

The mitochondria are the main source of cellular energy production and have an important role in development, fertility, and thermal limitations. Adaptive mitochondrial DNA mutations have the potential to be of great importance in determining aspects of the life history of an organism. Phylogenetic analyses of the globally invasive marine ascidian *Didemnum vexillum* using the mitochondrial cytochrome *c* oxidase 1 (COX1) coding region, revealed two distinct clades. Representatives of one clade (denoted by 'B') are geographically restricted to *D. vexillum's* native region (north-west Pacific Ocean, including Japan), whereas members of the other clade (denoted by 'A') have been introduced and become invasive in temperate coastal areas around the world. Persistence of clade B's restricted distribution may reflect it being inherently less invasive than clade A. To investigate this we sought to determine if the two clades differ significantly in other mitochondrial genes of functional significance, specifically, alterations in amino acids encoded in mitochondrial enzyme subunits. Differences in functional mitochondrial genes could indicate an increased ability for clade A colonies to tolerate a wider range of environmental temperature. Full mitochondrial genomic sequences from *D. vexillum* clades A and B were obtained and they predict significant sequence differences in genes encoding for enzymes involved in oxidative phosphorylation. Diversity levels were relatively high and showed divergence across almost all genes, with *p*-distance values between the two clades indicating recent divergence. Both clades showed an excess of rare variants, which is consistent with balancing selection or a recent population expansion. Results presented here will inform future research focusing on examining the functional properties of the corresponding mitochondrial respiration enzymes, of A and B clade enzymes. By comparing closely related taxa that have differing distributions it is possible to identify genes and phenotypes suited to particular environments. The examination of mitochondrial genotypes, and associated enzyme functioning, across populations may aid in our understanding of thermal tolerance and environmental adaptation.

1. Introduction

The role of mitochondrial functioning in shaping the demographics and adaptive evolution of both populations and species is poorly understood and mitochondrial genome sequences have typically been assumed to evolve neutrally (Balloux et al., 2009; Galtier et al., 2009; Ballard and Melvin, 2010). However, more recently it has been suggested that adaptive changes in mitochondrial DNA sequences are potentially of importance in influencing the life history of an organism (Ballard and Pichaud, 2014). The central role of mitochondria, the main source of cellular energy production, means that their functioning will profoundly influence many organism-level traits such as development, fertility and thermal limits (Gershoni et al., 2009). Therefore, mitochondrial bioenergetics associated pathways such as the electron transport system (ETS) and oxidative phosphorylation (OXPHOS) have increasingly been examined for evidence of eco-physiological adaptation (Mishmar et al., 2006; Luo et al., 2008; Pichaud et al., 2011; Evans and Bernatchez, 2012; Fields et al., 2012). Selection on coding sequences within the mitochondrial genome can potentially shape variations in the functioning of associated proteins (Foote et al., 2011; Evans and Bernatchez, 2012; Cheviron and Brumfield, 2012). In addition, the five enzyme complexes operating the OXPHOS system are comprised of multiple proteins, some encoded by the mitochondrial genome and some by the nuclear genome (Bar-Yaacov et al., 2012). For the last three decades individual genes from the mitochondrial genome, particularly the cytochrome *c* oxidase 1 (COX1) gene, have been used for examining genetic structuring and

* Corresponding author at: Cawthron Institute, Private Bag 2, Nelson 7042, New Zealand.
E-mail address: kirsty.smith@cawthron.org.nz (K.F. Smith).

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phylogenetic relationships at both the population and species level (Hajibabaei et al., 2007). While in more recent years, with the development of new sequencing technologies, whole mitochondrial genome sequences have been used for similar studies (Iannelli et al., 2007; Morin et al., 2010; Heller et al., 2012; Jacobsen et al., 2012; Shamblin et al., 2012). DNA sequence variation and genome-level structural features, such as gene presence or absence and order rearrangements, have been used to resolve phylogenetic relationships (Boore and Fuerstenberg, 2008; Gissi et al., 2010) and for studying recent intraspecific divergence and reproductive isolation (Morin et al., 2010; Jacobsen et al., 2012; Shamblin et al., 2012).

The establishment of invasive species into new locations and environments can have severe impacts on the established biodiversity and functioning of recipient ecosystems (Halpern et al., 2008). DNA sequence-based phylogenies have revealed that many invasive species are comprised of distinct clades, across both their native and expanded ranges (Tsutsui and Case, 2001; Iannelli et al., 2007; Winkler et al., 2008; Bock et al., 2012), and that only some such clades become invasive (Lee and Gelembiuk, 2008). While it is likely that physiological differences between invasive and non-invasive clades may account for differences in their invasive potential experimental evidence supporting this idea is still scarce (Prentis et al., 2008). One example is provided by the copepod *Eurytemora affinis* where distinct COXI clades have overlapping distributions in several marine coastal areas yet only one clade has invaded freshwater reservoirs with other clades remaining restricted to their native marine environment (Lee, 2000; Winkler et al., 2008). Increased hemolymph osmolality in the invasive freshwater populations of *E. affinis* appears to be associated with its ability to colonize freshwater environments (Lee et al., 2011; Lee et al., 2012).

Marine organisms are exposed to a wide range of environmental stressors and temperature is arguably the most significant environmental parameter influencing the distribution and abundance of marine organisms (Johnston and Bennett, 1996). The ability to tolerate a wide range of temperatures is hypothesized as a trait that enhances the successful introduction and establishment of invasive species (Zerebecki and Sorte, 2011). Additionally, the catalytic capacity of whole mitochondria and key enzymes of mitochondrial respiration are strongly affected by environmental temperature variations in ectotherms (Ballard and Pichaud, 2014). Mutations in mitochondrial genes have been associated with adaptive differences in aerobic capacity or thermal tolerance in a number of different taxa (Dalziel et al., 2006; Martinez-Fernandez et al., 2010; Scott et al., 2011; Pichaud et al., 2012). Evolutionary change within a species is thought to contribute to invasiveness (Prentis et al., 2008) but studies examining the functional factors involved in the evolution of invasive populations or clades are rare. Mitochondrial mutations and their effect on mitochondrial enzyme function may provide explanations that are useful for understanding temperature tolerances of invasive species.

Didemnum vexillum is a highly invasive colonial ascidian that has become established in temperate coastal waters around the world (Lambert, 2009; Smith et al., 2012; Stefaniak et al., 2012) where it has become a significant biofouling pest (Minchin and Sides, 2006; Valentine et al., 2007; Denny, 2008). Population genetic studies of *D. vexillum* have identified two distinct clades based on COX1 sequences (Smith et al., 2012; Stefaniak et al., 2012) with some, albeit limited, nuclear sequence data also supporting this division (Smith, 2012; Smith et al., 2012). One COXI clade (referred to as clade A) has expanded into temperate coastal areas around the world while the other (clade B), to date appears to remain restricted to its probable native region (northwest Pacific Ocean; Smith et al., 2012; Stefaniak et al., 2012). In Japan the distributions of clades A and B overlap so that at several sites the two clades occur in sympatry (Smith et al., 2012; Stefaniak et al., 2012). Potentially, the restricted distribution of clade B may reflect its being inherently less invasive than clade A (Smith et al., 2012). While the distinction of *D. vexillum's* two clades is based on only a short region (586 bp) of the mitochondrial genome, it is possible that these sequences are linked to functionally significant differences elsewhere in the mitochondrial genome. To investigate this possibility we sequenced complete mitochondrial genomes from multiple individuals belonging to clades A and B. Mitogenomes were sequenced from colonies collected within the native range (Japan) in order to avoid sampling introduced populations with reduced genetic diversity (Smith et al., 2012; Stefaniak et al., 2012).

2. Materials and methods

2.1. D. vexillum sample collection and DNA extraction

D. vexillum colony samples were collected in two years (2009 and 2010) from five sites around the coast of Japan (Table 1). Total genomic DNA was extracted (i-Genomic CTB DNA extraction mini kit, animal tissue protocol; Intron, Gyeonggi-do, South Korea) and stored at −20 °C. Each DNA sample was assigned to either the A or B clade on the basis of COX1 partial coding sequence data as described in Smith et al. (2012).

2.2. Generation of a draft D. vexillum mitochondrial genome sequence

D. vexillum genomic sequence data were generated using 454 pyrosequencing from a COX1 clade A colony from British Columbia, Canada (Abbott et al., 2011). Reads were assembled into contigs using Newbler (Margulies et al., 2005) and BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to determine contigs of mitochondrial origin. Two mitochondrial genome derived contigs (9985 bp, 3022 bp) were identified and PCR primers designed at their ends to amplify the two regions required to generate a complete circular mitochondrial genome: DVmt_9961 (5′-ACC ATC TTA TTA AGC TAC AAG-3′) with DVmt_81 (5′-TAT TAG TGT TCA TTT AGG TAT A-3′), and DVmt_2707 (5′-GCG CTG TTA TCC CTA GGG-3′) with ux1F (Gissi et al., 2010) (5′-CCD GAT ATR GCK TTY CCT CG-3′). Polymerase chain reactions (PCR) were carried out in 50 μl reaction volumes containing 25 μl of i-Star Taq 2× PCR supermix (Intron), 0.4 μM of both forward and reverse primers, 0.8 μg of non-acetylated bovine serum albumin (BSA, cat no. B8667; Sigma Aldrich, MO, United States), 2 mM of MgCl2, and 1 μl of *D. vexillum* genomic DNA (ca. 50–150 ng). Thermocycling conditions were 94 °C for 2 min, 1 cycle; 94 °C for 20 s, 50 °C for 20 s, 68 °C for 5 min, 35 cycles; and 68 °C for 5 min, 1 cycle. The primer pair DVmt_991 and DVmt_81 generated a product ca. 0.3 kb in length and the primer pair DVmt_2707 and DVmt_2707 generated a product ca. 2.8 kb in length. Amplification products were purified using AxyPrep PCR cleanup kits (Axygen, California, United States) and sequenced in one direction, using the respective PCR primers, DVmt_9961 or DVmt_2707, by an external contractor (Genetic Analysis Services, University of Otago, Dunedin, New Zealand). The two PCR product sequences were aligned with the 9985 and 3022 bp contigs from the *D. vexillum* genomic sequence data to generate a draft *D. vexillum* circular mitochondrial genome sequence of 13,378 bp.

2.3. Sequencing and annotation of multiple, pooled D. vexillum mitochondrial genomes

The draft *D. vexillum* mitochondrial genome was used to design PCR primers which, when used in combination with previously published ascidian mitochondrial genome primers (Stefaniak et al., 2009; Gissi et al., 2010), allowed *D. vexillum* mitochondrial genomes of both COX1 clades to be amplified in five overlapping fragments of 2.9–4.2 kb overlapping by ≥300 bp at both the 5′ and 3′ ends (Table S1A, B). Genomic DNA samples for full mitochondrial genome sequencing were selected to provide ten samples from each COX1 clade, representing different COX1 haplotypes and a range of locations around the Japan coast (Table 1). PCR were carried out in 50 μl reaction volumes containing 25 μl of MyFi 2× PCR supermix (Bioline, London, UK), 0.4 μM